

Nuclear organisation of NIPP1, a regulatory subunit of protein phosphatase 1 that associates with pre-mRNA splicing factors

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SUMMARY

Protein phosphatase-1 (PP1) is complexed to many proteins that target it to particular subcellular locations and regulate its activity. Here, we show that ‘nuclear inhibitor of PP1’ (NIPP1), a major nuclear PP1-binding protein, shows a speckled nucleoplasmic distribution where it is colocalised with pre-mRNA splicing factors. One of these factors (Sm) is also shown to be complexed to NIPP1 in nuclear extracts. Immunodepletion of NIPP1 from nuclear

extracts, or addition of a ‘dominant negative’ mutant lacking a functional PP1 binding site, greatly reduces pre-mRNA splicing activity in vitro. These findings implicate the NIPP1-PP1 complex in the control of pre-mRNA splicing.

Key words: Protein phosphatase, Splicing, Immunolocalisation, Targeting subunit, NIPP1

INTRODUCTION

Reversible protein phosphorylation catalysed by protein kinases and protein phosphatases regulates almost all aspects of cell life. Protein kinases and phosphatases frequently phosphorylate many proteins in vitro, raising the question of how they find their specific targets in vivo. In recent years evidence has been accumulating that these enzymes do not find their substrates by simple diffusion but are directed to them by specific ‘targeting’ subunits (Hubbard and Cohen, 1993). These accessory proteins bind to the catalytic subunit of the kinase or phosphatase, and determine its subcellular localisation and ability to be regulated by extracellular signals.

The paradigm for this phenomenon is protein phosphatase 1 (PP1), one of the major serine/threonine-specific protein phosphatases of eukaryotic cells (Hubbard and Cohen, 1993). PP1 interacts with a variety of targeting subunits, of which the best characterised are the cytosolic proteins that direct PP1 to glycogen (Stralfors et al., 1985) and to myosin (Alessi et al., 1992). Structural analysis of PP1 co-crystallized with a small PP1-binding peptide from the muscle glycogen-targeting subunit revealed that a hexapeptide sequence present in the latter (Arg-Arg-Val-Ser-Phe-Ala) interacts with a small hydrophobic channel on the surface of PP1 (Egloff et al., 1997). Similar sequences are present in many other PP1-targeting subunits, and synthetic peptides containing this motif disrupt their interaction with PP1 (Egloff et al., 1997; Kreivi et al., 1997). The same PP1-binding motif was identified independently by a different

approach in which random peptide libraries were passed through PP1-affinity columns (Zhao and Lee, 1997).

Although the cytosolic forms of PP1 have been studied in greatest detail, PP1 activity is located, and even enriched, in the nucleus (Kuret et al., 1986; Jakes et al., 1986). Many nuclear events are controlled by reversible protein phosphorylation, including pre-mRNA splicing (Mermoud et al., 1992; Tazi et al., 1992; Misteli and Spector, 1996; Cao et al., 1997; Duncan et al., 1997; Xiao and Manley, 1997; Kanopka et al., 1998) and there is evidence that PP1 plays a critical role prior to the first catalytic step of splicing (Mermoud et al., 1992) and in spliceosome assembly (Mermoud et al., 1994). However, the form(s) of PP1 involved in regulating pre-mRNA splicing and the substrates that it must dephosphorylate to regulate this process are unknown. Fractionation of rat nuclear extracts by anion exchange chromatography revealed two major forms of PP1 (Jagiello et al., 1995). The species eluting at a lower concentration of NaCl comprised PP1 complexed to a 41 kDa protein termed ‘nuclear inhibitor of PP1’ (NIPP1), while the species eluting at higher NaCl comprised PP1 complexed to a much larger protein, subsequently termed p99 (Kreivi et al., 1997) or PNUTS (Allen et al., 1998).

