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## **SARP, a new alternatively spliced protein phosphatase 1 and DNA interacting protein**

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## ABSTRACT

Protein phosphatase 1 (PP1) is a ubiquitously expressed serine/threonine-specific protein phosphatase whose activity towards different substrates appears to be mediated via binding to specific proteins that play critical regulatory and targeting roles. We report here the cloning and characterization of a new protein, termed several ankyrin repeat protein (SARP), which is shown to interact with all isoforms of PP1 by a variety of techniques. A region encompassing a consensus PP1 binding motif in SARP (<sup>354</sup>KVHF<sup>357</sup>) modulates endogenous SARP-PP1 activity in mammalian cells. This SARP PP1 interaction motif lies partially within the first ankyrin repeat in contrast to other proteins (53BP2, MYPT1/ M<sub>110</sub>/ MBS and TIMAP) where a PP1 binding motif precedes the ankyrin repeats. Alternative mRNA splicing produces several isoforms of SARP from a single human gene at locus 11q14. SARP1 and/or SARP2 (92-95 kDa) are ubiquitously expressed in all tissues with high levels in testis and sperm, where they are shown to interact with both PP1 $\gamma_1$  and PP1  $\gamma_2$ . SARP3 (65 kDa) is most abundant in brain where SARP isoforms interact with both PP1 $\alpha$  and PP1 $\gamma_1$ . SARP is highly abundant in the nucleus of mammalian cells, consistent with the putative nuclear localisation signal at the N-terminus. The presence of a leucine zipper near the C-terminus of SARP1 and SARP2, and the binding of mammalian DNA to SARP2, suggests that SARP1 and SARP2 may be transcription factors or DNA associated proteins that modulate gene expression.

Keywords: protein serine/threonine phosphatase; protein phosphatase 1; ankyrin repeat; leucine zipper; DNA binding protein.

Short running title: SARP, a novel PP1 interacting protein

Abbreviations: PP1, protein phosphatase 1; SARP, several ankyrin repeat protein; 53BP2, p53 binding protein 2; TIMAP, TGF- $\beta$  inhibited, membrane associated protein; MYPT1/M<sub>110</sub>/MBS, myosin binding protein of PP1; NIPP1, nuclear inhibitor of PP1; PNUTS, phosphatase1 nuclear targeting subunit; ZAP, putative RNA binding protein; PSF, polypyrimidine tract-binding protein associated splicing factor; SIPP1, splicing factor that interacts with PQBP-1; Rb, retinoblastoma protein; HCF, human factor C1, host cell factor; GST, glutathione-S-transferase; MBP, maltose binding protein; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate.

## INTRODUCTION

Protein phosphatase 1 (PP1) is a serine/threonine-specific phosphatase that controls many aspects of mammalian cell physiology. It is expressed ubiquitously and found in multiple subcellular locations [1]. In mammalian genomes, three genes encode the PP1 catalytic subunits: PP1 $\alpha$ , PP1 $\beta$  (also termed PP1 $\delta$ ) and PP1 $\gamma$ , and alternatively spliced isoforms of PP1 $\alpha$  and PP1 $\gamma$  have been described [2]. Although the PP1 isoforms exhibit differential tissue and subcellular distribution [3-6], they have broad substrate specificities *in vitro* and their cellular actions are largely determined by binding of the catalytic subunit to many different proteins that act as regulatory and targeting subunits [1, 7, 8]. Several recent studies confirm that the PP1 targeting subunits direct the catalytic subunit to specific subcellular locations, thus restricting the actions of the catalytic subunit to the substrates present there [9, 10]. Most PP1 binding proteins interact with the PP1 catalytic subunit through a common PP1 binding motif termed the RVxF motif with the consensus  $-(R/K)X_{A(0-1)}(V/I)X_B(F/W)-$ , where  $X_A$  is any amino acid and  $X_B$  is any amino acid except proline [1, 11-13]. Although this canonical motif occurs in numerous proteins, limited accessibility to the motif may prevent PP1 binding. A refinement of the motif has been suggested which decreases the sequence variation of the motif in order to identify good PP1 interactors [14]. The interaction of PP1 with the RVxF motif is believed to initiate binding and additional weaker interactions may then stabilize the PP1 complexes but in some cases a second binding site may also retain interaction with PP1 [1, 15]. The existence of a common interaction site explains why the binding of most regulatory subunits is mutually exclusive; however, trimeric and multicomponent complexes of PP1 can also occur [16-18]. Since the regulatory and/or targeting subunits are responsible for controlling PP1 activity and determining the disparate physiological roles of the different PP1 complexes, the key to understanding the function of PP1 lies in identifying and studying the cellular roles of the PP1 binding subunits. We have focused on the identification of novel PP1 regulatory proteins through yeast two-hybrid methodology using PP1 $\gamma$  isoforms as baits [19, 20]. The gene encoding PP1 $\gamma$  undergoes alternative splicing to generate a ubiquitous PP1 $\gamma_1$  protein and a testis-enriched PP1 $\gamma_2$  variant, which differ solely at their extreme C-termini [4, 21, 22]. We report here a novel protein possessing several ankyrin repeats (SARP), which forms a complex not only with both PP1 $\gamma_1$  and PP1 $\gamma_2$ , but also with PP1 $\alpha$  and PP1 $\beta$  and interacts with DNA.

## **MATERIALS AND METHODS**

### **Yeast two hybrid analysis**

Microbial strains and methods for yeast two hybrid screening of human peripheral lymphocyte or human testis cDNA libraries using human PP1 have been described [19, 20]. Positive colonies were obtained on selective media from which pACT plasmids were recovered into *E. coli* and their cDNA inserts sequenced. Screening of human Universal (Stratagene, La Jolla, USA) and Marathon-Ready (Clontech, Palo Alto, USA) cDNA libraries was performed according to the manufacturers' protocols. Automated DNA sequencing was performed by the sequencing services at the Universities of Dundee and Aveiro, using oligonucleotide primers. Analyses of protein sequence domains were performed using the Simple Modular Architecture Research Tool algorithm (SMART, <http://smart.embl-heidelberg.de/>) and ExPASy Proteomic PSORT II prediction algorithm (<http://www.expasy.org/>).

### **Cloning and bacterial expression of epitope-tagged SARP**

To construct the fusion vector for expression of GST-SARP1(240-832), the H2 cDNA was cleaved out of pACT using *Bgl*III and ligated into the *Bam*HI restriction site of the vector pGEX-3X (Pharmacia, Uppsala, Sweden) and pMAL-HA {plasmid pMAL-c2 (New England Biolabs, Inc., Beverly, USA) modified to contain a HA-tag and additional restriction sites). Fusion vectors for the expression of SARP1(708-832) and SARP1(240-333) were constructed by polymerase chain reaction (PCR) using appropriate oligonucleotide primers and H2 template, followed by ligation into pCR2.1 TOPO (Invitrogen, Groningen, Netherlands) and subcloning into pGEX and pMAL vectors. The cDNA encoding the 40Q3 cDNA was directionally subcloned into *Eco*RI/*Xho*I digested pET28a to express His<sub>6</sub>-SARP2(66-847). Another construct [His<sub>6</sub>-SARP2(331-847)] was produced by PCR amplification using a primer starting at Met331. SARP1(240-832) was subcloned into the pEBG vector for expression in mammalian cells, and mutagenesis to generate pEBG-SARP1(F357A) was performed using Stratagene's QuikChange II site directed mutagenesis kit. All constructs were verified by DNA sequencing and expressed in *E. coli* BL21 (DE3) pLysS. Soluble GST-fusion and MBP-fusion proteins were affinity purified on glutathione-Sepharose (Amersham Biosciences, Piscataway, USA) and amylose resin (New England Biolabs) respectively. Insoluble fusion proteins located in *E. coli* inclusion bodies were washed in 1% Triton X-100/1M NaCl, solubilised using either 1% sodium dodecyl sulphate (SDS) or 8M urea with subsequent removal of the urea by dialysis.

### **Northern Blot Analysis**

The 40Q3 cDNA was purified by low-melting point agarose gel electrophoresis, labelled by the random primed method using a commercial kit (Roche, Basel Switzerland) and [ $\alpha$ - $^{32}$ P]dCTP (Amersham Biosciences) and purified by passage through a NucTrap column (Stratagene) to remove unincorporated nucleotides. A Multiple Tissue Northern blot (Clontech) was incubated in the presence of the denatured radiolabeled DNA and hybridizing mRNAs were detected using a PhosphorImager (Bio-Rad Laboratories, Inc.). After probe stripping in 0.5% SDS at 90-100°C for 10min, the same blot was re-used with a  $\beta$ -actin probe as a control. Complete probe removal was verified before use.

