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Identification of the human testis protein phosphatase 1 interactome

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ABSTRACT

Protein phosphorylation is a critical regulatory mechanism in cellular signalling. To this end, PP1 is a major eukaryotic serine/threonine-specific phosphatase whose cellular functions, in turn, depend on complexes it forms with PP1 interacting proteins—PIPs. The importance of the testis/sperm-enriched variant, PP1γ2, in sperm motility and spermatogenesis has previously been shown. Given the key role of PIPs, it is imperative to identify the physiologically relevant PIPs in testis and sperm. Hence, we performed Yeast Two-Hybrid screens of a human testis cDNA library using as baits the different PP1 isoforms and also a proteomic approach aimed at identifying PP1γ2 binding proteins. To the best of our knowledge this is the largest data set of the human testis PP1 interactome. We report the identification of 77 proteins in human testis and 7 proteins in human sperm that bind PP1. The data obtained increased the known PP1 interactome by reporting 72 novel interactions. Confirmation of the interaction of PP1 with 5 different proteins was also further validated by co-immunoprecipitation or protein overlays. The data here presented provides important insights towards the function of these proteins and opens new possibilities for future research. In fact, such diversity in PP1 regulators makes them excellent targets for pharmacological intervention.

1. Introduction

Eukaryotic cell viability is dependent on dynamic post-translational modifications among them reversible protein phosphorylation. The sequencing of entire genomes has revealed that approximately 3% of all eukaryotic genes encode protein kinases or protein phosphatases [1]. Surprisingly, there appear to be 2–5 times fewer protein phosphatases as compared to protein kinases. This imbalance is even more pronounced when the analysis is limited to Ser/Thr phosphatases and kinases. In the human genome, for instance, the latter are approximately 20 fold more abundant. During the past decade it has become apparent that different strategies can be employed to achieve the required functional diversity of mammalian kinases and phosphatases. Ser/Thr phosphatases diversified not only through the evolution of new catalytic subunits, as evident with kinases, but also by the ability of a single catalytic subunit to interact with multiple regulatory subunits. A striking example is protein phosphatase 1 (PP1) that in vivo possesses exquisite specificities, both in terms of its substrates and cellular localization. The control mechanism for such 'fine tuning' results from the nature of the proteins to which it binds. Hence, the functional diversity of PP1 activity and cellular localization is controlled via its interaction with a wide range of regulatory proteins, so called PP1 interacting proteins (PIPs). An increasing number of PIPs regulating the catalytic activity of protein phosphatases are being identified in various cell types [2]. PIP diversity not only explains the need for few catalytic subunit types but also makes them attractive targets for pharmacological intervention [3]. From a clinical perspective, non-selective or marginally selective phosphatase 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 relatively non-specific inhibitors (e.g. calyculin A, microcystin, okadaic acid or cantharidin) into therapeutic agents for systemic use seems unlikely. Still, the development of phosphatase type- and isoform-specific inhibitors is highly promising [2,4]. Furthermore, and supporting the possibility of developing highly targeted therapeutic interventions, are some recent studies showing that it is possible to activate a specific PP1

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complex in vivo by targeting the PP1 regulatory subunit [5,6]. Consequently there is an urgent need for all the putative PIPs to be made available to the public domain, particularly given the fact that these are highly likely to be tissue specific.

In mammals, PP1 [7] is encoded by three separate genes (PP1α, PP1β and PP1γ), PP1γ undergoes tissue-specific splicing, giving rise to a ubiquitously expressed isoform, PP1γ1, and a relatively poorly characterized testis-enriched isoform, PP1γ2 [8–10]. PP1γ2 is highly abundant on a protein level in mammalian spermatozoa and has been shown to be involved in the regulation of motility in all mammalian spermatozoa studied thus far. It is also involved in the onset of hyperactivated motility and the acrosome reaction [11–16]. Immotile spermatozoa contain higher activity levels of PP1γ2 compared to motile spermatozoa, while expressing similar protein levels. Inhibition of protein phosphatase activity by okadaic acid or calyculin A initiates motility in caput epididymal sperm without requiring a change in cAMP levels [13]. Based on these data, a role for PP1γ2 in the control of sperm motility was suggested. The evolutionary conservation and the importance of Ser/Thr-phosphatases in regulating flagellar motility are also highlighted by the involvement of a PP1 homolog in the regulation of rooster sperm motility [17,18]. In addition, the involvement of Ser/Thr-phosphatases in the regulation of microtubule sliding velocity in *Paramecium* and *Chlamydomonas* is functionally relevant [19–23]. Other studies implicate PP1 in the spermiogenesis process. PP1γ2 is expressed during germ cell differentiation in testis [8–10] and the knock-out of the PP1γ gene in mice resulted in sterile males due to arrest of spermatogenesis at the spermatid stage [24].

Spermatozoa leave the testis incapable of progressive motility and unable to fertilize an egg. Motility is acquired during transit through the epididymis [25], and the ability to fertilize eggs is achieved after incubation in the female reproductive tract, a process referred to as capacitation [26]. There, an acrosome reaction occurs and spermatozoa acquire a hyperactivated state. A variety of experimental approaches have involved a huge number of proteins in mediating the interaction between a spermatozoon and its environment [27]. However, the nature of the molecular events leading to acquisition of progressive motility, capacitation, hyperactivation of motility, directed motility or induction of acrosome reaction remains unresolved.

Previous studies [11–13] have clearly placed PP1γ2 as a key component in regulating spermatozoa motility and male fertility. The work described here was designed to determine the identity of the protein regulators of PP1γ2 expressed in human testis and sperm, and subsequently to provide the molecular tools capable of modulating PP1γ2 activity in a tissue-specific manner. In order to identify PIPs three separate and exhaustive Yeast Two-Hybrid (YTH) screens of a human testis cDNA library, using as baits PP1γ1, PP1γ2 and PP1γ2C, were performed. The approach allowed us to identify proteins responsible for transient interactions and significantly those involving proteins of low abundance. Among the members of the PP1γ2 testis-specific interactome identified, were proteins previously characterized, but also novel PIPs. The latter are important from a physiologically relevant perspective as they place PP1γ2 at functionally critical positions. Additionally immunoprecipitates from human testis using a PP1γ2-specific antibody were carried out and proteins isolated were subsequently analysedanalysed by mass spectrometry. Some PIPs were validated by Co-immunoprecipitation and overlay. Our results provide new insights into PP1 function in human testis and sperm physiology, and reveal possible targets for pharmacological intervention, particularly regarding infertility and contraception.

2. Materials and methods

2.1. Library screening

The PP1γ1 cDNA was directionally subcloned into *Sal*I/*Sma*I digested pAS2-1 (GAL4 binding domain expression vector) to produce pAS-PP1γ1. The 200 bp PP1γ2-specific C-terminal-containing *Pst*I fragment was transferred from pTacTacγ2 [28] into *Pst*I digested pAS-PP1γ1 to produce pAS-PP1γ2 or into *Pst*I digested pAS2-1 to produce pAS-PP1γ2C. These expression vectors were first used to confirm the expression of the resulting fusion proteins (GAL4-PP1γ1, GAL4-PP1γ2 and GAL4-PP1γ2C) in yeast strain AH109. For library screening, the yeast strain AH109 transformed either with pAS-PP1γ1, pAS-PP1γ2, or pAS-PP1γ2C, was mated with yeast strain Y187 expressing the human testis cDNA library in the pACT-2 vector (Gal4 activation domain expression vector, Clontech, Saint-Germain-en-Laye, France). Half the mating mixture was plated onto high stringency medium (quadruple dropout medium: SD/-Ade/-His/-Leu/-Trp) and the other half onto low stringency medium (triple dropout medium: 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 colour). All the YTH reagents were purchased from Clontech, Saint-Germain-en-Laye, France. All other non-specified reagents were purchased from Sigma–Aldrich, Portugal.