NIPP1 was cloned (Van Eynde et al., 1995) and shown to be an RNA-binding protein (Jagiello et al., 1997). Its C-terminal 127 residues are identical to ‘activator of RNA decay’ (ARD1), the product of a mammalian gene that complements mutations in *Escherichia coli* caused by deleting the gene encoding *E. coli* ribonuclease E (Wang and Cohen, 1994). ARD1 was

subsequently reported to possess endoribonuclease activity (Claverie-Martin et al., 1997) and may be produced from the NIPP1 gene by an alternative splicing event. NIPP1 binds tightly to PP1 and prevents it from dephosphorylating a number of phosphoproteins (Van Eynde et al., 1995; Jagiello et al., 1995). This inhibition can be relieved by phosphorylation of NIPP1 in vitro with cyclic AMP-dependent protein kinase and CK2 (Vulsteke et al., 1998). p99 also suppresses the activity of PP1 towards glycogen phosphorylase (a phosphoprotein frequently used to assay PP1 activity) and contains RNA-binding motifs (Kreivi et al., 1997; Allen et al., 1998). NIPP1 and p99 both contain sequences rich in basic amino acids that may allow the import of these proteins into the nucleus.

The function of the NIPP1-PP1 complex is unknown, but in this paper we provide evidence for an important role in the control of pre-mRNA splicing. We find that NIPP1 colocalises and interacts with pre-mRNA splicing factors and that its depletion from nuclear extracts or the addition of a dominant-negative mutant to nuclear extracts greatly reduces pre-mRNA splicing activity in vitro. In addition, we show that loss of either PP1 or RNA binding has no effect on the localisation of the protein within the cell.

MATERIALS AND METHODS

Materials

Tissue culture reagents were obtained from Life Technologies Inc. (Paisley, UK), and Protein G-Sepharose, CH-Sepharose and glutathione-Sepharose from Pharmacia (Milton Keynes, UK). NIPP1 peptides corresponding to residues 175-190 (NIPP1a) and residues 127-140 (NIPP1b) were synthesised on an Applied Biosystems 430A peptide synthesizer by Mr F. B. Caudwell in the MRC Protein Phosphorylation Unit.

Antibodies

Polyclonal antibodies were raised in sheep against NIPP1 peptides NIPP1a and NIPP1b (see above) and bacterially expressed hexahistidine-tagged ARD1 (residues 225-351 of NIPP1). The antibodies were affinity purified on peptide or protein CH-Sepharose columns and used for immunoblotting and immunocytochemistry, respectively, at the following concentrations: anti-NIPP1a (0.5 µg/ml and 100 µg/ml), anti-NIPP1b (0.5 µg/ml and 100 µg/ml) and anti-ARD1 (0.1 µg/ml and 10 µg/ml). Monoclonal anti-c-myc antibodies were purified from ascites fluid from mice injected with 9E10 hybridoma cells by chromatography on Protein G-Sepharose and used for immunocytochemistry at 1 µg/ml, while goat polyclonal anti-GST antibodies (Pierce) were used at 5 µg/ml. Affinity purified sheep polyclonal antibodies against PP1 and antisera against PP2A were gifts from Dr P. T. W. Cohen and Dr G. Moorhead (MRC Protein Phosphorylation Unit, Dundee), respectively. They were used for immunoblotting at the following dilutions: anti-PP1 (0.5 µg/ml) and anti-PP2A (1:2000).

Antibodies to nuclear antigens used for immunocytochemistry included mouse monoclonal antibodies to Sm proteins (anti-Y12, dilution 1:500; Pettersson et al., 1984), U2AF65 (anti-MC3, dilution 1:10; Gama-Carvalho et al., 1997), SR proteins (anti-3C5 and anti-SC35, both diluted 1:10; Turner and Franchi, 1987; Fu and Maniatis, 1990) and survival of motor neuron protein (anti-2B1, dilution 1:10; Liu and Dreyfuss, 1996), rabbit polyclonal antibodies to U1A (anti-856, dilution 1:500; Kambach and Mattaj, 1992) and p80 coilin (anti-204/10, dilution 1:500; Bohmann et al., 1995), and a human autoimmune serum that recognises the SP100 protein in PML bodies (anti-Krieb, dilution 1:10; Lamond and Carmo-Fonseca, 1993). For immunoblotting, antibodies/antisera were used at the following

dilutions: anti-Y12 (1:500), anti-856 (1:2000) and anti-MC3 (1:500). HRP-conjugated (Pierce) and FITC- and Texas Red-conjugated (Jackson Laboratories) secondary antibodies were used according to the manufacturers' recommendations.

Microinjection of FITC-coupled NIPP1 antibodies

Affinity purified antibodies to NIPP1 were directly coupled to fluorescein isothiocyanate (FITC; Molecular Probes) and dialysed into 100 mM glutamic acid, pH 7.2 (with citric acid), 140 mM KOH, 1 mM MgSO₄ and 1 mM DTT prior to microinjection into living HeLa cells using an Eppendorf 5242 microinjector. The medium was changed and the cells allowed to recover before examining the staining pattern with a Bio-Rad MRC-600 Laser Scanning Confocal Imaging System.