### **Production of antibodies and immunoblotting**

Antisera were raised in sheep against GST-SARP1(240-832) at the Scottish Antibody Production Unit (Carluke, Lanarkshire, UK) and affinity purified against maltose binding protein (MBP)-SARP1(240-832), MBP-SARP1(240-708), MBP-SARP1(708-832) or SARP1(240-333) from *E.coli* inclusion bodies. Affinity purified anti-PP1 $\alpha$ , anti-PP1 $\beta$ , anti-PP1 $\gamma$ , anti-PP1 $\gamma_1$  and anti-PP1 $\gamma_2$  antibodies have been described previously [4, 23-25]. For immunoadsorption studies, anti-PP1 $\gamma_1$  antibodies (200  $\mu$ g) raised in sheep were non-covalently coupled to protein G-Sepharose (200  $\mu$ l) by incubation at 4°C for 1 hour in 1 ml 50 mM Tris-HCl pH7.5, 0.15 M NaCl, followed by washing twice with the same buffer. Alternatively anti-PP1 antibodies and anti-SARP(240-333) antibodies were covalently coupled to protein G-Sepharose using dimethylpimelidate [26]. Immunoblotting was performed using anti-SARP or anti-PP1 primary antibodies and horseradish peroxidase conjugated (HRP)-anti-sheep or anti-rabbit secondary antibodies coupled to enhanced chemiluminescence (ECL) detection.

### **Blot Overlay Analysis**

A single colony expressing His-tagged SARP2(331-847) was selected and grown overnight in 3 ml of LB/Ampicillin at 37°C. Aliquots (0.5 ml) were transferred to 50 ml LB/Ampicillin until the optical density at 600nm reached 0.6. Expression was induced with 50  $\mu$ l of 1M isopropyl- $\beta$ -D-thio-galactopyranoside at 21°C, for different periods of time with shaking. Samples (1 ml) were collected every hour, the cells were recovered by centrifugation, resuspended in 0.5 ml 1%SDS and 30  $\mu$ l of the bacterial extracts were loaded onto 12% SDS-PAGE gels. The proteins were subsequently transferred to a nitrocellulose membrane that

was then overlaid with purified PP1 $\gamma_2$  protein, prepared as described [27]. After washing to remove excess protein, bound PP1 $\gamma_2$  was detected by incubating the membrane with a rabbit anti-PP1 $\gamma_2$  antibody, raised against a specific C-terminal peptide [4, 24]. Immunoreactive bands were revealed by incubating with a HRP-conjugated secondary antibody followed by ECL detection [20].

### **Immunocytochemical analysis of COS-7 cells**

COS-7 cells were grown as previously described [28] in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal calf serum (Invitrogen). After fixation with 4% paraformaldehyde, cells were permeabilized with methanol for 2 min and washed with phosphate buffered saline. Cells were incubated with the anti-SARP primary antibodies for 1 hr at room temperature, followed by FITC-conjugated anti-sheep secondary antibody. After thorough washing with phosphate buffered saline, preparations were mounted using Fluoroguard (Biorad, Portugal) and visualized in an Olympus IX-81 inverted epifluorescence microscope (Lisboa, Portugal).

### **Preparation of tissue and cell extracts**

Tissue and cultured cell lysates for assays were prepared as previously described [29]. For immunoblotting, tissues were ground under liquid nitrogen and cell cultures were harvested and heated at 100°C for 5 min in gel loading buffer containing 1% SDS or 4% lithium dodecyl sulphate before being analysed by denaturing PAGE. HeLa nuclear extract was obtained commercially from Computer Cell Culture Centre (Mons, Belgium).

### **Protein phosphatase assays**

Rabbit skeletal muscle glycogen phosphorylase was phosphorylated to a stoichiometry of 1 mol phosphate per mol subunit using phosphorylase kinase and [ $\gamma$ <sup>32</sup>P]ATP. PP1 assays were performed in the presence of 3 nM okadaic acid and either 0.5 mM Mn<sup>2+</sup> (PP1 $\gamma_1$ ) or in the absence of divalent cations (endogenous PP1) as described in [30]. Immunoabsorption and sedimentation of endogenous SARP-PP1 complexes was performed using anti-SARP(240-333) antibodies covalently coupled to Sepharose. Subsequent phosphatase assays were carried out in a shaking incubator in the presence of 2  $\mu$ M PP1-complex dissociating peptide (GKRTNLRK TGSERIAMGMRVKFNPLALLLDSC) or 2  $\mu$ M control peptide (NLRKTGSERIAMGMRVKANPLALLLDSC) as previously described [29]. Trypsin treatment of pellets prior to some assays was performed by incubating each pellet with 14U

trypsin (Sigma-Aldrich, Poole, UK) for 5 min at 30°C, followed by addition of 1 mg Soybean trypsin inhibitor (Type 1-S). One unit of phosphatase activity was the amount of enzyme that catalysed the release of 1  $\mu$ mol [ $^{32}$ P]-phosphate/min from [ $^{32}$ P]-labelled phosphorylase *a* in the assay.

### **DNA binding assays**

SARP2(331-847) protein was generated by in vitro translation (IVT) from the pET-SARP2(331-847) expression vector using the TnT-coupled transcription/translation kit (Promega) according to manufacturer's instructions. For radioactive detection of proteins, reactions were carried out in the presence of [ $^{35}$ S]-methionine. For the DNA binding assays, aliquots of labelled protein were mixed with immobilized DNA (ssDNA cellulose, Sigma) in 150  $\mu$ l Tris buffered saline (TBS) [31]. The specificity of binding was tested in the absence or presence of bacterial and human competitor DNA. After gently mixing for 3h at 4°C, the cellulose was pelleted in a microcentrifuge at 10 000 *g* for 1 min and washed three times with 500  $\mu$ l TBS before bound proteins were eluted in SDS-PAGE sample buffer. The eluted fractions were separated by electrophoresis on 10% SDS-PAGE gels that were subsequently dried and exposed to X-ray films at -70°C.

## **RESULTS**

### **Identification of a novel ankyrin repeat-containing PP1-binding subunit**

In order to identify proteins capable of interacting with PP1, PP1 $\gamma$ 1 was used as bait to screen human B-lymphocyte and human testis cDNA libraries by the yeast two hybrid approach [20, 32]. One of ten positive clones (H2) identified from the human B-lymphocyte library and one of 120 clones (40Q3) isolated from the human testis library encoded part of a novel protein, termed SARP. H2 comprised an open reading frame of 1,779nt that encoded 593 amino acids followed by a stop codon and a short 3' untranslated region, while 40Q3 encoded 782 amino acids in an open reading frame of 2,346nt, followed by a stop codon and a different 3' untranslated sequence (Supplementary Fig. 1). The H2 encoded protein sequence (SARP1) contained eight ankyrin repeats preceded by a potential PP1 binding motif (KVHF), and the 40Q3 encoded protein (SARP2) represented a splice variant differing in its C-terminal sequence (Fig. 1A). The absence of a consensus start codon (specifying an initiating methionine) indicated that both clones were incomplete. Screening of a human universal cDNA library (Stratagene) using a 300 bp fragment from the 5' end of H2 resulted

in the identification of clone 1G07 with overlapping sequence but a different 3' end. 1G07 contained an open reading frame encoding SARP3 (591 amino acids), but still no initiating methionine codon. Several PCR based cDNA cloning strategies were followed to attempt to find the 5' end of the clone, including, 5'-RACE carried out on total RNA from MCF 7 cells and the Marathon-Ready human testis cDNA system (Clontech). Although these methods technically worked and confirmed the known sequence (including the KVHF putative PP1 binding domain), none were able to add to the existing sequence information generated from the 1G07 cDNA. Consequently, a 475 bp fragment from the 5' end of this clone was sent to Genome Systems Inc. (St Louis, USA) and a genomic clone identified (BAC 23122) was sequenced 3' to 5' starting from the known sequence. Analysis of the genomic sequence combined with the cDNA sequence using the Sanger Centre FGENESH gene-finder programme predicted the exon1-intron1 structure (Supplementary Fig. 1). Exon 1 encoding the initiating methionine and five additional amino acids was located 728 base pairs 5' to the start of exon 2 that contained previously determined cDNA sequence (Supplementary Table1). The intron/exon structure for exon2-exon14 of the SARP gene was determined by comparison of the cDNA sequences with information in the National Center for Biotechnology Information human genomic sequence database (Supplementary Fig. 1 and Table 1, Genbank nos. DQ508815, EF041819, DQ508934, AP000873,). The chromosomal location for the entire SARP coding sequence was identified at chr11q14.

The amino acid sequence for SARP3 predicted from the cDNA sequence is depicted in Fig. 1A and the calculated molecular mass is 66 kDa, while those for SARP1 and SARP2 assuming the same N-terminal section are 92.5 kDa and 94.3 kDa, respectively. These molecular masses for SARP1, SARP2 and SARP3 are consistent with the sizes of endogenous SARP determined by SDS-PAGE (see below). The domain structures of the SARP proteins are depicted in Fig. 1B. Six ankyrin repeats are identified in SARP3, while SARP1 and SARP2 besides containing eight ankyrin repeats, also possess a putative Ca<sup>2+</sup>-binding EF hand and a putative leucine zipper domain. A possible nuclear localisation sequence was predicted at amino acids 14-18 in the N-terminus of SARP suggesting that the protein isoforms may be localised in the nucleus (Fig. 1A).

### **Interaction of SARP with PP1 in vitro and in vivo**

The binding of SARP1 to PP1 $\gamma$ 1 was confirmed by immunoadsorption analysis of bacterially expressed MBP-SARP1 in the presence of bacterially expressed PP1 $\gamma$ 1 using anti-PP1 $\gamma$  antibodies non-covalently coupled to Sepharose. Detection of proteins present in the

pellets and supernatants by Coomassie Blue staining showed that all the PP1 $\gamma_1$  was in the pellets and that as the MBP-SARP concentration was increased, higher levels of MBP-SARP were present in the pellets. In the absence of PP1 $\gamma_1$ , the MBP-SARP1 remained in the supernatant (lane 4, Fig2A).