2.2. Recovery of plasmids from yeast and sequence analysis

Yeast plasmid DNA was recovered and used to transform *E. coli* XL1-Blue. Plasmid DNA was obtained from each resulting bacterial colony and digested with the restriction enzyme *Hind*III (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—TACCACTACAATGGATG (Clontech, Saint-Germain-en-Laye, France). The DNA sequences obtained were compared to the GenBank database, using the BLAST algorithm, to identify the corresponding encoded proteins.

2.3. Antibodies

Anti- PP1γ2 antibody–antisera were raised in rabbits against the specific PP1γ2 C-terminal peptide, VGSGLNPSIQKASNYRNNTVLYE, and affinity purified [10].

Anti-PP1γ antibody (CBC3C)–antisera were raised in rabbits against the PP1γ C-terminal peptide, KKPNATRPVTPPRGMITKQAKK, and affinity purified, which detects both γ isoforms, at the Centre for Cell Biology at University of Aveiro.

Anti-SARP antibody–antisera were raised in sheep against GST-SARP1 (aa240-832) and affinity purified against MBP-SARP1 (aa708-832) [29].

Anti-GFP antibody–JL-8 was obtained from Clontech, Saint-Germain-en-Laye, France.

2.4. Immunoprecipitation of PP1γ2 from human sperm

Human semen samples were collected from healthy adult donors. All samples were scored as normal according to the World Health Organization (WHO) criteria [30]. Each sample was processed within a maximum of 1 h after collection. Semen samples were centrifuged at 350 × *g* for 7 min at room temperature and the pellet resuspended in 1 ml lysis buffer (see below) for 15 min on ice and then sonicated for 30 s. The lysates were

precleared with 25 μl of Protein A Sepharose slurry (Amersham, Uppsala, Sweden) for 1 h at 4 °C, with agitation. After centrifuging the samples for 1 min at 4 °C at 10,000 × *g*, the supernatant was transferred to a new tube. Protein A Sepharose slurry (25 μl) and 2 μl of Anti-PP1γ2 antibody were added and the mixture incubated overnight at 4 °C with agitation. Subsequently, the mixture was centrifuged for 1 min at 4 °C at 10,000 × *g* and the pellet washed four times for 15 min with 500 μl of 50 mM Tris–HCl (pH 8.0), 120 mM NaCl. Finally, two washes were performed with 50 mM Tris–HCl (pH 8.0). To each pellet 100 μl of 2D rehydration solution (8 M Urea, 4% CHAPS, 0.002% of bromophenol blue) was added and the samples were further analysed by 2D-gel electrophoresis.

2.5. 2D-analysis and mass spectrometry

Samples from the sperm immunoprecipitation were supplemented with 1.25 μl of IPG buffer (in the 3–10 pH range) and 0.7 mg of DTT in a final volume of 250 μl. The samples were transferred into a strip holder and electrophoresis was performed as follows (1 h at 30 V, 2 h at 150 V, 1 h at 500 V, 1 h at 1000 V and 2 h at 8000 V). For the second dimension, 11% gels were casted into a 14 cm × 16 cm gel apparatus (Hoeffer SE600 system) and the strips from the first dimension were placed on top of the casting gel. Electrophoresis was performed at 90 mA until the tracking dye reached the bottom of the gel. Immunoprecipitated proteins were visualized by silver staining. Gels were incubated overnight in fixing solution (40% methanol, 10% acetic acid) and then transferred into sensitizing solution (30% methanol, 0.2% sodium thiossulfate, 6.8% sodium acetate) for 30 min. Subsequently gels were washed three times with distilled water and incubated for 20 min in silver solution (2.5% $AgNO_3$), in the dark. Following two washes with distilled water to remove excess silver nitrate, the gels were placed in developing solution (2.5% sodium carbonate, 0.04% formalin) until the protein spots became visible. The reaction was stopped with stop solution (1.46% EDTA). Finally, gels were washed with distilled water and scanned in a GS-710 calibrated imaging densitometer and analysed with Quantity One software (Biorad, Herts, UK). Protein spots were excised and analysed in the Proteomics facility of the Medical Research Council Protein Phosphorylation Unit in Dundee as described in Stubbs et al. [31].

2.6. Validation of interactions by yeast co-transformation

Yeast cells (AH109 strain) were co-transformed with PP1α, PP1γ1, PP1γ2 or PP1γ2C in pAS2-1 [32], and Tctex1d4, or SARP2, or C11ORF66s, or R15B or RANBPM in pACT-2 using the Li-Ac method (Clontech, Saint-Germain-en-Laye, France). After growing in selective media, in order to confirm the interaction, colonies were transferred to plates with X-α-Gal.

2.7. Validation of interactions by co-immunoprecipitation of PP1 containing complexes

2.7.1. SARP2

Endogenous SARP was immunoprecipitated from human sperm with an anti-SARP antibody in the presence of Protein G Sepharose (Amersham, Uppsala, Sweden). Human sperm samples were centrifuged at 350 × *g* for 7 min and the pellet was washed three times with phosphate buffer saline (PBS). The pellet was resuspended in lysis buffer with a cocktail of protease inhibitors [50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 4% CHAPS, 0.1 mg/ml Pepstatin A, 0.03 mM Leupeptin, 145 mM Benzamidine, 0.37 mg/ml Aprotinin and 4.4 mM PMSF] and sonicated twice for 15 s. The lysates were pre-cleared with Protein G Sepharose slurry for 1 h at 4 °C with shaking. After centrifugation, Protein G Sepharose and primary antibody were added to the supernatant, followed by overnight incubation at 4 °C with agitation. Subsequently the beads were washed three times with 50 mM Tris–HCl (pH 7.5), 120 mM NaCl and resuspended in loading buffer. Samples were boiled and centrifuged to pellet the beads, separated by SDS–PAGE (7.5%), transferred to a nitrocellulose membrane following standard procedures [33], and then incubated with anti-PP1γ2 antibody.

2.7.2. RanBPM and R15B

COS-7 cells were transfected with RanBPM-pEGFP-C2 (kindly provided by Dr. Hideo Nishitani) or with R15B-pEGFP-N1, and were then harvested in lysis buffer (see above). The lysates were sonicated and the procedure to immunoprecipitate RanBPM-GFP and R15B-GFP was similar to that described above. Briefly, anti-PP1γ antibody and protein A sepharose (Amersham, Uppsala, Sweden) were used to immunoprecipitate PP1γ1 and PP1γ2 from the cells transfected with R15B-GFP. The corresponding immunoblot was probed with JL-8 anti-GFP antibody. To immunoprecipitate RanBPM-GFP the JL-8 antibody and agarose beads were used, followed by detection of PP1γ. Negative controls were performed without the primary antibodies for both proteins. Immunoreactive bands were revealed by incubating with horseradish peroxidase conjugated secondary antibody and developed by ECL (Amersham, Uppsala, Sweden).

2.8. Validation of interactions by blot overlay analysis

2.8.1. C11ORF66s and Tctex1d4

The cDNAs encoding C11ORF66s and Tctex1d4 were subcloned into pET28 vector (Novagen, Nottingham, UK) to produce pET-C11ORF66s and pET-Tctex1d4. These plasmids and the empty pET28 vector were transformed into *E. coli* Rosetta (DE3) bacteria (Novagen, Nottingham, UK). A single colony was selected and allowed to grow in 3 ml of LB medium containing kanamycin (LB/kan) at 37 °C overnight. Then, 0.5 ml were transferred to 50 ml LB/