Immunolocalisation of NIPP1

Cells were grown to near confluency on glass coverslips, washed with phosphate-buffered saline (PBS) and fixed for 5 minutes with a -20°C methanol solution (90% methanol, 10% 100 mM MES, pH 6.9, 1 mM MgCl₂, 1 mM EGTA). After several washes with PBS the cells were further permeabilised for 10 minutes with PBS containing 1% (by vol) NP-40, washed several times with PBS containing 0.1% (by mass) Tween-20, and blocked for 10 minutes in the same solution containing 3% BSA. After 1 hour in primary antibody, the cells were washed extensively in PBS containing 0.1% (by mass) Tween-20 before being exposed to secondary antibody for 1 hour. In some experiments DNA was stained for 5 minutes with 0.25 µg/ml propidium iodide (Sigma). When two primary antibodies were used for double labelling experiments they were applied sequentially as described above, with extensive washes in between. After staining, cells were mounted in Mowiol/Dabco and allowed to dry before examination on either a Bio-Rad MRC-600 or a Zeiss LSM 410 Confocal Laser Scanning Microscope with excitation wavelengths of 488 nm (FITC) and 543 nm (Texas Red).

Site-directed mutagenesis of NIPP1

The Val and Phe residues of the PP1-binding motif in NIPP1 (residues 201 and 203) were changed to Ala using PCR mutagenesis of a pKS+ vector containing a cDNA encoding full length human NIPP1. This V201A/F203A double mutant was produced by two-step recombinant PCR as described (MacKintosh et al., 1995). The DNA constructs were confirmed by restriction analysis and their DNA sequences checked on an Applied Biosystems 3T3A DNA sequencer using specific oligonucleotide primers.

Expression and purification of recombinant NIPP1

For bacterial expression of the protein, the wild-type and mutant NIPP1 DNA was subcloned into the pGEX-4T3 vector using unique *EcoRI* and *SalI* sites. The truncated construct which lacked the C-terminal ARD1 domain was generated by PCR of the full-length clone from the EGFP-C1 construct with N-terminal *KpnI* and C-terminal *BamHI* restriction sites. *BamHI* digestion released nucleotides 675-1051 (amino acid residues 215-351) and the resulting fragment containing nucleotides 1-674 (residues 1-214) was subcloned into pGEX-4T3. All constructs were transformed into *E. coli* BL21(D3). The cells were grown to an A₆₀₀ of 0.6 in 500 ml of Luria-Bertani (LB) broth containing ampicillin and chloramphenicol selection, induced for 4 hours at 37°C with 1 mM isopropylthiogalactoside, and GST-NIPP1 purified by affinity chromatography on glutathione-Sepharose as described (Helps et al., 1995).

Preparation of DIG-PP1 and Far Western analyses

Human PP1γ (Barker et al., 1993; Alessi et al., 1993) was labelled with digoxigenin (DIG-PP1) and used in Far Western assays to study the binding of the enzyme to wild-type and mutant NIPP1 (Alessi et al., 1992; Kreivi et al., 1997).

Subcloning of NIPP1 into mammalian expression vectors

Expression of wild-type and mutant NIPP1 in mammalian cells was

achieved by subcloning the DNA into a variety of vectors. pSG8M expresses the 9E10 c-myc epitope at the N terminus of the protein (Bohmann, 1996). NIPP1 was subcloned from pKS+ into pSG8M using unique *EcoRI* and *SalI* restriction sites. NIPP1 constructs were also subcloned using the same unique sites into the pEGFP-C1 and pEGFP-N3 vectors (Clontech), which express at the N terminus or C terminus of NIPP1, respectively, a fusion of the enhanced version of the green fluorescent protein from the jellyfish *Aequorea victoria*. ARD1 was subcloned into pEGFP-C1 using unique *Asp718* and *BamHI* restriction sites.

GST-NIPP1 constructs were subcloned from the pGEX-4T3 bacterial vector into the mammalian pCMV5 vector using PCR to transfer the GST coding sequence in frame with the NIPP1 cDNA. To facilitate this transfer, oligonucleotides were synthesised which added a unique *HindIII* site upstream of the GST coding region along with a Kozak sequence, and maintained the unique *SalI* site at the C terminus of NIPP1 while adding a stop codon immediately upstream of this site.