PP1 $\gamma_2$  interaction with SARP in vitro was demonstrated by blot overlay. His-tagged SARP2(331-847) expression was induced in bacteria and aliquots taken at several times after induction. After SDS-PAGE analysis, samples were transferred to a nitrocellulose membrane that was incubated successively with recombinant PP1 $\gamma_2$ , rabbit anti-PP1 $\gamma_2$  antibody and HRP-conjugated anti-rabbit secondary antibody followed by ECL detection. Increased expression of His<sub>6</sub>-SARP2(331-847) with time (Fig. 2B), resulted in proportionately increased binding of PP1 $\gamma_2$ , thus demonstrating its interaction with SARP2.

Although SARP was identified by interaction with PP1 $\gamma$  isoforms, SARP was found to bind PP1 $\alpha$  and PP1 $\beta$  in tissues or cell lines where these PP1 isoforms were more abundant. Fig. 2C (left panel) shows that anti-SARP(708-832) immune complexes from rat cortex lysates contained both PP1 $\alpha$  and PP1 $\gamma_1$ , While Fig. 2C (right panel) shows that in HEK293 cells anti-PP1 $\alpha$ , antiPP1 $\beta$  and antiPP1 $\gamma_1$  immune complexes contained both SARP1/2 and SARP3.

### **Immunodetection of endogenous SARP**

Immunoblotting of lysates from rodent and human tissues with anti-SARP (708-832) antibodies identified a protein band of approximately 95 kDa, consistent with the predicted sizes of 92.5 kDa and 94.3 kDa for full length SARP1 and SARP2 (Fig. 3A and 3C). SARP1/2 was present in all mouse tissues, with appreciable levels being present in testis, liver, spleen, lung and ovary compared with the GAPDH or tubulin controls. High levels of SARP1/2 detected in testis (Fig. 3A) are consistent with the mRNA analysis (Fig. 3F). However, the high sensitivity of SARP isoforms to proteolytic degradation may lower detection in some tissues samples. SARP1/2 appeared extremely abundant in human sperm (Fig. 3E). An immunoreactive protein of similar size was present in several human tumour cell lines (Fig. 3D) and also in mammary gland carcinoma epithelial MCF7, epidermis carcinoma epithelial A431 and fibroblast MRC5 cell lines (data not shown). The highest levels of SARP3 were found in mouse brain (Fig. 3B). Lower levels of SARP3 in other tissues were often difficult to differentiate from non-specific bands (data not shown). Immuno cross-reactivity of brain SARP3 could be specifically blocked by the antigen SARP(240-708), which does not block detection of SARP1/2 (Fig. 3B). Immunocytological

localisation of SARP using two different anti-SARP antibodies showed that endogenous SARP-isoforms are highly enriched in the nucleus of COS-7 cells (Fig. 4), although SARP also appears to be present at low levels in the cytoplasm. Since the anti-SARP (708-832) antibodies recognize SARP1/2 but not SARP3, the nuclear localisation indicates that SARP1/2 are likely to possess the same (or similar) N-terminal sequence as SARP3 containing the nuclear localisation motif.

### **SARP is associated with and modulates the phosphatase activity of PP1**

In order to ascertain if SARP was associated with PP1 activity *in vivo*, anti-SARP(240-333)-antibodies coupled to Sepharose beads were mixed with HeLa cell lysates. After centrifugation, the immunoadsorbed proteins on the Sepharose were analysed for phosphatase activity using phosphorylase as substrate. A small but significant amount of phosphatase activity is found associated with SARP (Fig. 5A). The phosphatase activity increased approximately tenfold either upon mild trypsin digestion of the immune complex or on incubation with a specific PP1-complex 'dissociating' peptide (derived from the PP1 binding domain of 53BP2, containing an -RVxF- motif) prior to the assay, indicating that the -<sup>354</sup>KVHF<sup>357</sup>- motif is a major site of interaction of SARP with PP1. Pre-incubation with a control peptide, where the phenylalanine residue of the PP1 binding domain of the peptide was substituted with an alanine residue, had no stimulatory effect on the phosphatase activity. Dissociation of PP1 from its regulatory subunit by the dissociating peptide and digestion of the regulatory subunit with trypsin have been used previously to determine the effects of glycogen and other regulatory subunits on PP1 [25, 29]. The results described here indicate that the phosphorylase phosphatase activity in the anti-SARP(240-333)-immune complex is due to PP1 and that the PP1 in the immune complex is inhibited by SARP towards the assay substrate phosphorylase *a*. To examine whether the -<sup>354</sup>KVHF<sup>357</sup>- motif was solely responsible for maintaining binding to SARP with PP1 we expressed GST-SARP1 and GST-SARP1(F357A), in which the phenylalanine of the <sup>354</sup>KVHF<sup>357</sup>- motif was mutated to alanine, in HEK293 cells. However, mutation of the Phe357 was insufficient to disrupt the interaction of SARP1 with PP1. Furthermore, addition of the 'dissociating' peptide, although clearly modulating the activity of endogenous SARP-PP1 complexes (Fig. 5A), did not disrupt the binding of PP1 to GST-SARP1 or GST-SARP1(F357A) indicating that there is more than one motif that maintains SARP1 interaction with PP1 (data not shown).

Immunoprecipitation of SARP complexes was also carried out from a HeLa nuclear extract using anti-SARP(240-333)-antibody coupled Sepharose beads and the phosphorylase

phosphatase activity measured after mild trypsin digestion (Fig. 5B). The trypsin revealed phosphorylase phosphatase activity of this nuclear fraction was found to be  $\sim 0.2$  mU/mg lysate protein, approximately twice that observed in the whole HeLa cell lysate, consistent with enrichment of SARP in the nucleus.

A range of rat tissues were examined for anti-SARP(240-333) immunoadsorbed phosphatase activity that could be revealed by subsequent trypsin treatment (Fig. 5C). The highest levels of SARP associated phosphorylase phosphatase activity are found in rat testis while the brain contains the next highest levels. These studies are consistent with the immunoblotting, which indicated that high levels of the SARP protein are present in testis.

### **SARP is highly abundant in the nucleus and binds to mammalian DNA**

The presence of a nuclear localisation signal in the SARP sequences and the observed enrichment of SARP in the nucleus suggest that SARP functions within the nucleus. The identification of a leucine zipper domain near the C-terminus of SARP1/2 (Fig. 1A), a domain found in many transcription factors, raised the question of whether SARP1/2 is a transcription factor or cofactor. We therefore examined whether SARP1/2 was able to bind to DNA. Fig. 6 shows [ $^{35}$ S]-labelled SARP2(331-847) synthesized by in vitro transcription/translation binds to mammalian single stranded(ss)-DNA immobilized on cellulose in the presence of *E. coli* competitor DNA but not in the presence of human competitor DNA. These studies indicate that SARP2(331-847) binds specifically to mammalian DNA.

## **DISCUSSION**

PP1 participates in many distinct cellular processes by virtue of interaction with numerous regulatory subunits. We describe here a novel protein in which a PP1 binding motif precedes an ankyrin repeat domain. At least three isoforms have been identified, which are derived by alternative splicing from a single gene on chromosome 11 of at least 65 kb and 14 exons. One isoform has six ankyrin repeats, while the other two possess eight ankyrin repeats and therefore we have termed the protein several ankyrin repeat protein (SARP). Binding of SARP to PP1 was demonstrated by a number of techniques. Not only was SARP identified in two different yeast two hybrid screens using PP1 $\gamma$ 1 as bait, but bacterially expressed SARP was found to bind bacterially expressed PP1 $\gamma$ 1 and PP1 $\gamma$ 2, by immunoadsorption and blot

overlay experiments respectively and endogenous PP1 $\gamma$ 2 was found in complex with SARP in human sperm. In addition, endogenous SARP was also found associated with PP1 $\alpha$  and PP1 $\beta$  in immune complexes. A putative PP1 binding motif <sup>354</sup>KVHF<sup>357</sup> was identified in SARP by sequence comparison with the consensus PP1 interaction motif  $-(R/K)X_{A(0-1)}(V/I)X_B(F/W)-$ . The interaction of SARP with PP1 via this canonical motif was supported by modulation of SARP-PP1 activity with a peptide covering this region, the PP1 activity increase being virtually identical to that obtained by trypsin digestion. However, neither the mutation of SARP357 Phe to Ala within the <sup>354</sup>KVHF<sup>357</sup> motif, nor the addition of a peptide covering this region, resulted in the complete disruption of the binding of SARP to PP1, indicating the existence of one or more further SARP interaction sites with PP1. Other PP1-regulator complexes with more than one interaction site have been reported previously [1, 15].