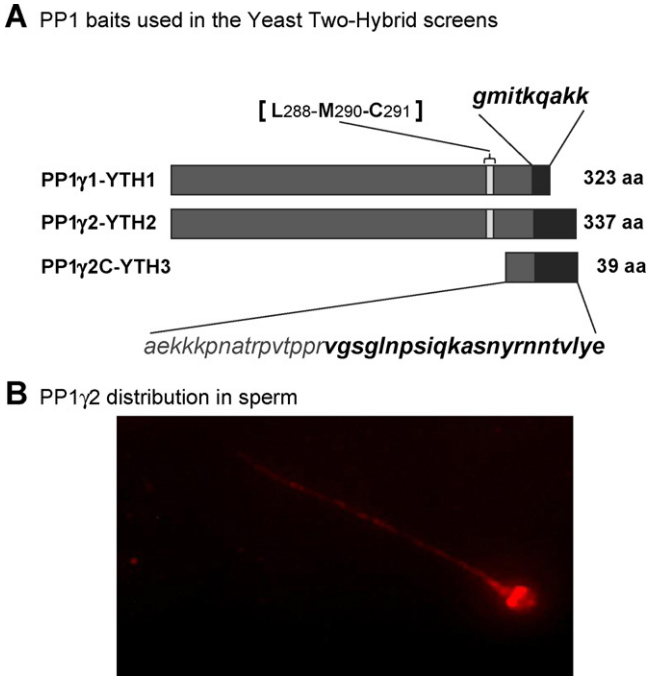

Fig. 1. (A) PP1 baits used in the Yeast Two-Hybrid screens. The light grey box highlights the most important amino acids that make contacts with the PP1 binding motif present in most PP1 binding proteins. (B) Immunocytochemistry of PP1γ2 in human sperm. PP1γ2 localizes to the sperm tail and to the equatorial region of the head (60× magnification).

kan and grown either at 18 °C (C11ORF66s) or 26 °C (Tctex1d4) until the O.D. reached 0.5–0.6. The expression of the proteins was induced with 0.1 mM IPTG, without changing the temperature, for 2 h. The cells were centrifuged at 8000 × *g* for 10 min and the pellet resuspended in 1 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, 0.5% Tween-20, pH 8.0). The translucid lysates obtained by sonication of 10 s cycles (3×), were centrifuged at 15,000 × *g* for 20 min. Aliquots of 0.5, 1 and 5 μl of the Tctex1d4 and C11ORF66s supernatants, and 5 μl of the pET28 supernatant were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The Tctex1d4 and C11ORF66s blots were overlaid with purified recombinant PP1γ1 and PP1γ2, respectively, followed by incubation with anti-PP1γ1 and anti-PP1γ2 antibodies. Immunoreactive bands were revealed by incubating with horseradish peroxidase conjugated secondary antibody and developed by ECL plus (Amersham, Uppsala, Sweden).

Table 1
PP1 interacting proteins.

	Protein designation	Accession number	YTH1	YTH2	YTH3	PP1 BM (putative)
A: Known PIPs	C9ORF75[a]	NM_173691.1	4			KISF/RAIRW
	KIAA1949/phostensin	BC066644	9	2		KISF
	KPI-2[a]	NM_014916	5			VTF
	Nek2A	NM_002497	29	65		KVHF
	Nek2A-T (Nek2C)	AY863109	18	6		KVHF
	PPP1R13A (53BP2/ASPP2)[a]	NM_005426	2	1	1	RVKF
	PPP1R13B (ASPP1)[a]	NM_015316	9	2		RVRF
	PPP1R15B	NM_032833	2	17		KVTF
	PPP1R8-T (NIPP1-T)	DQ223931		1		RVTF
	RIF1[a]	NM_018151.3	10	11		RVSF
	SARP2	EF041819	1			KVHF
	ZAP3	NM_019589	3			KKRVRW
	APAF-1[sa]	NM_015957.2			1	
B: Known proteins but novel PIPs	ASH2[sa]	NM_004674			1	
	AXUD1[a]	NM_033027		1		KRKF/RVAF/RILSF/RVEF
	C1QA[a]	NM_015991		8		RSLGF/KGLF
	COPS5	NM_006837			8	
	CTSL	NM_001912			1	
	CYFIP1[a]	NM_014608			1	
	DAPPER1	NM_016651		1		KILRF
	DYRK4[sa]	NM_003845			1	
	GL1BL[s]	NM_024506			1	
	GSTZ1[a]	NM_145870.1			1	
	HLCDPG1[s]	AF447582			1	
	JAK2[s]	AF058925	1			
	JMJD2D[a]	NM_018039			1	*RVTF*
	MAP4K4[a]	NM_004834		1		RVHF
	NMT2[s]	BC005232			9	
	NRBP1[s]	NM_013392			1	*KVYF*
	RANBPM	NM_005493		1	13	RMIHF
	RRM1[a]	NM_001033			1	
	SGCE[s]	BC021709			1	
	SMG6	NM_017575	1			RVTF
	Tctex1d4	NM_001013632			4	*RVSF*
	TMEM120A[s]	NM_031925			1	
	TOPBP1[s]	NM_007027			1	
	TRA2A[a]	NM_013293			1	
	TWISTNB[a]	NM_001002926.1			1	
	BC001205[a]	BC001205	1			
C: Uncharacterized proteins which are novel PIPs	C10ORF122	BC062717		3		KVRF
	C10ORF72[s]	NM_001031746			1	
	C11ORF66[s]	NM_145017		1		KVHF
	C11ORF74[s]	NM_138787			1	
	C15ORF59[a]	NM_001039614	1			
	C16ORF48[a]	NM_032140			1	
	C1ORF71	NM_152609		1		RVRF
	C21ORF56[a]	BC084577		1	3	KVCF
	C7ORF47	NM_145030	1	3		RQVRF
	C9ORF50	NM_199350	14	9		RVRF
	DKFZp686I1129	AL833488			1	
	FLJ11904	AK021966	1		1	
	KRCC1	NM_016618		2	2	
	MXRA6	BC063142		2	2	
	NKLAM[a]	BC062374			2	KIEF/KILW
	PPP1R2-L (I2-L)[a]	NT_007592.15	1			KLHY
	SDR39U1	BC000989			1	
	SH3RF2	BC073914	1	4		KTVRF
	SPOCD1[a]	NM_144569		1		KMVSF
	STARD9	XM_001129482	1			RVSF
	TBC1D19	NM_018317			1	RISF
	TESTIS-809	AY726579			1	

Numbers indicate the count of isolated cDNA clones for the respective YTH screen; 's' possible splice variant of the indicated GenBank entry; BM, PP1 binding motif.
[a] Clone out of frame.

3. Results

3.1. Identification of 77 proteins by differential Yeast Two-Hybrid screening of a human testis cDNA library

In order to identify PIPs expressed in human testis, three separate YTH screens of a human testis cDNA library were carried out using full-length human PP1γ1 (YTH1), full-length human PP1γ2 (YTH2) and the C-terminal 39 amino acids specific for PP1γ2 [termed PP1γ2C] (YTH3) as baits (Fig. 1A). PP1γ2 is an isoform particularly enriched in sperm, where it is distributed along the tail and in the equatorial region of the head (Fig. 1B). PP1γ1 is ubiquitously expressed, and PP1γ2C lacks the surface hydrophobic pocket (Fig. 1A, L288-M290-C291) [34] that is the typical docking site for the PP1 binding motif present in the vast majority of PIPs, Given that this hydrophobic pocket is absent in PP1γ2C, the inclusion of this bait in the YTH screens can potentially unravel hitherto unknown PIPs.

The screens were performed as described above, yielding 126 positive clones in YTH1, 156 positive clones in YTH2 and 83 positives in YTH3, from a total of 9×10^7, 5×10^7 and 2×10^6 clones screened, 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 their identification and classification into five separate groups. Four groups are listed in Table 1A–C and Table 2. The fifth group corresponds to false positive hits (see below). Table 1A (known PP1 interacting proteins) lists positives encoding previously identified PP1 interacting proteins, such as Nek2 [35] and NIPP1 [36]. Table 1B (known proteins but novel PIPs) lists positives encoding known proteins which were not previously associated with PP1; thus, novel in terms of PP1 binding. Table 1C (uncharacterized proteins which are novel PP1 interactors) lists positives encoding uncharacterized proteins which are present in the database; this group constitutes potential novel PIPs. Table 2 (clones putatively encoding novel PIPs with homology to genomic sequences) lists positives where the GenBank sequence similarity, although apparent, did not correspond to an annotated gene. These cDNA clones may correspond to transcripts derived from novel, previously unidentified genes. Thus, Tables 1 and 2 correspond to novel PIPs.