Expression of wild-type and mutant NIPP1 in mammalian cells

The constructed plasmids were transiently transfected into 10 cm dishes of human embryonic kidney 293 cells using a modified calcium phosphate-mediated transfection procedure with 1 µg/ml DNA/plate (Alessi et al., 1996; Chen and Okayama, 1988). The cells were incubated for 48 hours prior to microscopic analysis or cell lysis. For preparation of total cell lysates, cells were first washed twice with ice-cold PBS and then lysed in 0.5 ml of ice-cold 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1% (by vol) Nonidet P-40, 1% (by mass) sodium deoxycholate, 0.1% (by mass) SDS, 2 mM EDTA plus Complete protease inhibitor cocktail (Boehringer Mannheim, one tablet per 25 ml). The lysate was repeatedly passed through a 21G hypodermic needle to break up the DNA and then cleared by centrifuging at 4°C for 15 minutes at 13,000 g.

The EGFP fusions of NIPP1 were also expressed in HeLa (human cervical carcinoma) and MCF7 (human breast carcinoma) cells. These cells were transfected using the FuGENE transfection reagent (Boehringer Mannheim) and 2 µg DNA/10 cm dish. After 48 hours the live cells were either examined by microscopy or fixed for cell staining.

Immunoprecipitation of transiently expressed c-myc-tagged NIPP1 and endogenous NIPP1 from mammalian cells

A lysate from 293 cells expressing wild-type or mutant myc-NIPP1 (0.5 ml, 6 mg/ml protein) was incubated for 1 hour on a shaking platform with 20 µl Protein G-Sepharose coupled to 5 µg anti-c-myc monoclonal antibody. 50 µl Protein G-Sepharose coupled to 100 µg anti-ARD1 antibody was then added to the supernatant to immunoprecipitate endogenous NIPP1. The beads were washed with 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, electrophoresed on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes for immunoblotting.

Isolation of GST-NIPP1 and associated proteins from nuclear extracts

293 cells overexpressing GST-NIPP1 were scraped into ice-cold PBS containing Complete protease inhibitors, followed by homogenisation with 10 strokes of a Dounce homogeniser and centrifugation for 5 minutes at 1,000 g to pellet the nuclei. The nuclei were resuspended in 25 mM Tris-HCl, pH 7.5, 0.14 M NaCl, 5 mM KCl, 0.5% (by mass) Triton X-100 and sonicated to release the nuclear contents. After centrifugation for 10 minutes at 13,000 g, the supernatant was decanted and incubated for 1 hour at 4°C with glutathione Sepharose on an end-over-end shaker to deplete GST-NIPP1. The resin was washed several times with 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl. In some experiments the bound proteins pulled down on 50 µl of resin from 400 µg total protein were solubilised in SDS and subjected to polyacrylamide gel electrophoresis followed by staining with Coomassie blue or immunoblotting. In other experiments the bound

proteins were eluted from the beads by multiple washes with Tris-HCl, 50 mM glutathione, pH 8.0. The washes were combined and dialysed overnight at 4°C against Nuclear Extract Buffer (20 mM Hepes, pH 7.9, 10% (by vol) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride and 0.5 mM dithiothreitol).

Immunodepletion of endogenous NIPP1 from nuclear extracts

HeLa nuclear extracts were prepared as described (Mermoud et al., 1992) and NIPP1 depleted using affinity purified anti-NIPP1a conjugated to Protein G-Sepharose. Depleted extracts were subjected to immunoblotting to determine the efficiency of NIPP1 depletion.

In vitro splicing assays

Splicing assays were performed using uniformly labelled, capped pre-mRNAs incubated with nuclear extracts using the in vitro splicing conditions described by Lamond et al. (1987). Adenovirus major late precursor (adeno pre-mRNA) was transcribed from *Sau3A*-digested plasmid pBSAd1 (Konarska and Sharp, 1987). Splicing products were separated on 10% polyacrylamide/8 M urea and denaturing gels run in Tris-borate/EDTA electrophoresis buffer. In some experiments 10 µg of recombinant GST-tagged protein or GST alone was incubated with pre-mRNA in the absence of nuclear extract (and hence splicing) for 30 minutes at 30°C. The proteins were purified on glutathione-Sepharose, washed three times with PBS, and then treated as splicing reactions and run on acrylamide gels to visualise any associated pre-mRNA.