Although most regulatory subunits of PP1 share little or no obvious sequence similarities unless they are isoforms, the coupling of a canonical PP1 binding motif and an ankyrin repeat domain in the manner found in SARP is observed in three other known PP1 associated proteins: the p53 binding protein 53BP2 [32] and the TGF- $\beta$  inhibited, membrane associated protein TIMAP, which also binds to a laminin receptor [33, 34], both with 4 ankyrin repeats, and the myosin binding protein MYPT1/M<sub>110</sub>/MBS with 7 ankyrin repeats [35, 36] (Fig. 7A). The PP1 binding motif immediately precedes the start of the first ankyrin repeat in 53BP2, TIMAP and MYPT1, but in SARP the <sup>354</sup>KVHF<sup>357</sup> PP1 binding motif lies partially within the first ankyrin repeat. Thus the precise location of the PP1 interaction with respect to the ankyrin repeat appears to be flexible, suggesting that the ankyrin repeat domain folds favourably to present an exposed RVXF motif that can initiate the binding to PP1, whether it lies immediately preceding or at the start of the first ankyrin repeat. Secondary interactions similar to those observed in PP1-MYPT1 may stabilise the complex [37-39]. The crystal structure of PP1 bound to the N-terminal domain of MYPT1 shows that ankyrin repeat domains possess many secondary interactions with PP1 [40]. Since the ankyrin repeats are degenerate sequences and there is considerable variation between those in SARP and MYPT1, TIMAP and 53BP2, there exists a real potential for differential binding and/or modulation of secondary interactions. In addition, localisation of the canonical PP1 binding motif partially within the first ankyrin repeat of SARP may aid stabilisation of the SARP-PP1 complex and perhaps allow an ankyrin repeat interaction with PP1 that is stable to disruption of the canonical PP1 binding site.

SARP is widely distributed in different tissues and PP1 activity was observed in endogenous SARP immunocomplexes from a variety of tissues. When phosphorylase was used as substrate, SARP inhibited the bound PP1 catalytic activity, but one might expect PP1 to be less inhibited or even activated towards an *in vivo* substrate of the PP1-SARP complex, as observed with PP1-Nek2 [25]. Overall, the highest levels of SARP mRNA, protein and PP1 activity bound to SARP were observed in testis. Previous studies showed that mice homozygous for a targeted mutation of the PP1 $\gamma$  gene, which disrupts expression of both PP1 $\gamma$ 1 and PP1 $\gamma$ 2 isoforms, exhibit a defect in spermiogenesis but are otherwise healthy, suggesting that the function of PP1 $\gamma$  in testis cannot be substituted by other PP1 isoforms, but the functions of PP1 $\gamma$ 1 present in all other tissues can be compensated for by PP1 $\alpha$  [41]. Therefore one would not necessarily expect the function(s) of SARP, which interacts with PP1 $\gamma$ 1, PP1 $\gamma$ 2, PP1 $\alpha$  and PP1 $\beta$ , to be compromised in these PP1 $\gamma$  null mice, unless the interaction of SARP1/2 with PP1 $\gamma$ 2 serves a unique function in testis.

Endogenous SARP appears to be highly enriched in the nucleus, consistent with the putative nuclear localisation signal at the N-terminus. A variety of PP1 binding proteins with predominantly nuclear localisation and/or nuclear functions have been identified [1, 17, 42, 43]. The demonstration here that SARP2 specifically binds to mammalian DNA suggests a role for SARP2, and therefore probably also SARP1, in the regulation of transcription. Several studies have indicated that PP1 may interact with proteins that modulate transcription. PP1 interacts with the homeodomain transcription factor Hox11 [44], host cell factor HCF [45], the retinoblastoma protein p10Rb, which regulates the transcription factor E2F [46, 47] and 53BP2, which associates with p53 transcription factor [32, 48].

SARP1 and SARP2 possess a putative leucine zipper motif near the C-terminus suggesting that they may function as transcription factors or cofactors. An alignment of the SARP1/2 C-terminal region with the leucine zipper regions of representative members of transcription factor families is shown (Fig. 7B). However, leucine zipper domains may also serve as protein-protein interaction domains and the SARP1/2 C-terminus is also related to the KE2 family of leucine zipper proteins, which encompass proteins involved in tubulin folding [49]. Nevertheless, the specific binding of SARP2 to mammalian DNA supports a role for SARP1/2 as transcription factors or cofactors and suggests that PP1 may participate in the regulation of transcription in a wide variety of tissues via SARP1/2. The shorter isoform SARP3, which may be particularly important in brain where it is most abundant, terminates before the leucine zipper motif and therefore may perform a different function from SARP1 and 2.

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## FIGURE LEGENDS

### Fig. 1 SARP protein isoforms

- A** Comparison of the SARP1, SARP2 and SARP3 amino acid sequences derived from the three cDNAs (DQ508815, EF041819, DQ508934). The six N-terminal amino acids of SARP3, not encoded in cDNA but predicted from the gene sequence, are shown in italics. The putative nuclear localisation signal (amino acids 14-18) is underlined. The molecular masses of SARP1 and SARP2 on SDS-PAGE are consistent with these isoforms possessing the same N-terminal sequence as SARP3. The PP1 binding motif is underlined with asterisks. The eight ankyrin repeats (ANK1-ANK8) are indicated by a single line above the sequences. The dotted line above the sequences indicates a putative EF hand in SARP1 and SARP2. A double underline indicates a putative leucine zipper domain present in SARP1 and SARP2. Note that only one clone of SARP3 was sequenced and the encoded PP1 binding motif was KMHF. Since PCR of this region in several libraries (including a human brain library) yielded only sequence coding for KVHF, we presume that a mutation may have arisen during the preparation or cloning of SARP3 cDNA. Methionine has never been reported or predicted at position 2 [11, 12].
- B** Schematic comparison of the protein domains structures of SARP1, SARP2 and SARP3.

### Fig. 2 In vitro interaction of SARP with PP1 $\gamma_1$ and PP1 $\gamma_2$ .

- A** Co-immunoadsorption of bacterially expressed MBP-SARP1 with bacterially expressed PP1 $\gamma_1$  using anti-PP1 $\gamma_1$  antibodies bound to Sepharose. The quantities of SARP1 and PP1 $\gamma_1$  in each immune reaction are indicated. Washed anti-PP1 $\gamma_1$  immune pellets (upper panel) and the supernatants after removal of the immune pellets (lower panel) were subjected to SDS-PAGE. Protein bands were detected with Coomassie Blue. Molecular size markers in kDa are indicated.
- B** Overlay of bacterially expressed His-tagged SARP2(331-847) with recombinant PP1 $\gamma_2$ . Bacterial cultures were collected at different times after induction of recombinant protein expression and equal amounts of protein were separated by SDS-PAGE and immunoblotted. Interaction was demonstrated as described in the methods section. Lane 1, control; Lane 2, 1h induced expression; Lane 3, 2h induced expression; Lane 4, 3h induced expression.
- C** Co-immunoadsorption of PP1 $\alpha$  and PP1 $\gamma_1$  from lysates of rat brain cortex with anti-SARP(708-832) antibodies coupled to protein G-Sepharose (left panel). Co-immunoadsorption of SARP from HEK293 cell lysates with anti-PP1 $\alpha$ , anti-PP1 $\beta$  and anti-PP1 $\gamma_1$  antibodies coupled to protein G-Sepharose (right panel).

### Fig. 3 Detection of SARP in cell lines and tissue lysates.

- A** SDS-PAGE separation and immunoblot analysis of SARP1/2 in mouse tissues using anti-SARP(708-832) antibody. In order to prevent proteolytic degradation ground tissue samples were heated directly at 100<sup>o</sup>C in gel sample buffer and equal volumes were loaded on the gel. The levels of SARP are therefore presented relative to the levels mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same samples.
- B** Lysates (20  $\mu$ g protein) from mouse brain were separated by SDS-PAGE and analysed by immunoblotting using anti-SARP(240-832) antibody in the absence (-) and presence (+) of SARP (240-708) protein.

- C** Immunoblot analysis of SARP1/2 in human testis and rat tissues (100  $\mu$ g) using anti-SARP(708-832) antibody.  $\beta$ -tubulin was used as a control.
- D** Lysates (20  $\mu$ g protein) from tumour cell lines were analysed by SDS-PAGE and immunoblotting using anti-SARP(708-832) antibody. HeLa, human cervical carcinoma cell line; HEK293, human embryonic kidney cell line; SHSY5Y, human neuroblastoma SHSY5Y epithelial cell line, COS-7, monkey kidney COS-7 fibroblast cell line. Human GAPDH was employed as a control.
- E** SDS-PAGE separation and immunoblot analysis of SARP1/2 in human testis and sperm (50  $\mu$ g). Molecular size markers in kDa are indicated in panels A-E
- F** Northern blot analysis of SARP mRNA from rat tissues. A single mRNA of approximately 3.6 kb was particularly abundant in rat testis. Actin mRNA was employed as a control.

**Fig. 4 Immunocytochemical localisation of endogenous SARP in COS-7 cells.**

COS-7 cells were cultured as described in the methods section and fixed in paraformaldehyde. The left panels show the phase contrast image of same cells analysed for endogenous SARP on the right. For the detection of SARP, anti-SARP(240-708) and anti-SARP(708-832) antibodies were used, followed by a fluorescein isothiocyanate-conjugated secondary antibody. Microscopic visualization and image acquisition were performed using a F-View digital monochromatic camera (Soft Imaging Systems, USA) coupled to an Olympus IX-81 motorized inverted microscope (Olympus, Portugal). All images are at 60X magnification.