Taken together the three YTH screens yielded 365 positive clones (Table 1) which correspond to 77 different proteins (Fig. 2A), some with more than one hit. Careful analysis of the different screens revealed that diverse proteins were identified with the various baits (Fig. 2A and B). The largest number (35) of encoded proteins (all of them novel and unique for this bait) was identified in YTH3. The YTH1 and YTH2 resulted in clones which encoded 12 and 14 proteins, respectively, of those 4 and 1 had previously been reported as PIPs (Fig. 2A). Ten proteins were common to both the YTH1 and YTH2, likewise, of those, 6 had already been identified. Proteins common to YTH3 and the other two baits were few in number; namely 4 for YTH3 and YTH2 (Fig. 2B) and 2 proteins for YTH3 and YTH1. Only 1 protein was identified with all 3 baits, PPP1R13A (53BP2/ASPP2), this is a protein that has been previously associated with PP1 [37] (Table 1A). In summary the total number of proteins identified for each of the baits are, 25 (YTH1), 28 (YTH2) and 42 (YTH3), taking into account the overlap of proteins identified by each of the baits as exemplified in the Venn diagram, depicting the protein expression profiles for each of the baits used (Fig. 2B).

Interestingly, the most abundant interactions detected for both PP1 isoforms (YTH1 and YTH2) were with the same kinase (Table 1A, Fig. 3A). Thus, 47 positives out of the 126 detected in YTH1 and 71 positives out of the 156 detected in YTH2 (Fig. 3, blue colour) encoded either Nek2A or Nek2A-T/Nek2C (alternatively spliced variants of the centrosomal Nek2 kinase) [32,35,38]. This novel isoform was submitted, by us, to the GenBank database (AY863109). Another common interaction detected in YTH2 (17 positives) was with a protein identified as R15B in the GenBank database [39,40]. Thus named because it shares a region of homology with R15A (also known as GADD34); it is also known as CReP [32]. Only 2 positives were obtained for R15B with PP1γ1. Given that this protein binds PP1, it has been termed PPP1R15B [41]. Another abundant clone detected in both the YTH1 and YTH2 screens (Table 1A and Fig. 3) was ASPP1 (Apoptosis Stimulating Protein of p53), an important tumour-suppressor, shown to localize to the nucleus of human germ cells [42]. An alternatively spliced form of ASPP1 (53BP2/ASPP2), containing an alternative exon and using a downstream start codon compared to variant 1, thus having a shorter N-terminus, was detected in all 3 YTHs. In fact, this was the only protein found in the 3 different screens. RIF1 (Receptor Interacting Factor) [43], was likewise an abundant positive, 10 and 11 clones were obtained in YTH1 and YTH2, respectively. This protein interacts with retinoic acid receptors (RARs) and other nuclear receptors that are ligand-dependent transcription factors, playing critical roles in cell differentiation, embryonic development, and tumour suppression [44]. It binds aberrant telomeres and aligns along anaphase midzone microtubules. It is regulated by ATM and 53BP1, and functions in the S-

Table 2
Clones putatively encoding novel PIPs with homology to genomic sequences.

Designation of the genomic clones	Accession number	YTH1	YTH2	YTH3	Putative PP1 BM	i.nt	cDNA (bp)
RP11-114H24	AC104758.12	4	6		RVWW	89511	161
RP11-282K6	AC093420.2		3			170763	∼800
RP11-294018	AC122176		1			150852	437
RP11-792D24	AL591408		1		RVRF	147962	∼800
RP11-405I21	AC073468	1		1		51421	∼800
RP1-223E5	AL441883	1			KFVSF	46951	∼1400
RP13-39P12	AL450306		1			10333	∼2300
RP11-288B23	AC090000.17			1	RKRNIQWN	70385	∼900
RP5-902P15	AL096888.30			2		9463	∼800
RP11-249I1	AP003777			1		45601	401
RP11-475023	AC119424.2			1		160139	∼700
RP11-134J21	AC116038.3			1		57855	∼900
CTD-2125J1	AC100834.3			1		78497	397
Chr1 contig	NT_032977.9	2				46545789	∼900
RP11-12A16	AL162584.9			1		72462	∼1300
RP11-268J15	AL591845.27			1		73288	252

i.nt, initial nucleotide in the correspondent GenBank entry; cDNA (bp), corresponds to the length of the clones in base pairs; the clones with the sign '∼' were not fully sequenced and the size is estimated based on the agarose gel fragment after restriction analysis of the positive YTH prey plasmid.

A

	Known PIPs (table 1A)	Known proteins Novel PIPs (table 1B)	Novel proteins Novel PIPs (table 1C)	Positives corresponding to genomic clones (table 2)	Total
YTH 1	4	2	4	2	12
YTH 2	1	5	4	4	14
YTH 3	-	19	8	8	35
YTH 1+2	6	-	3	1	10
YTH 2+3	-	-	3	-	3
YTH 1+3	-	-	1	1	2
YTH 1+2+3	1	-	-	-	1
TOTAL	12	26	23	16	77

B

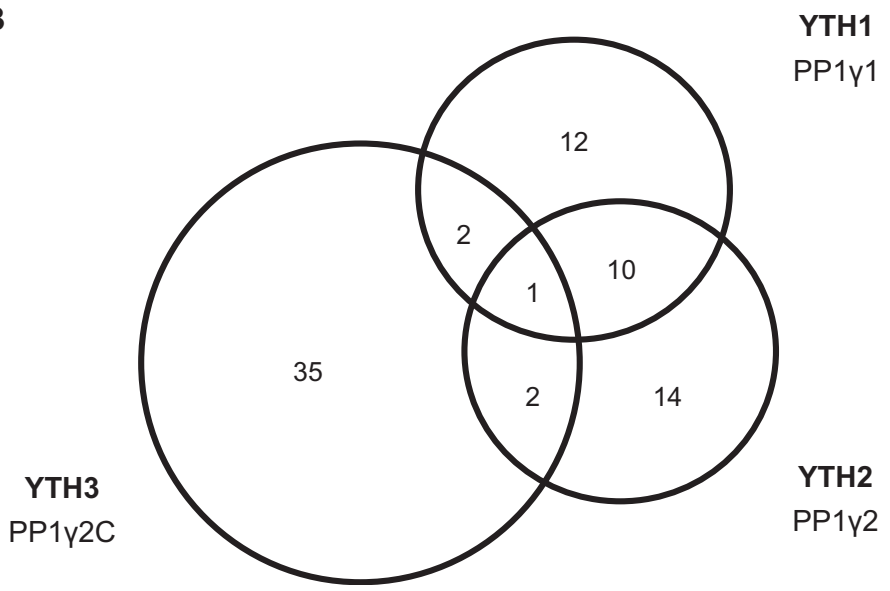

Fig. 2. (A) The proteins encoded by the cDNAs obtained for each of the screens were determined (365cDNAs encoded 77 proteins). These proteins were subdivided with respect to their incidence in the different screens. YTH1 denotes proteins encoded by cDNAs resulting from the screen with PP1γ1 alone. Likewise, for YTH2 with PP1γ2 alone and YTH3 with PP1γ2C alone. YTH1 + 2 denotes proteins encoded by cDNAs resulting from the screen with PP1γ1 as well as from the screen with PP1γ2. Similarly for the other double hits. Only one protein, PPP1R13A (53BP2/ASPP2), was picked up a total of four times (Table 1A) by all three screens (YTH1 + 2 + 3). (B) Venn diagram summarizing proteins obtained in the YTH screens.

phase checkpoint, being highly expressed in germ cells and embryo-derived pluripotent stem cells [45–47]. Among the previously identified PP1 interacting proteins (Table 1) were KIAA1949 and C9ORF75 (FLJ90254), reported to bind PP1 in HeLa cells by Trinkle-Mulcahy and co-workers [43]. Recently, KIAA1949 was reported to be a PP1 F-actin cytoskeleton targeting subunit and named phostensin [48]. C9ORF75, renamed Taperin, was associated with autosomal-recessive non-syndromic hearing loss by target genome capture combined with next-generation capture [49] and by homozygosity mapping [50]. Immunolocalization studies of mouse cochlea by Rehman et al. [49] 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.