RESULTS

Localisation of endogenous NIPP1 in mammalian cells

The localisation pattern of endogenous NIPP1 was examined in live HeLa cells by directly conjugating two separate NIPP1

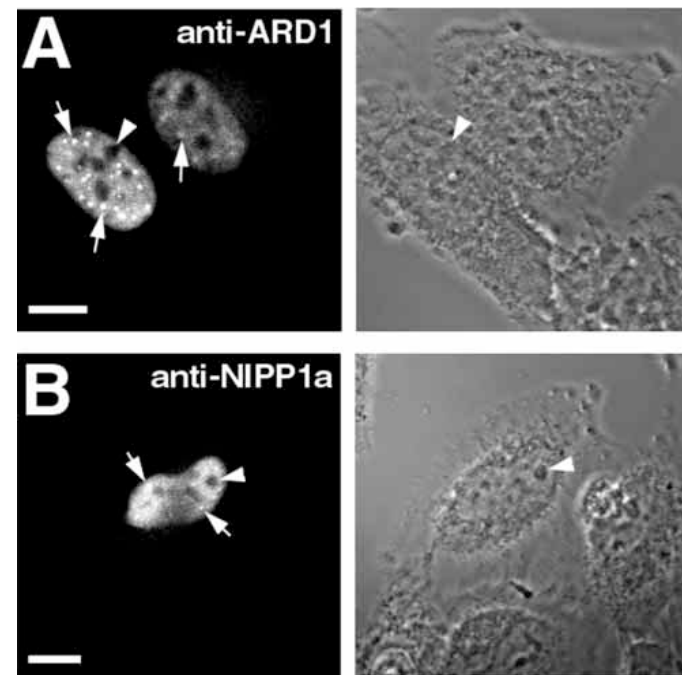


Fig. 1. Confocal images of live HeLa cells microinjected with affinity-purified FITC-conjugated NIPP1 sheep polyclonal antibodies. Nuclear speckles stained by the antibodies are indicated by arrows, and nucleoli by arrowheads. (A) Anti-ARD1 and accompanying phase image. (B) Anti-NIPP1a and accompanying phase image. Bar, 5 µm.

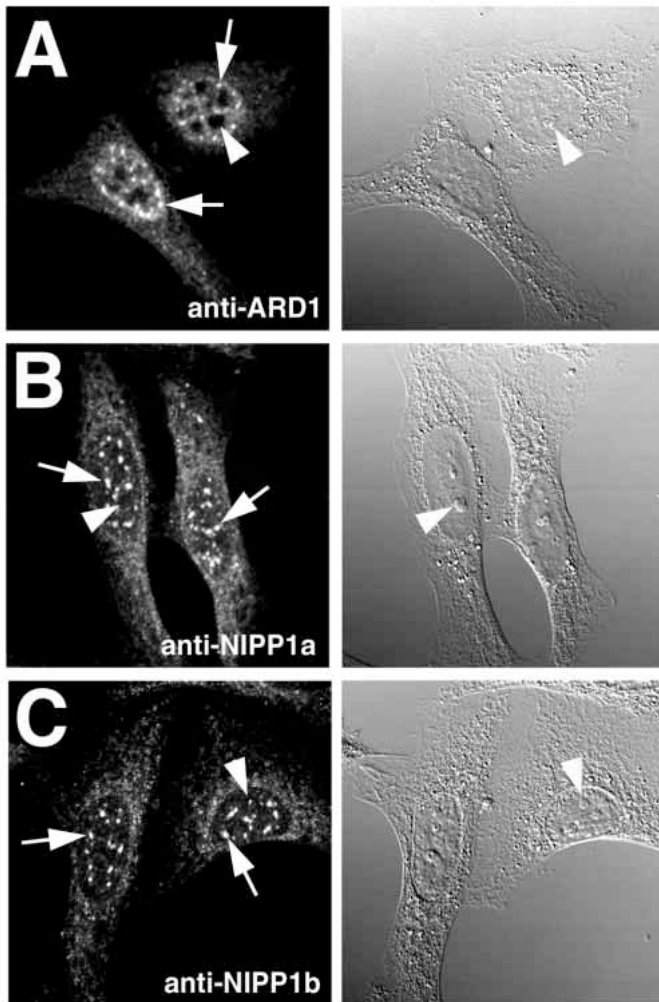


Fig. 2. Confocal images of methanol-fixed HeLa cells stained with anti-NIPP1 antibodies. The antibodies were detected using FITC-conjugated anti-sheep IgG secondary antibody. Nuclear speckles stained by the antibodies are indicated by arrows and nucleoli by arrowheads. In each panel a DIC image of the cell structure is shown to the right of the antibody image. (A) Anti-ARD1 antibody; (B) anti-NIPP1a antibody; (C) anti-NIPP1b antibody. Bar, 10 μ m.