**Fig. 5 Analysis of SARP-PP1 phosphatase activity.**

- A** Protein phosphatase activity of endogenous SARP-PP1 complexes in HeLa cell lysates. Anti-SARP(240-333)-antibody coupled to protein-G-Sepharose beads was mixed with HeLa cell lysates. Immune pellets were assayed for phosphorylase phosphatase activity either in the presence or absence of trypsin or in the presence of PP1-complex dissociating peptide or control peptide. The immunopelleted activity is expressed as mU per mg of total protein in the HeLa cell lysate. Error bars indicate the standard deviation of the mean of triplicate immuno adsorptions. Assays: (1) pre-immune IgG pellet + trypsin treatment, (2) anti-SARP(240-333)-antibody pellet, (3) anti-SARP(240-333)-antibody pellet + control peptide, (4) anti-SARP(240-333)-antibody pellet + dissociating peptide, (5) anti-SARP(240-333)-antibody pellet + trypsin treatment.
- B** Protein phosphatase activity of endogenous SARP-PP1 complexes in HeLa cell nuclei. Anti-SARP(240-333)-antibody coupled to protein-G-Sepharose beads was mixed with HeLa cell nuclear lysates. Immune pellets were assayed for phosphorylase phosphatase activity either in the presence (+) or absence (-) of trypsin.
- C** Endogenous trypsin-revealed SARP-PP1 phosphatase activity in different rat tissues as indicated. Anti-SARP(240-333)-antibody immune pellets were prepared from rat tissue lysates and assayed for trypsin-revealed phosphorylase phosphatase activity. The activity is expressed as mU/mg lysate protein. Error bars indicate the standard deviation of the mean of triplicate immunoprecipitations. The assays were carried out in the presence of 2 nM okadaic acid.

**Fig. 6 Analysis of SARP DNA binding**

SARP DNA binding assay. (A) Lane 1, 1  $\mu$ l in vitro translated (IVT) SARP2(331-847); Lanes 2-4, DNA binding assay with 1 mg ssDNA-cellulose with increasing amounts of IVT SARP2(331-847): 1  $\mu$ l (lane 2), 3  $\mu$ l (lane 3) or 5  $\mu$ l (lane 4). (B) DNA binding assay using competitor DNA. Lane 1, IVT negative control; lane 2, 1  $\mu$ l IVT SARP2(331-847); Lanes 3-7, DNA binding assay with 1 mg ssDNA-cellulose plus 3  $\mu$ l of SARP2(331-847) in the absence (lane 3) or presence of 10-fold or 100-fold excess of competitor bacterial DNA (lanes 4 and 5) and human placental DNA (lanes 6 and 7).

**Fig. 7 Sequence comparison of the ankyrin repeat and leucine zipper motifs of SARP to other family members.**

- A Alignment of the first four ankyrin repeats of human SARP with those present in other human PP1 binding proteins (53BP2, MYPT1 and TIMAP). Only the three contiguous ankyrin repeats of TIMAP are shown. Note that the PP1 binding motif of SARP (indicated in bold) lies partially within the first ankyrin repeat, while the PP1 binding motif of 53BP2, MYPT1 and TIMAP (indicated in bold) precede the start of the first ankyrin repeat. Isoforms of these proteins have been identified; ASPP2 is an isoform of 53BP2 [50], MYPT2 an isoform of MYPT1, while MYPT3 is probably an isoform of TIMAP [33].
- B Alignment of the SARP C-terminal putative leucine zipper domain with the leucine zipper domains of representative human members of different families: prefoldin-6 (PFD) in the KE2 leucine zipper family, neural retina leucine zipper (NRL) in the bZIP Maf transcription factor family, JUN-B in the bZIP1 and hepatic leukemia factor (HLF) in the bZIP2 transcription factor families and the MYC transcription factor.

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SARP1 -----  
SARP2 -----  
SARP3 1 **MPLEGSTSEVYSFPRRRPCLSSQVAGVGRLERDSEKVPAEGGRYGDSQGVATEDGGRR**

SARP1 -----  
SARP2 66 -----**WRINGLHGHS GAESRERVSDDGERKCEESDRRSRVAAGSL SADAGFSRSDLRRVS**  
SARP3 61 **TSHHVWRINGLHGHS GAESRERVSDDGERKCEESDRRSRVAAGSL SADAGFSRSDLRRVS**

SARP1 -----  
SARP2 121 **ENLGHCRSVSRERVRGDSGHS SHSCSWERVRGDSTFCSSDVNFSEVGGHQTTDSRGTARE**  
SARP3 121 **ENLGHCRSVSRERVRGDSGHS SHSCSWERVRGDSTFCSSDVNFSEVGGHQTTDSRGTARE**

SARP1 240 -----**D**  
SARP2 181 **RGLRLSGPWEGVSDIRDPRTSDFGDRVSDDRSRFFSGSWEEGGSVEGGH SVGSSWEEVSGD**  
SARP3 181 **RGLRLSGPWEGVSDIRDPRTSDFGDRVSDDRSRFFSGSWEEGGSVEGGH SVGSSWEEVSGD**

SARP1 241 **RGYAASDSSGVSGSEDASYRFSGFWERESEDEGFRC SFWERAREDLGPRPSDDGEEGR**  
SARP2 241 **RGYAASDSSGVSGSEDASYRFSGFWERESEDEGFRC SFWERAREDLGPRPSDDGEEGR**  
SARP3 241 **RGYAASDSSGVSGSEDASYRFSGFWERESEDEGFRC SFWERAREDLGPRPSDDGEEGR**

**ANK1**

SARP1 301 **CSGSWVRASEDRRSIRGLDSTPPQSRRCAMPGVANSGPSTSSRETANPCSRKKVHFGSI**  
SARP2 301 **CSGSWVRASEDRRSIRGLDSTPPQSRRCAMPGVANSGPSTSSRETANPCSRKKVHFGSI**  
SARP3 301 **CSGSWVRASEDRRSIRGLDSTPPQSRRCAMPGVANSGPSTSSRETANPCSRKKVHFGSI**

**ANK2** \*\*\*\*

SARP1 361 **HDAVRAGDVKQLSEIVVRGASINELDVLHKFTPLHWAHSGSLECLHWLLWHGADI THVT**  
SARP2 361 **HDAVRAGDVKQLSEIVVRGASINELDVLHKFTPLHWAHSGSLECLHWLLWHGADI THVT**  
SARP3 361 **HDAVRAGDVKQLSEIVVRGASINELDVLHKFTPLHWAHSGSLECLHWLLWHGADI THVT**

**ANK3** **ANK4**

SARP1 421 **TRGWTASHIAAIRGQDACVQALIMNGANLTAQDDRGCTPLHLAATHGHSFTLQIMLRSGV**  
SARP2 421 **TRGWTASHIAAIRGQDACVQALIMNGANLTAQDDRGCTPLHLAATHGHSFTLQIMLRSGV**  
SARP3 421 **TRGWTASHIAAIRGQDACVQALIMNGANLTAQDDRGCTPLHLAATHGHSFTLQIMLRSGV**

**ANK5** **ANK6**

SARP1 481 **DPSVTDKREWRPVHYAAFHGRLGCLQLLVKWCSTIEDVDYNGNLPVHLAAMEGHLHCFKF**  
SARP2 480 **DPSVTDKREWRPVHYAAFHGRLGCLQLLVKWCSTIEDVDYNGNLPVHLAAMEGHLHCFKF**  
SARP3 481 **DPSVTDKREWRPVHYAAFHGRLGCLQLLVKWCSTIEDVDYNGNLPVHLAAMEGHLHCFKF**

**ANK7**

SARP1 541 **LVSRMSSATQVLKAFNDNGENVLDLAQRFFKQNILQFIQGAIEYEGKDLEDQETLAFPGHV**  
SARP2 540 **LVSRMSSATQVLKAFNDNGENVLDLAQRFFKQNILQFIQGAIEYEGKDLEDQETLAFPGHV**  
SARP3 541 **LVSRMSSATQVLKAFNDNGENVLDLAQRFFKQNILQFIQGS PRPATDLQGETGATELAPR**

**ANK8**

SARP1 601 **AAFKGDLGMLKKLVEDGVININERADNGSTPMHKAAGQGHIECLQWLIKMGADSNITNKA**  
SARP2 600 **AAFKGDLGMLKKLVEDGVININERADNGSTPMHKAAGQGHIECLQWLIKMGADSNITNKA**  
SARP3 601 **ES**

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SARP1 661 **GERPSDVAKRFAHLAAVKLLEELQKYDIDDENEIDENDVKYFIRHGVEGSTDAKDDLCLS**  
SARP2 660 **GERPSDVAKRFAHLAAVKLLEELQKYDIDDENEIDENDVKYFIRHGVEGSTDAKDDLCLS**

SARP1 721 **DLDKTDARMRAYKKIVELRHILLEIAESNYKHLGGITEEDLKQKKEOLESEKTIKELQGO**  
SARP2 720 **DLDKTDARMRAYKKIVELRHILLEIAESNYKHLGGITEEDLKQKKEOLESEKTIKELQGO**