Table 1B presents the list of known proteins that have not been previously associated with PP1 (Fig. 3, purple shades), and thus represent potential novel PP1 interacting proteins. Among them, RanBPM (also known as RanBP9) was the most abundant positive in YTH3 (13 hits) and was also identified in YTH2. RanBPM was previously found to bind Ran, a small GTP binding protein belonging to the Ras superfamily, essential for the translocation of RNA and proteins through the nuclear pore complex. RanBPM is

A YTH cDNA clones encoding PIPs

		YTH 1	YTH 2	YTH 3	TOTAL cDNAs	Total Proteins	Average cDNAs/ protein	Key for pie chart
Known PIPs (table 1A)	Nek2A or Nek2A-T/Nek2C	47	71	-	118	2	59	
	R15B	2	17	-	19	1	19	
	ASPP1 and ASPP2	11	3	1	15	2	7	
	RIF1	10	11	-	21	1	21	
	Others	22	3	-	25	6	4	
	Sub-total	**92**	**105**	**1**	**198**	**12**	**17**	
Novel PIPs (table 1B)	RANBPM	-	1	13	14	1	14	
	Others	2	11	37	50	25	2	
	Sub-total	**2**	**12**	**50**	**64**	**26**	**3**	
Novel Proteins and PIPs (table 1C)	C9ORF50	14	9	-	23	1	23	
	Others	7	18	17	42	22	2	
	Sub-total	**21**	**27**	**17**	**65**	**23**	**3**	
Genomic clones (table 2)	RP11-114H24	4	6	-	10	1	10	
	Others	4	6	10	20	15	1	
	Sub-total	**8**	**12**	**10**	**30**	**16**	**2**	
False positives		3	-	5	8	4	2	
TOTAL cDNAs		**126**	**156**	**83**	**365**	**77**	**5**	

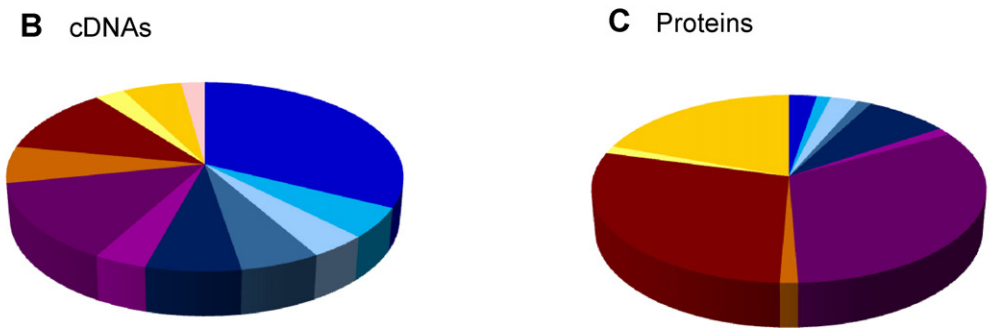

Fig. 3. (A) Comparison of the positive cDNA clones obtained in each of the groups generated (Tables 1 and 2) and corresponding cDNAs and proteins, Pie charts (B and C). Colour codes are indicated in (A).

the Ran binding protein in the microtubule-organizing centre. Unlike most other identified Ran binding proteins, RanBPM appears to have no effect on nuclear trafficking. Recent reports indicate that RanBPM may act as a scaffolding protein since it interacts with several signalling molecules, including cell surface receptors, nuclear receptors, transcription factors and cytosolic kinases [51]. The novel PIPs identified in Table 1B represent a large variety of proteins linking PP1 to numerous cellular events. However, of the 26 proteins in this group (Table 1B and Fig. 2A) the majority, 73% (n = 19), were only isolated in the YTH3, and only 3 of these (underlined and shaded in Table 1B) appear to possess a putative RVxF-PP1 binding motif. Of the remaining 7 proteins, 2 were identified in YTH1 only, 4 in YTH2 only and 1 (RanBPM) in both YTH2 and YTH3. All these 7 clones, except JAK2, contain a

putative PP1 binding motif. Overall, for this group of known proteins but novel PIPs (Table 1B) there are 9 proteins that possess a RVXF-type motif, and besides RanBPM and Tctex1d4 (see below), the other proteins in this category are C1QA, DAPPER1, AXUD1, MAP4K4, JMJD2D, NRBP1 and SMG6 (Table 1A). Some of the identified positives may represent alternatively spliced variants of the known proteins, since analysis of their sequence (data not shown), revealed them to exhibit an alternative open reading frame (e.g. APAF-1^s, ASH2^s and DYRK4^s) or the sequence isolated aligns with the 3′-UTR of the known mRNA (e.g. GL1BL^s, HLCDPG1^s, JAK2^s, NMT2^s, NRBP1^s, SGCE^s, TMEM120A^s and TOPBP1^s). In this respect, it should be stressed that although some may represent false positive interactors, they should not be excluded from a more careful analysis, since alternative splicing is a common event in the testis that may provide for interesting functional regulatory mechanisms [38]. The alternatively spliced C-terminal sequence of PP1γ2 is a good example of alternative splicing that aligns with the 3′-UTR of the more common PP1γ1 variant. Nevertheless, the occurrence and physiological significance of such alternatively spliced variants needs to be confirmed experimentally.

Twenty-two open reading frames, potentially encoding uncharacterized proteins, were shown to bind PP1 in the YTH screens (Table 1C). A consensus PP1 binding motif was identified in approximately half (12) of these putative PIPs, considerably strengthening their putative role as PP1 binding proteins. Among them, the most abundant was C9ORF50 (Fig. 3, light brown shade) with a total of 23 hits (14 from YTH1 and 9 from YTH2). This protein contains a canonical PP1 binding motif (RVRF). Although little information is available for the proteins listed in this category, 4 deserve special mention: SPOCD1, STARD9, TESTIS-809 and C11ORF66^s (highlighted in grey in Table 1C). Since SPOCD1 contains a SPOC domain (Spen paralogue and orthologue C-terminal) normally involved in developmental signalling, STARD9 has a kinesin motor domain and TESTIS-809 was obtained directly from testis, they may all be involved in physiologically testis relevant processes. A possible splice variant in this list is C11ORF66^s, whose interaction with PP1 is further analysed below. Additionally, another positive in this category is I2-L, an isoform of the well characterized PP1 inhibitor 2 (I2) that lacks the critical regulatory GSK-3 phosphorylation site and whose functional relevance is currently being addressed (manuscript in preparation).

Table 2 lists positives whose nucleotide sequence did not align with mRNA or cDNA sequences in the GenBank database. Thus, since the positives identified were derived from the testis cDNA library, the 16 genomic sequences listed probably contain hitherto unidentified genes encoding putative novel PP1 interacting proteins. Indicated are also the first nucleotides of the GenBank entry correspondent to the first nucleotides of the positive clones (Table 2), the cDNA lengths are also specified. For some clones an approximate length is given, based on the fragment of the YTH prey plasmid, analysed by restriction with *HindIII* and separated on an agarose gel. Of those, by translation of the nucleotide sequences obtained, a PP1 binding motif was detected in four (Table 2). Further, bioinformatic analysis of deduced amino acid sequences, for the presence of signalling motifs of functional significance [52], identified several interesting domains in most of the genomic clones. Consequently one can allocate a putative function for the PP1 holoenzyme complex. For instance, the sequence similarity detected with genomic clone RP11-12A16 may encode a protein containing a RRM (RNA recognition motif) that appears in RNA binding proteins, including hnRNPs (proteins that regulate alternative splicing), snRNPs and also in single stranded DNA binding proteins. This sequence bears similarities to hnRNPA1 (NM_002136, chr12) that is involved in pre-mRNA processing and other aspects of mRNA metabolism and transport.