antibodies, anti-ARD1 and anti-NIPP1a, to FITC and microinjecting them individually into the cell nuclei (Fig. 1). Both antibodies showed a widespread nucleoplasmic staining pattern, with additional accumulation in 'speckled' structures (Fig. 1, arrows). NIPP1 antibodies were primarily excluded from the nucleoli, although staining within the nucleoli was observed in approximately 20% of the cells.

A similar staining pattern was observed when HeLa cells (Fig. 2), as well as MCF7, KB, 293 and GM38 cells (data not shown), were methanol-fixed and stained with the same anti-ARD1 and anti-NIPP1a antibodies, or with anti-NIPP1b (Fig. 2). Fixation of HeLa cells permitted counter-staining with antibodies to various nuclear antigens which also show punctate or speckled staining patterns. Antibodies against p80 coilin (Fig. 3B), the survival of motor neuron protein and the PML body-associated protein SP100 (data not shown), each of which produce punctate nuclear staining, did not colocalise with NIPP1. However, a clear

colocalisation was observed between the staining pattern for the anti-Y12 monoclonal antibody, which recognises core Sm proteins of the small nuclear ribonucleoproteins (snRNPs), and the staining pattern for anti-NIPP1a (Fig. 3A). Because these proteins are integral components of the spliceosome complex in mammalian cells, colocalisation implies that NIPP1 may be associated with splicing factors *in vivo*. The Sm proteins are found in both interchromatin granule clusters (speckles) and in coiled bodies (see below).

Preabsorption of the NIPP1 antibodies with their respective antigens blocked all staining (data not shown), indicating that the pattern is specific for these epitopes. Varying levels of diffuse cytoplasmic staining were observed with all three anti-NIPP1 antibodies in HeLa (Fig. 2) and other cells. However, cytoplasmic staining was not observed with any of the antibodies used to localise tagged versions of NIPP1 (see below), and NIPP1 is absent from cytosolic extracts as judged by immunoblotting (Van Eynde et al., 1995), so this cytoplasmic staining is probably not NIPP1, and likely to be artefactual.

Localisation of EGFP-NIPP1 in mammalian cells

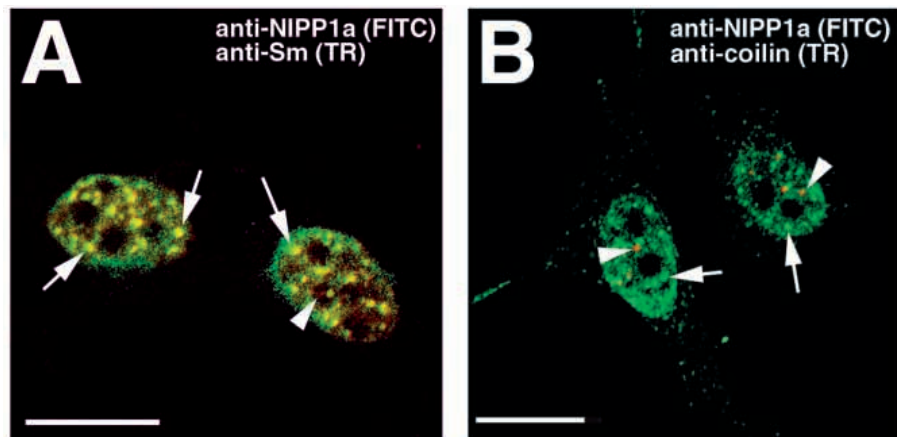
In order to examine further the localisation of NIPP1, we expressed it as a fusion with the enhanced green fluorescent protein (EGFP). EGFP was fused to either the N terminus or C terminus of NIPP1, and expressed in a variety of cell types by transient transfection. Fig. 4 shows the expression pattern of EGFP-NIPP1 in live HeLa cells (A) and live MCF7 cells (C and E). Both N and C terminal fusions showed identical widespread nucleoplasmic distributions with additional accumulation in speckles (compare C and E). EGFP-NIPP1 fusions were not observed in the cytoplasm. Like the endogenous protein, EGFP-NIPP1 was occasionally observed in the nucleolus. This was more prevalent in HeLa cells than in other cell types, and when cells were dividing more actively (e.g. at lower confluencies). In addition, NIPP1 fusions were more likely to accumulate in the nucleoli of cells showing the highest overexpression of this protein.