SARP1 781 **EYERLRREKLECOLDEYRAEVDQLRETLEKIQVPNFVAMTALLVSQTKRRGE**  
SARP2 780 **EYERLRREKLECOLDEYRAEVDQLRETLEKIQVPNFVAMEDSASCESNKEKRRVKKKVSS**

SARP2 840 GGVFVRRY

Figure 1A

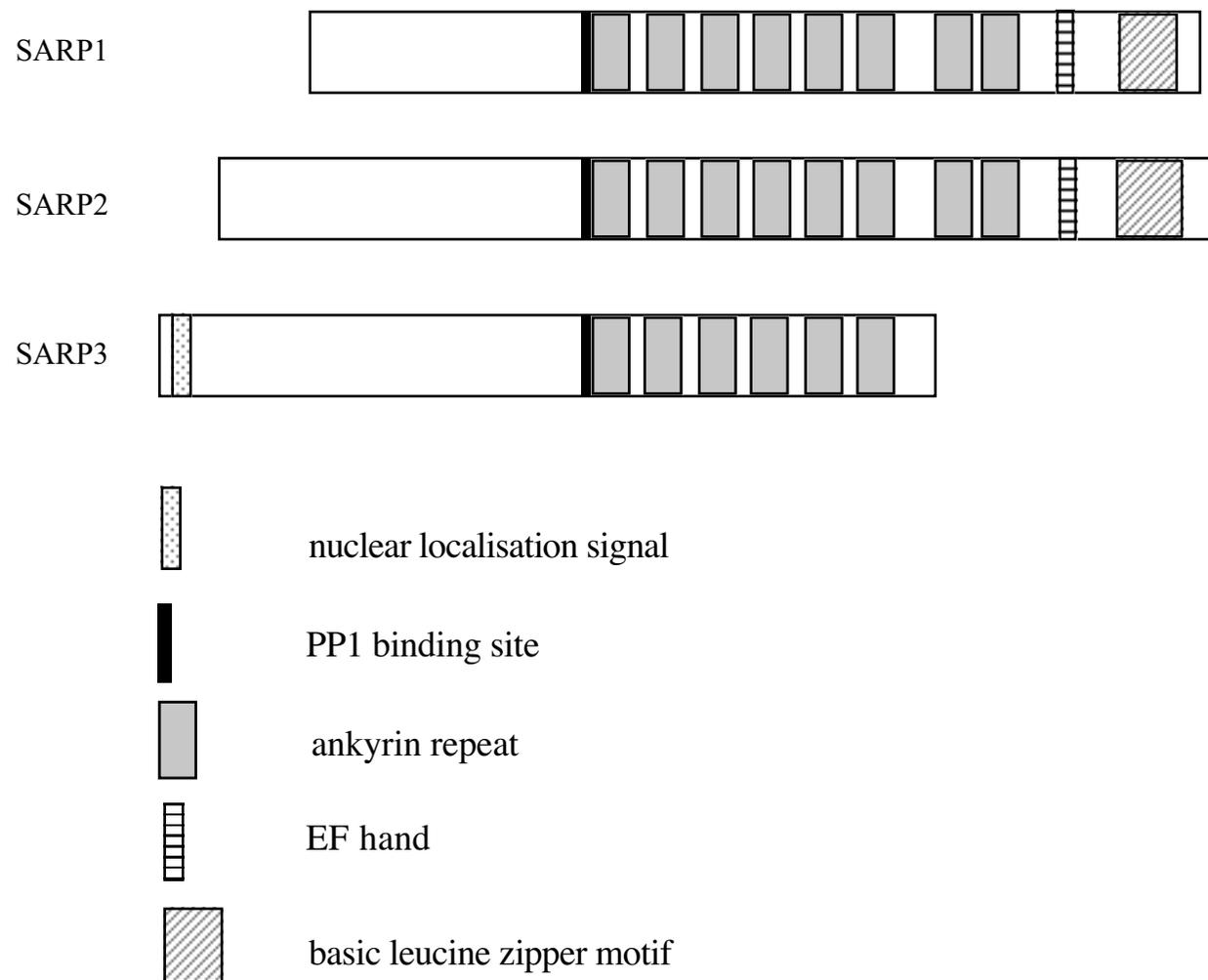
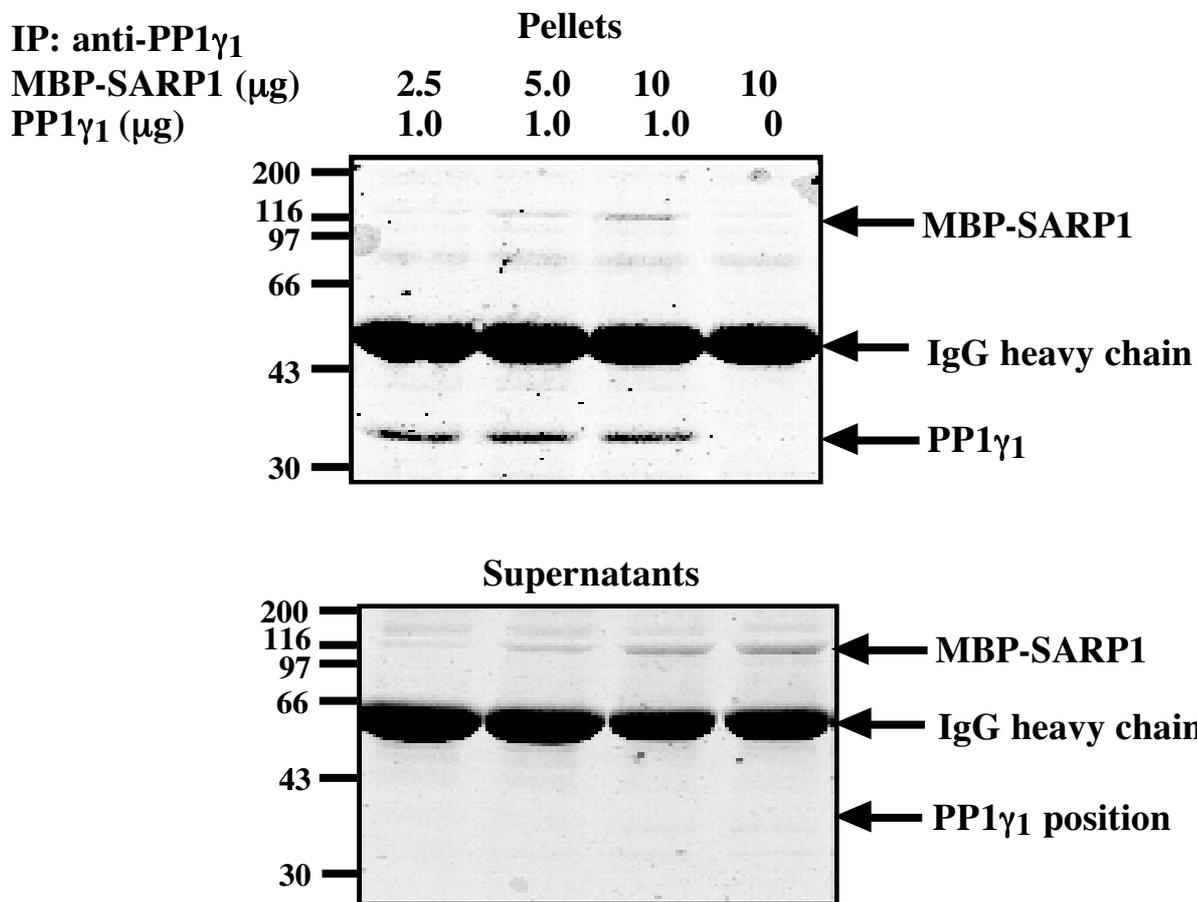