Only four different possible false positive interactions were detected in our screens. Three correspond to mitochondrial clones: four independent clones codified for 16s ribosomal RNA (AM263191), one independent clone codified for MT-ND4 (NC_001807.4) and two independent clones codified for MT-CO1 (NC_001807.4). The fourth false positives contained DNA derived from both chromosomes 2 and 10, RGPD5 + RP11-222G7 (NM_005054 + AC063962). These clones might be an artefact from the cDNA library.

3.2. Identification of PP1γ2 binding proteins in human sperm

In order to further characterize the human sperm PP1 interactome, a totally different and independent approach was pursued. To this end, human sperm PP1γ2 complexes were immunoprecipitated with the highly specific anti-PP1γ2 antibody [10], separated by 2D-electrophoresis and the interacting proteins identified by MS–MS. Protein visualization was achieved by silver staining. Many protein spots were observed, consistent with the multiple interactions postulated for PP1. The more strongly staining spots were excised from the gel and submitted to mass spectrometry analysis, resulting in the unequivocal identification of 7 proteins (Table 3). Spot *A* comprised two different protein members of the HSP70 family: HspA2 and Hsp-71. Even though it lacks a canonical PP1 binding motif, the latter was previously reported to bind PP1 [53,54]. Both these proteins and the protein identified in spot *E*, TCP-1β, are chaperones. Two cytoskeletal proteins were also identified: tubulin β in spot *B* and cytoplasmic

Table 3
Human sperm proteins that bind PP1γ2 identified by mass spectrometry.

Spot ID	Spot name	Protein name	Accession number	Protein prospector score	Protein C.I. (%)	No. peptides used for protein ID	% protein covered	Mol. mass/p*I*	PP1 binding motif	Function
A	HspA2	HSP70 family member; human counterpart of the testis-specific rodent Hst70/Hsp70.2	AAH36107	216	100	15	29.3	69.90/5.63	Absent	Chaperone
A	Hsp-71	Heat shock 70 kDa protein 8	P11142	124	100	8	18	70.85/5.37	Absent	Chaperone
B	Tubulin	Tubulin β	AAH20946	178	100	10	27.5	49.64/4.75	Absent	Cytoskeleton
C	ATP synthase β	ATP synthase subunit β	P06576	232	100	16	49.5	56.52/5.26	Absent	Metabolism
D	actin	actin cytoplasmic 2(γ)//β	P63261//Q96HG5	82	100	5	15.5//15.8	40.19/5.55// 40.98/5.56	Absent	Cytoskeleton
E	TCP-1β	T complex protein 1 β	P78371	44	81	6	20.2	57.45/6.01	EDKLIHF	Chaperone
F	GST Mu-5	Glutathione S-transferase Mu 5	P46439	48	93	6	22.5	25.55/7.3	KITF	Metabolism

actin 2 γ or β in spot *D* (it was not possible to distinguish between the 2 actin isoforms based on the MS–MS data obtained). Three actin isoforms (α, β and γ) are known in vertebrates. While α actins are found in muscle tissue as major constituents of the contractile apparatus, β and γ actins occur in most cells as part of the cytoskeleton, mediating cell motility. There are several PP1 binding proteins that target PP1 to the actin cytoskeleton, including neurabin [55] and phostensin/KIAA1949 [48]. Interaction with actin could therefore be indirect and mediated through such proteins, which together would yield sufficient actin for analysis. Nevertheless, the possibility that PP1 may bind directly to actin cannot be excluded, although the proteins here identified did not exhibit a typical PP1 binding domain.

Two proteins identified by this method do contain a PP1 binding motif; T complex protein subunit β in spot *E* and GST Mu-5 in spot *F*. The former is a molecular chaperone that assists the folding of proteins upon ATP hydrolysis. As part of the BBS/CCT complex may play a role in the assembly of BBSome, a complex involved in ciliogenesis regulating transports vesicles to the cilia It is known to play a role, in vitro, in the folding of actin and tubulin in energy metabolism producing ATP from ADP in the presence of a membrane proton gradient [56]. GST Mu-5 is involved in detoxification by catalysing the conjugation of glutathione with a wide range of endogenous and xenobiotic alkylating agents, including carcinogens and therapeutic agents. Many weakly staining spots, indicative of many other PIPs, were apparent but did not provide sufficient protein to permit identification by mass spectrometry.

3.3. Functional classification of the identified PP1 binding proteins

The present work identified 77 different PP1 interacting proteins by the YTH technique and 7 by the proteomics approach. Of the 84 identified proteins, 68 have a plausible function and the functional distribution is shown in Fig. 4 (Table 1—supplementary information). The proteins encoded by the clones in Table 2 and the false positives were left out of this analysis and were considered to have unknown function. Thus, the proteins encoded by genomic clones (16) plus 23 proteins have unknown functions, although some have very important signalling motifs that could lead us to deduce putative functions. These clones represent 46% of the total clones identified. These clones provide a basis for future experimental designs to determine their functions and add to the already extensive functions of PP1. For the remainder 54% (corresponding to 45 proteins) the most abundant group corresponds to proteins involved in splicing and transcription (11%), followed by proteins involved in signalling (10%), metabolism and energy (7%) and proteins involved in the cell cycle (6%). The remaining proteins identified include components of the cytoskeleton, proteins involved in apoptosis, as well as chaperones and stress response proteins (Fig. 4).

3.4. Confirmation of interactions by yeast co-transformation

Considering the various and diverse proteins identified in the three YTH screens, five unrelated proteins were chosen to confirm the interaction with the different PP1 isoforms by yeast co-transformation. The chosen proteins were SARP2 [29] and R15B [32,39,57] (Table 1A), known to bind PP1 but still relatively poorly characterized, Tctex1d4 [58] and RanBPM [59] (Table 1B), not previously associated with PP1, and C11ORF66s [60] (Table 1C), which may represent a new splice variant of the known database entry. To this end, AH109 yeast strain was co-transformed with pAS-PP1α, pAS-PP1γ1, pAS-PP1γ2, or pAS-PP1γ2C [32], and pACT-R15B, pACT-Tctex1d4, pACT-SARP2, pACT-C11ORF66s, or pACT-RanBPM. After growth in selective media, the resulting colonies were plated onto X-α-Gal containing media in order to confirm the interaction. The results obtained (Fig. 5A) confirmed the interaction of Tctex1d4, SARP2, C11ORF66s and RanBPM with all three PP1 isoforms tested (PP1γ1, PP1γ2 and PP1α). R15B interaction with PP1α appeared to be weaker than with the other two PP1 isoforms. In addition, Tctex1d4 and RanBPM also appear to interact strongly with the specific PP1γ2 C-terminal fragment (PP1γ2C).

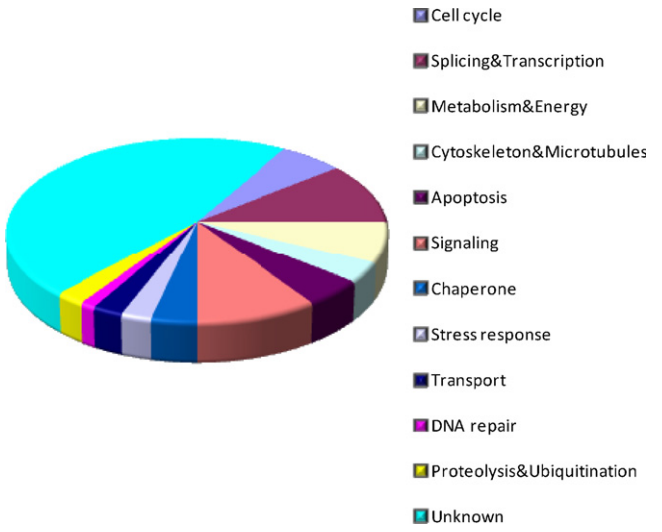

Fig. 4. Distribution of the PP1 regulators according to function. Functions attributed to PIPs identified, according to databases searches based on functional motifs present.