HeLa cells expressing EGFP-NIPP1 were also methanol-fixed and stained with antibodies to a variety of nuclear antigens. All three anti-NIPP1 antibodies recognised the EGFP fusions (data not shown). Much like the endogenous protein, EGFP-NIPP1 did not colocalise with p80 coilin, the survival of motor neuron protein or the SP100 protein found in PML bodies (data not shown). It did, however, colocalise with antibody staining for various pre-mRNA splicing factors, including Sm proteins (Fig. 5A), U1A (Fig. 5B), U2AF65 (Fig. 5C) and SR proteins (Fig. 5D). Colocalisation was observed for U1A, U2AF65 and SR proteins in both control cells and in cells treated with actinomycin D to inhibit transcription, which induces reorganisation of the splicing factors causing them to concentrate predominantly in large speckles. However, although every EGFP-NIPP1 speckle was labelled by the anti-Sm antibody, in control cells we observed some Sm-containing speckles that did not contain EGFP-NIPP1. The reasons for this are considered further in the Discussion.

RNA binding and localisation of NIPP1 constructs lacking the ARD1 domain

The fact that NIPP1 is known to bind RNA (Jagiello et al., 1997), and that this RNA binding is most likely mediated by the ARD1 domain which includes the C-terminal 225-351 residues of the

Fig. 3. Confocal images of methanol-fixed HeLa cells double-stained with anti-NIPP1 and either anti-Sm or anti-p80 coilin antibodies. Anti-NIPP1 stained cells were counter-stained with mouse monoclonal antibodies to either the Sm family of snRNP proteins (anti-Y12) or p80 coilin (anti-5P10) and Texas Red-conjugated mouse IgG secondary antibody. (A) Anti-NIPP1a and anti-Y12. Yellow speckles, such as those marked by arrows, represent areas within the nucleus where the two antibodies colocalise. The arrowhead marks an Sm speckle in the nucleolus which is not stained by anti-NIPP1a. (B) Anti-NIPP1a and anti-p80 coilin. Arrows indicate representative speckles stained with anti-NIPP1a, while arrowheads indicate coiled body structures stained with anti-p80 coilin but not anti-NIPP1a. Bar, 10 μ m.



protein (Claverie-Martin et al., 1997) led us to question whether it is this domain that is responsible for targeting NIPP1 to sites in the nucleus containing RNA splicing factors. EGFP-ARD1 shows a diffuse nuclear staining pattern in cells, however, and no accumulation in nuclear speckles (Fig. 6A). In contrast, EGFP-NIPP1 lacking this ARD1 domain shows an identical localisation to the full-length protein, accumulating in nuclear speckles (Fig. 6C) and colocalising with Sm proteins both before

(Fig. 6D) and after (Fig. 6E) treatment with actinomycin D. The loss of RNA binding is demonstrated by the fact that recombinant full-length GST-tagged NIPP1 binds pre-mRNA *in vitro* whereas the truncated GST-NIPP1 does not (Fig. 6B).