Figure 1B

**A**



**B**

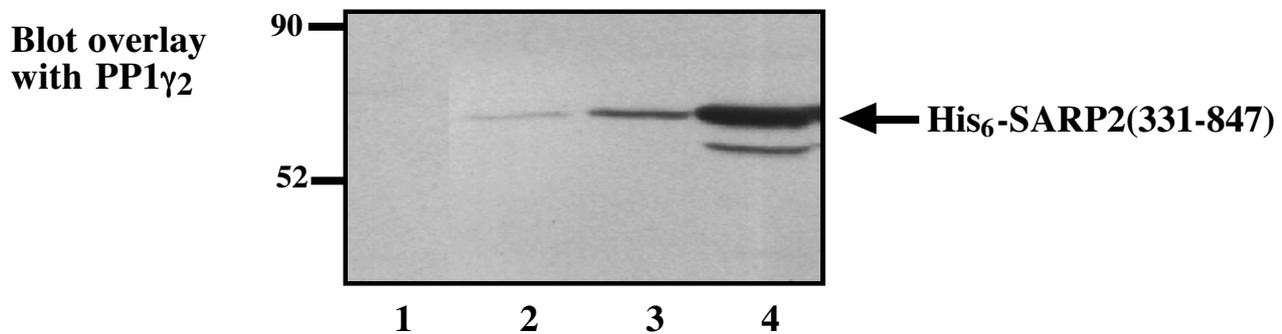


Figure 2A+B

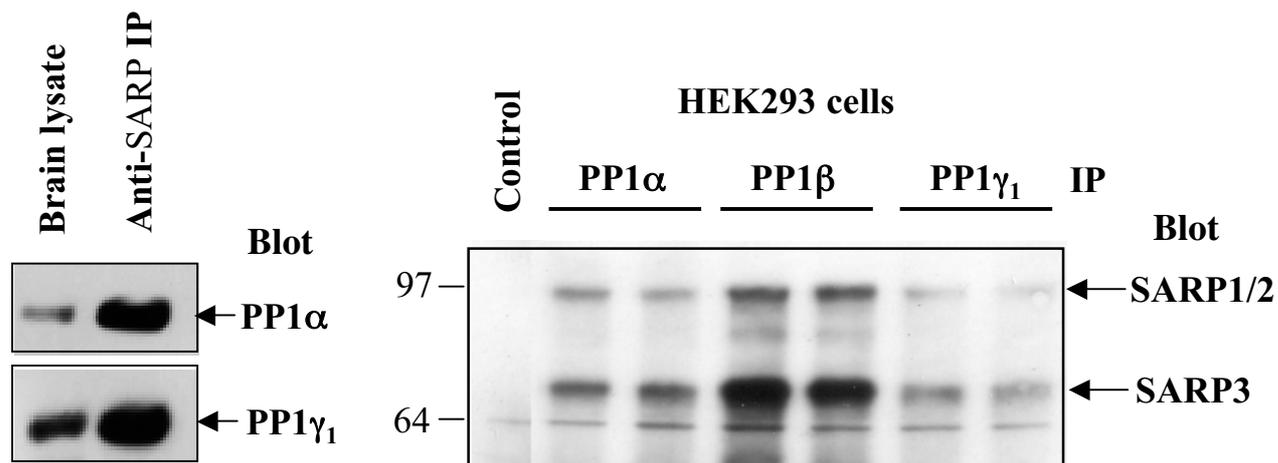


Figure 2C

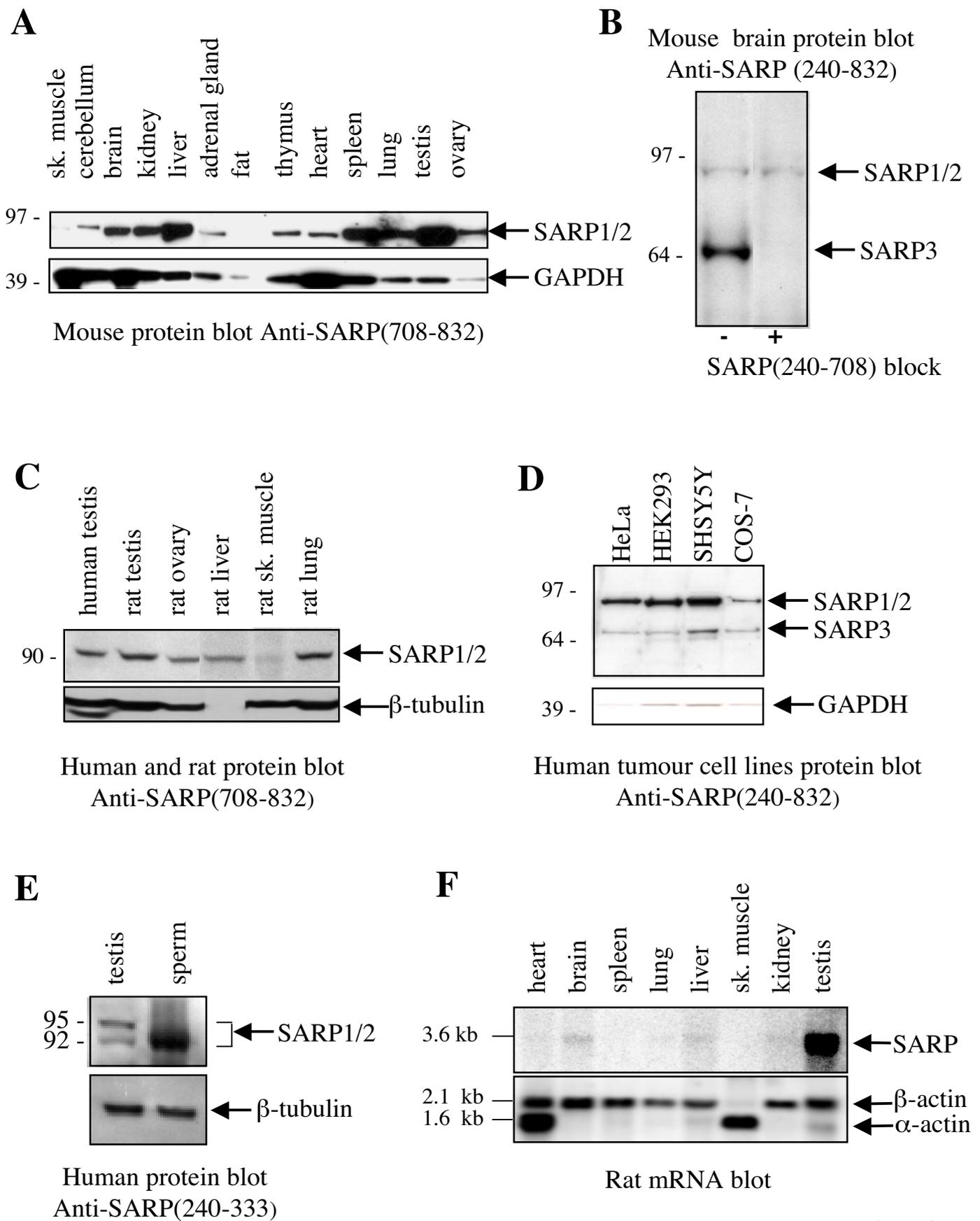


Figure 3

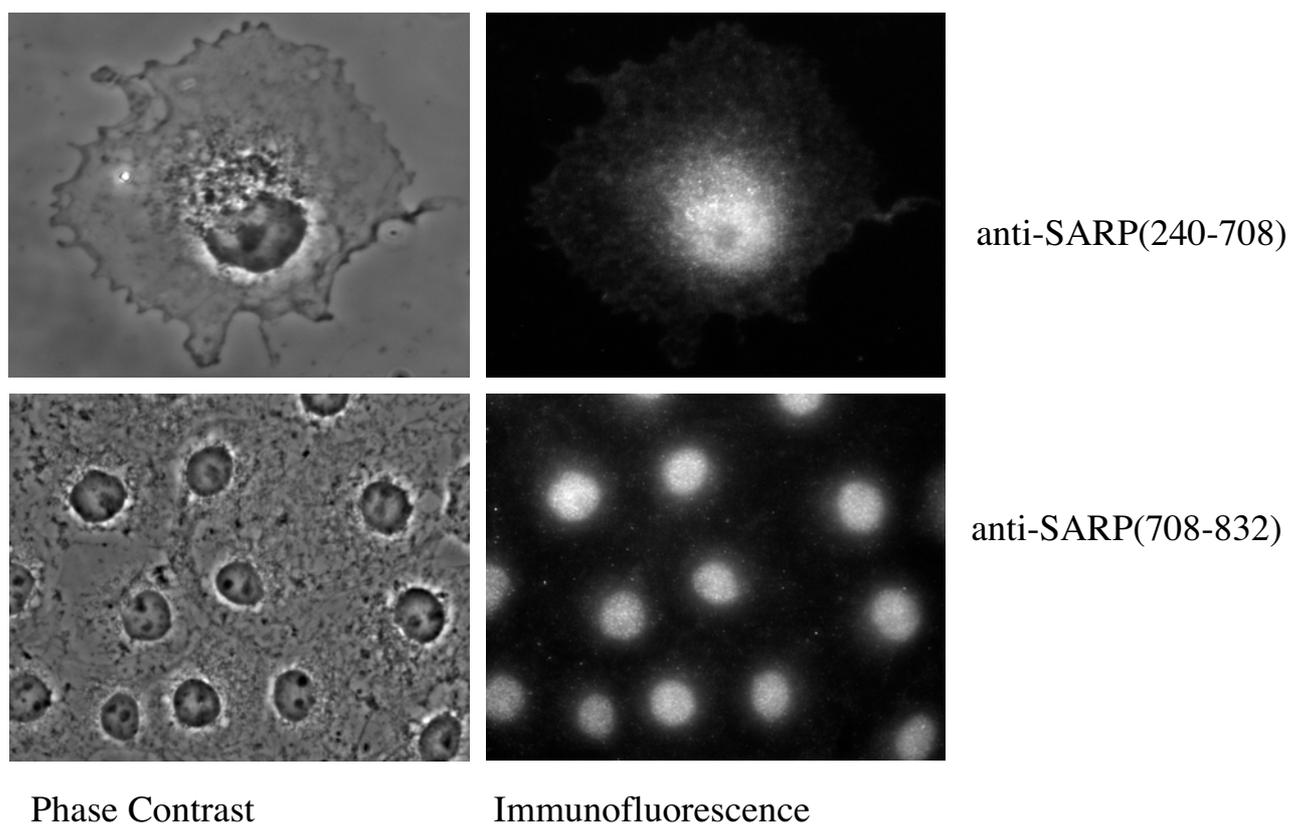
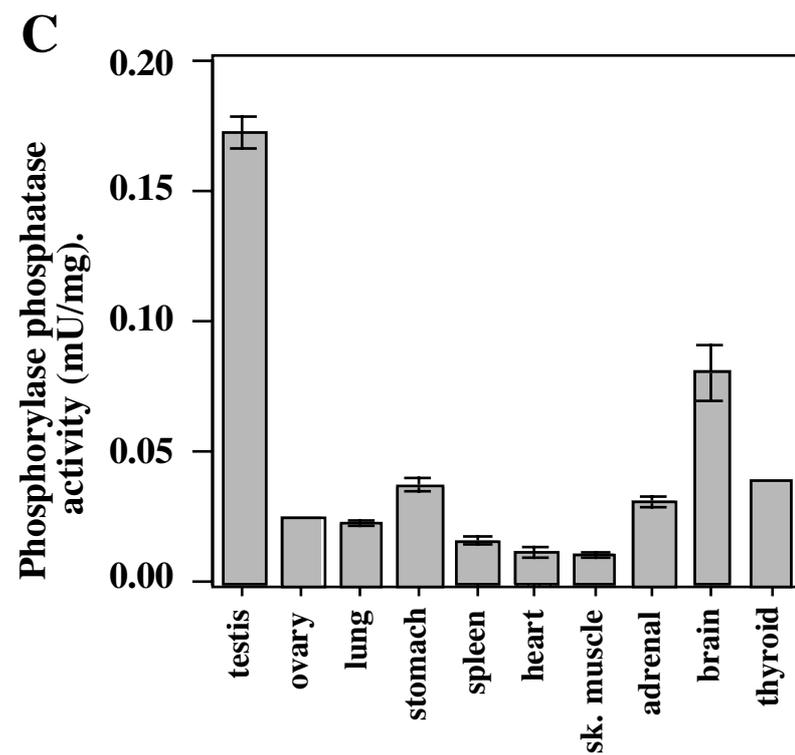
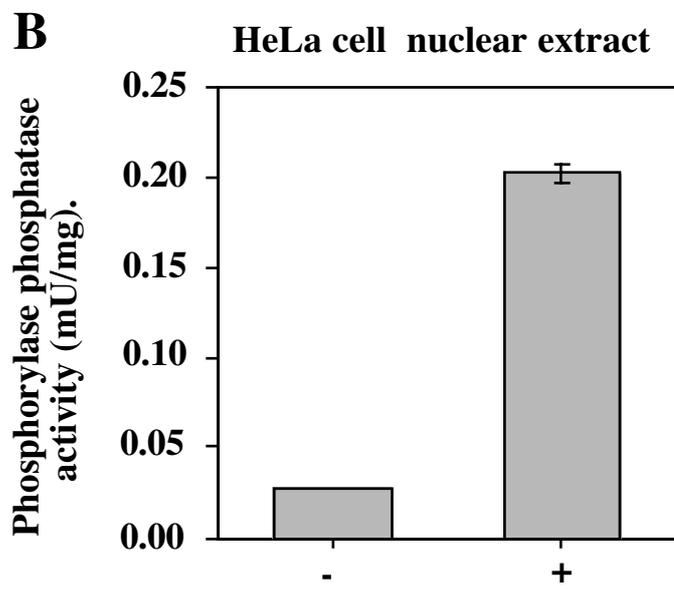
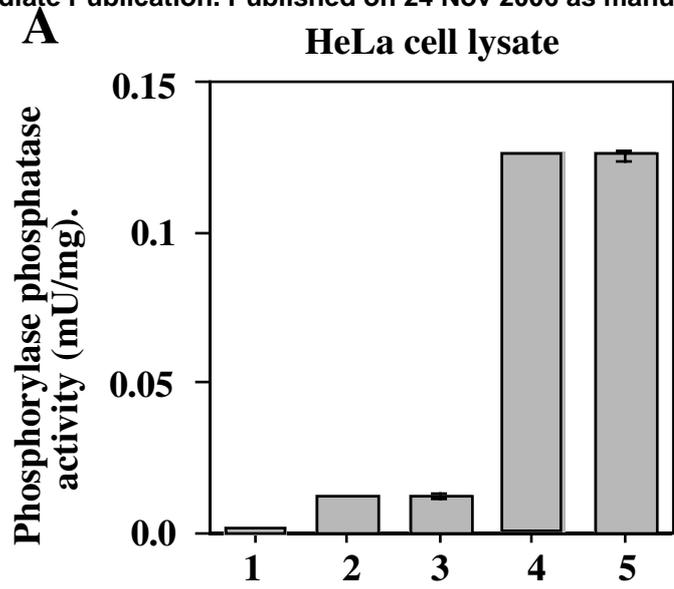


Figure 4



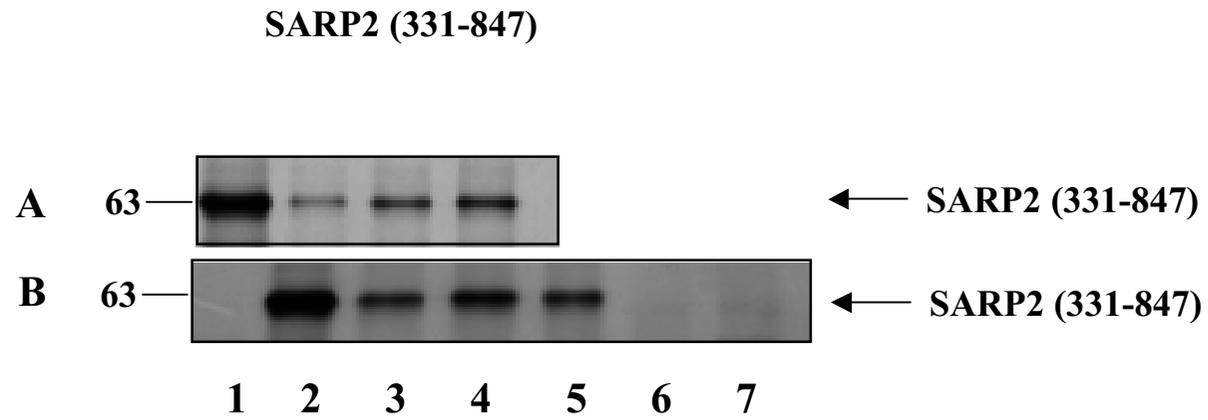


Figure 6

## A

SARP	348		NPCSR <b>KK</b>	354
53BP2	795		HGM <b>RVKF</b>	801
MYPT1	30		QKT <b>KVKF</b>	38
TIMAP	60		RRK <b>KVSF</b>	66
SARP	355	<b>VHFG</b> SIHDAVRAGDVKQLSEIVVRGASINE	LDVL	388
53BP2	802	NPLALLLDSSLEGEFDLVQRIIYEVDPSL	PND	834
MYPT1	39	DDGAVFLAACSSGDTDEVLKLLHRGADINY	ANV	71
TIMAP	67	EASVALLEASLRNDAEEVRYFLKNKVSPDL	CNE	99
SARP	389	HKFTPLHWAHSGSLECLHWLLWHGADITH	VTT	421
53BP2	835	EGITALHNAVCAGHTEIVKFLVQFGVNVNA	ADS	867
MYPT1	72	DGLTALHQACIDDNVDMVKFLVENGANINQ	PDN	104
TIMAP	100	DGLTALHQCCIDNFEEIVKLLLSHGANVNA	KDN	132
SARP	422	RDWTASHIAAIRGQDACVQALIMNGANLTA	QDD	454