3.5. Confirmation of the interaction with PP1 of selected novel and known PP1 interactors

The cDNAs encoding R15B and RanBPM were subcloned into the pEGFP vector and transiently transfected into COS-7 cells. The corresponding fusion proteins, R15B-GFP and RanBPM-GFP were co-immunoprecipitated with endogenous PP1γ1 (Fig. 5B, I and II), thus validating the interactions for both proteins in cell lysates; a known and a novel interactor. In order to test the physiological significance of SARP2 interaction with PP1, endogenous SARP2 immunoprecipitated from human sperm was found to co-immunoprecipitate with the endogenous and physiologically relevant PP1γ2 isoform (Fig. 5B, III). Thus, PP1γ2 binds SARP2 in human sperm. Finally, the cDNAs encoding two of the novel PP1 binding proteins, Tctex1d4 and C11ORF66s, were subcloned into the pET28 vector and expressed in bacterial cells. The corresponding lysate blots were overlaid with purified recombinant PP1γ1 and PP1γ2, and the interactions validated with our isoform-specific PP1 antibodies. Immunoreactive bands of the expected molecular weights were observed, corresponding to expressed Tctex1d4 (Fig. 5C, I) and C11ORF66s (Fig. 5C, II), again confirming these interactions.

4. Discussion

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, infertility etc.). Thus, protein phosphorylation systems represent alternative and attractive targets for diagnostics and therapeutics. Using the YTH system, new PP1 interactors from human testis were identified, thus providing some insight into the various roles of PP1. The results obtained validated the method as a promising

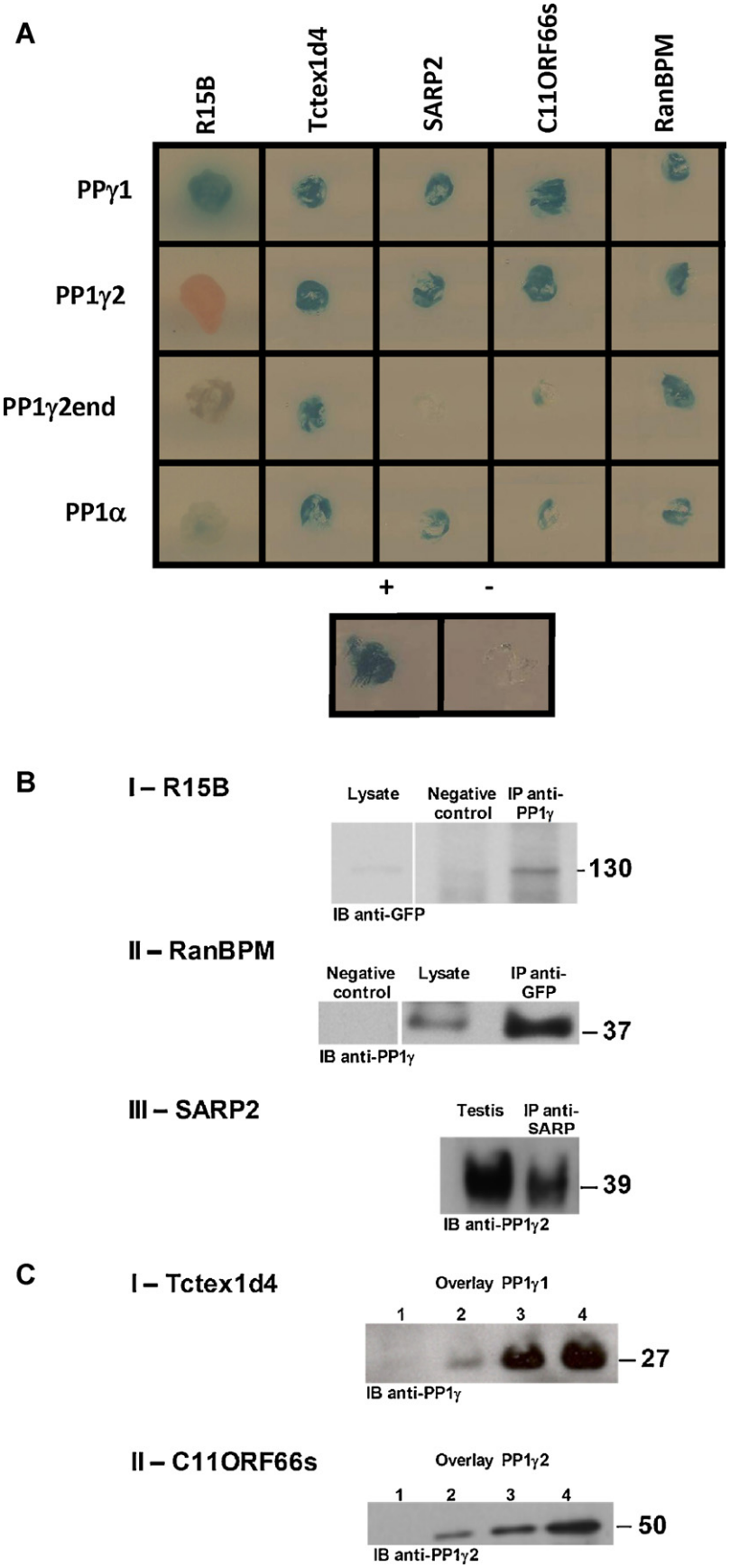

Fig. 5. Different PP1 regulators bind the different PP1 isoforms. (A) Yeast co-transformation of PP1α, PP1γ1, PP1γ2 or PP1γ2C-terminal [32] in pAS2-1 vector, with R15B, Tctex1d4, SARP2, C11ORF66s or RANBPM in pACT-2 using the Li–Ac method. The (+) control is co-transformed with the Clontech vectors pVA3-I and pTD1-I and a blue colony grows in selective media with X-α-Gal; The (−) control is co-transformed with the Clontech vectors with pAS2-1 and pACT-2 and there is no growth in selective media. (B.I) COS-7 cells were transiently transfected with R15B-GFP. Co-immunoprecipitation (IP) was carried out using anti-PP1γ antibody and R15B-GFP was detected using anti-GFP

approach to understanding the multiple functions of the PP1 catalytic subunit, being a method of choice to screen a large number of proteins for putative interactions. We set out to identify all the positive clones obtained in our YTH screens and this strategy proved to be fruitful since several new and interesting proteins were detected. Some occurred only once among the many positives isolated and would not have been identified if only the more abundant clones were characterized. Other previously characterized PP1 binding proteins, like the protein kinase Nek2, were also identified in our YTH screens, thus validating the physiological relevance of the YTH approach.

The differential YTH screens were fundamental, given the PP1 isoform specificities in terms of cellular localization and potential differences in interacting with specific regulators. PP1γ1 and PP1γ2 are expressed in the testis, but in sperm PP1γ2 is the abundant isoform whereas PP1γ1 is absent or highly diminished [10,11,13]. Previous studies suggest that the majority of PP1 regulatory subunits/PIPs bind to the PP1 catalytic subunit through a conserved PP1 binding motif, conforming to the previously characterized canonical [RK]{0,1}[VI][^P][FW] sequence, where [^P] is any amino acid except proline and {0,1}means none or one amino acid [37,61–64]. The PP1γ2C bait used in this study, lacks the docking site for interaction (Fig. 1A) with the above mentioned motif, but may have a different docking motif or account for secondary interactions with regulatory subunits [62]. Work by Dominguez and co-workers showed through the crystal structure of the PP1-MYPT complex how interactions involving the "RVxF" motif are important to the formation of the complex and to PP1-specific functions. Moreover, they suggest that some PIPs interact with the flexible C-termini of PP1 to mediate isoform specificity [62]. Curiously, the YTH screen carried out with PP1γ2C resulted in the greatest number of novel potential PIPs that are unique for this bait ($n = 35$). The fact described above, is in accordance to the absence of a typical PP1 binding domain in the majority of those clones obtained (Table 1B and C and Table 2). These proteins represent a putative group of PIPs which clearly deserve to be further studied.

Of the 77 proteins identified, 12 had already been reported to bind PP1 (Table 1A), although some of these proteins are alternatively spliced, and represent novel isoforms, such as NIPP1-T and Nek2-C. Bennett et al. reported the only PP1 interactome using the YTH system in *Drosophila*. In this large-scale screen 193 cDNAs were isolated encoding 24 PIPs. Some of the previously known PIPs (Table 1A) were also identified in studies performed using alternative strategies, such as, antibody arrays [54], in silico screening [64] and proteomics approaches [43,53]. These include ZAP [53], PPP1R13A, PPP1R15B, SARP2, KIAA1949, Nek2A [64], Nek2 [54], ZAP, RIF1, KPI-2, PPPR13A, KIAA1949 and C9ORF75 [43].

Alternative splicing appears to be a common strategy in the testis for generating diversity and, like PP1γ2, many other testis-expressed genes also undergo alternative splicing. Among these are Nek2 variants (Nek2A and Nek2C), as well as a new splice variant of NIPP1-β (designated NIPP1-T, GenBank database DQ223931), which was also identified among the positives. Nek2C derives from an alternative 3′splice-site of the pre-mRNA and NIPP1-T results from excision of part of exon 7 [32,38].

The differential YTH screens clearly revealed the variability between the frequency of the cDNAs isolated and the total number of PIPs identified. For example 54% of the cDNAs isolated account for 16% of the PP1 interacting proteins identified and are known PIPs (Fig. 3B and C, blue shades). Even more striking is that, 32% of all cDNAs/clones identified correspond to Nek2A or Nek2A-T/Nek2C which in fact account for only 2.6% of all proteins identified. Percentage representation was quite different for the other groups. Known proteins but novel PIPs (Table 1B and Fig. 3B and C, purple shades), novel proteins and putative novel PIPs (Table 1C and Fig. 3B and C, brown shades) and cDNAs corresponding to genomic clones (Table 2 and Fig. 3B and C, yellow shades) had, respectively, cDNAs which represented 18%, 18% and 8% of total cDNAs isolated and 34%, 30% and 21% of total proteins identified. This fact may be the explanation why these proteins were not yet characterized by others before, alternatively they may have been discarded given that they are less abundant clones in the YTH screens, or they could have just been missed. 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. For instance, for SARP2 [29] and NIPP1-T only a single positive clone was obtained, reflecting either the low abundance of those proteins in human testis or the rarity of the corresponding transcripts in the library used. The identification of both abundant and rare known PIPs in our screens confirms the specificity and reliability of the YTH approach.

Overall, in a total of 77 different proteins identified (not considering the putative false positives), only 12 (16%) are known PP1 regulators. Of note, a considerable percentage of proteins identified (35 proteins, corresponding to 45%) were obtained as unique clones in YTH3. This may explain why these PIPs had hitherto not been identified. Considering only the 65 previously unknown PP1 binding partners identified, at least 25 (38%) appear to possess a consensus PP1 binding motif. In fact, the number of proteins carrying a PP1 binding motif 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 testis. As discussed above, alternatively spliced isoforms appear to be frequent in testis.

It is interesting to note that some of the positives isolated corresponded to cDNAs fused out of frame with GAL4-BD. This was the case, for example, with some of the positives isolated for the well known PP1 binding proteins PPP1R13A (ASPP2) and PPP1R13B (ASPP1). Therefore, we would expect this to lead to the expression of an artificial protein that would not bind PP1 (a false negative) and therefore should not be detected in the screen. Thus, the fact that several positives encoding *bona fide* PP1 regulators were isolated fused out of frame to the GAL4-BD strongly suggests that the correct protein was still produced. In fact, this may be explained by a well established mechanism in yeast called programmed translational frameshift or translational recoding [65]. This is a directional and reading frame specific event, and given our observations we conclude that translational recoding may be a feasible explanation for the results obtained.

antibody (IB—immunoblot). As a control 50 μg of the lysate was loaded on the gel. Negative control was performed without primary antibody. Two different sections of the same membrane are presented. (B.II) COS-7 cells were transiently transfected with RanBPM-GFP. Co-immunoprecipitation (IP) was carried out using anti-GFP antibody and PP1γ was detected using anti-PP1γ antibody (IB). As a control 30 μg of the lysate was loaded on the gel. Negative control was performed without primary antibody. Two different sections of the same membrane are presented. (B.III) SARP2 was immunoprecipitated from human sperm extract using anti-SARP antibody and PP1γ2 was detected with the respective isoform-specific antibody. As a control 50 μg of human testis extract was also loaded on the gel. (C) Both Tctex1d4 (I) and C11ORF66s (II) were expressed in bacteria and the membranes incubated with purified recombinant PP1γ1 and PP1γ2 (overlay), respectively. The binding was detected with the respective anti-PP1γ and anti-PPγ2 antibodies (IB). Lane 1, 5 μl of soluble bacterial lysate of expressed pET vector alone. Lanes 2–4, respectively, 0.5, 1 and 5 μl of soluble bacterial lysate of expressed pET-Tctex1d4 (I) and pET-C11ORF66s (II). Molecular weights of the respective proteins are indicated in kDa.

Moreover, this mechanism could be enhanced during the screening procedure because of the need to use selective media without specific amino acids. As a result, the yeast translation machinery may favour the usage of alternative codons.

The proteins identified by MS–MS were quite different and fewer than those identified by YTH. However it is worthwhile noting that there is considerable overlap with the protein profile in human sperm previously identified [66]. Among the proteins described in both studies were HSPs, tubulin and also ATP synthase, although in the latter a different subunit (α) was found [66]. Additionally, in an attempt to study phosphopeptide changes during rat sperm capacitation, Aitken and co-workers [67] have placed Glutathione S-transferase Mu 5 in rat non-capacitated sperm. Also, Varmuza and co-workers [68] by performing a comparative phosphoproteomic approach identified 10 differentially phosphorylated proteins in the testis of *Ppp1cc* null mice, which are potential targets of PP1γ2. Interestingly, 2 of the hyperphosphorylated proteins identified were HspA2 and tubulin β, that we also obtained by MS–MS after co-immunoprecipitating PP1γ2 from human sperm. It has to be stressed that YTH system and co-immunoprecipitation coupled to MS–MS are complementary approaches in terms of identifying different interactions. YTH usually picks up binary interactions while co-immunoprecipitation coupled to MS–MS is capable of isolating large complexes that do not necessarily interact directly with each other. Those proteins may be PIPs (such as Hsp70), may bind to PIPs, or may be substrates of PP1 present in large protein complexes.

A random subset of 5 proteins was used to validate and confirm that the PIPs did in fact bind to PP1. All the proteins analysed bound to the three isoforms of PP1 tested, albeit to differing degrees, thus validating the procedure used to identify the PP1 interactome. The experiments on sperm are particularly important as PP1γ2 is enriched and therefore probably the most physiologically relevant isoform of PP1γ in human sperm. Examination of SARP2 interaction with PP1γ2 demonstrated that endogenous SARP2 from human sperm co-immunopelleted the endogenous PP1γ2 isoform (Fig. 5B, III). Thus, endogenous PP1γ2 and SARP2 form a complex in human sperm lysates. The physiological significance of this interaction is discussed elsewhere [29]. Further confirmation comes from the fact that previously identified PP1 binding proteins were also characterized and of particular note, the presence of the PP1 binding motif. In fact the latter was present in 35 (42%) out of the 84 proteins identified, by the proteomics approach and the YTH screens.

In closing, we report the identification of 77 proteins present in human testis and 7 proteins present in human sperm that bind PP1. The function of most of the identified proteins is not fully determined, therefore potentiating many avenues for further experimentation in distinct areas of research. The data obtained increased the known PP1 interactome by reporting 72 new interactions. Confirmation of the interaction of PP1 with 5 very different proteins was also shown by co-immunoprecipitation or overlay. Although subsequent studies need to be performed to confirm the physiological nature of the identified interactions, many of the identified proteins possess the canonical PP1 binding motif "RVXF". The present work provides the basis for more extensive studies on PP1 holoenzyme functions in testis and sperm in physiological and pathological conditions, and also for studying the relevance of the PP1 regulatory subunits as therapeutic targets for male contraception and male infertility treatments [32,69].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.02.018.

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