53BP2	868	DGWTPLHCAASCNNVQVCKFLVESGAAVFA	MTY	900
MYPT1	105	EGWIPLHAAAASCGYLDIAEFLIGQGAHVGA	VNS	137
TIMAP	133	ELWTPLHAAAATCGHINLVKILVQYGADLLA	VNS	165
SARP	455	RGCTPLHLAATHGHSFTLQIMLR-SGVDPS		483
53BP2	901	SDMQTAADKCEEMEEGYTQCSQFLYGVQEK		930
MYPT1	138	EGDTPLDIAEEEEAMEELLQNEVNRQGV DIE		167

## B

SARP1/2	780	LEYERLRREK <b>LE</b> COLDE <b>Y</b> RAEVDQ <b>L</b> RETLEK <b>I</b> QVPNFV <b>A</b>	818
KE2 (PFD)	66	KQELGEARAT <b>V</b> GKRLDY <b>I</b> TAEIKR <b>Y</b> ESQLRD <b>L</b> ERQSE <b>Q</b>	104
bZIP Maf (NRL)	177	RSKRLQQR <b>R</b> GLEAERAR <b>L</b> AAQLD <b>L</b> RAEVAR <b>L</b> ARERD <b>L</b>	215
bZIP1 (JUN-B)	286	RKRKLERIAR <b>L</b> EDKV <b>K</b> T <b>L</b> KAENAG <b>L</b> SSTAG <b>L</b> LREQVA <b>Q</b> <b>L</b>	324
bZIP2 (HLF)	244	RDARRLKEN <b>Q</b> I <b>A</b> IRAS <b>F</b> LEKENS <b>L</b> RQEVAD <b>L</b> RKLGK <b>C</b> K	282
MYC	403	ILSVQAE <b>E</b> Q <b>K</b> L <b>I</b> SEED <b>L</b> LKR <b>R</b> EQ <b>L</b> KHKLE <b>Q</b> L <b>R</b> NS <b>C</b> A	439

Figure 7