Identification of proteins associated with expressed NIPP1

We next examined whether the colocalisation of NIPP1 with

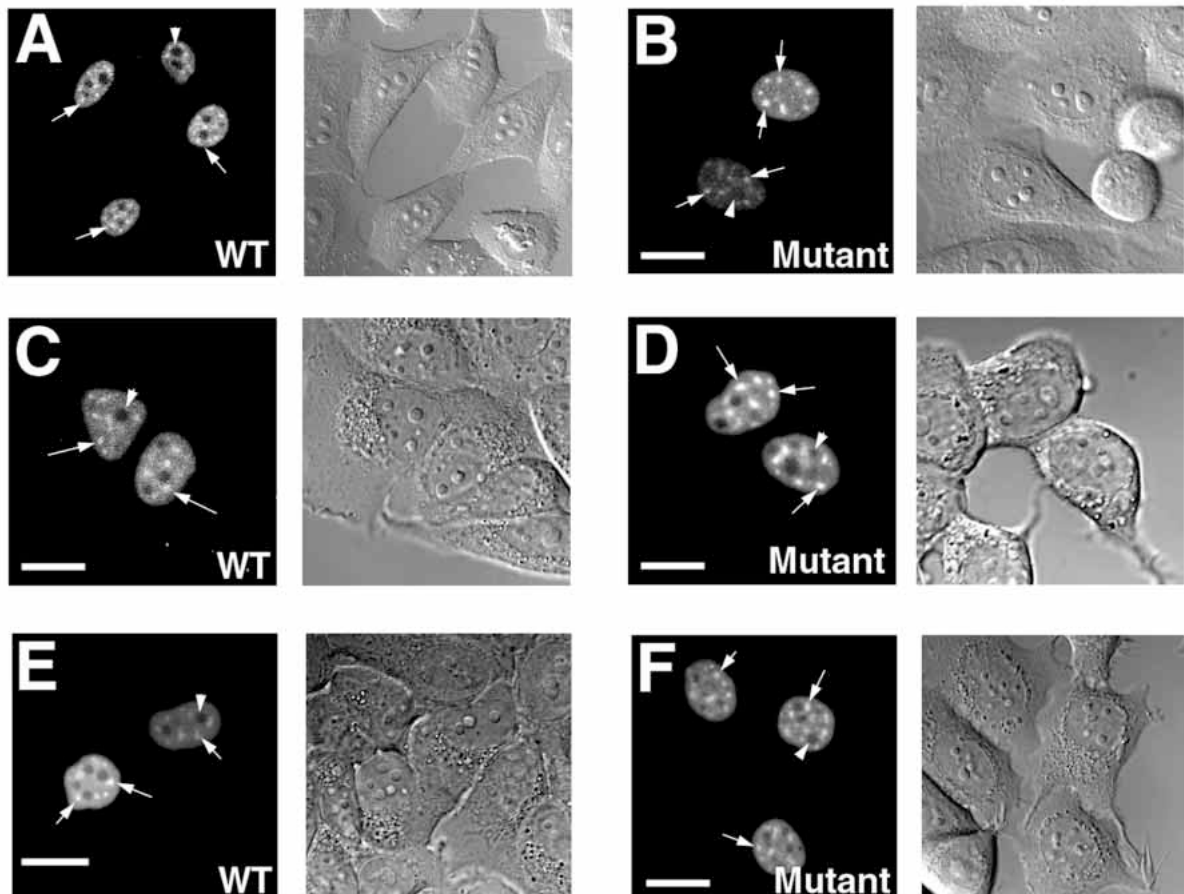


Fig. 4. Confocal images of live cells transiently transfected with EGFP-NIPP1. In each panel a DIC image of the cell structure is shown to the right of the EGFP image. (A) HeLa cells expressing EGFP-NIPP1. (B) HeLa cells expressing the EGFP-NIPP1(V201A/F203A) mutant that does not interact with PP1. (C) MCF7 cells expressing EGFP-NIPP1. (D) MCF7 cells expressing the EGFP-NIPP1 (V201A/F203A) mutant. (E and F) Same as C and D except that the EGFP is fused to the C terminus rather than the N terminus of NIPP1. Bar, 10 μ m.

splicing factors at the nuclear speckles may be due to the formation of a complex between NIPP1 and one or more components of the spliceosome. To assay for association of NIPP1 with splicing factors, 293 cells were transiently transfected with either plasmid pCMV5 encoding a GST-NIPP1 fusion protein or plasmid SVG8M encoding a c-myc-NIPP1 fusion protein (see Materials and Methods) and lysates prepared 36 hours following transfection. For control experiments lysates were prepared from cells expressing GST or c-myc alone. Immunofluorescence analysis indicated that the transfection efficiency was 90-100%, and the fusion proteins showed a distribution pattern similar to that of the endogenous protein (data not shown). Analysis of the total cell lysate by immunoblotting with anti-NIPP1 antibodies prior to purification revealed that the GST and c-myc fusion proteins were expressed in 10-fold and 2-fold excess, respectively, over the endogenous protein (data not shown). These expression levels facilitated the purification of NIPP1 and the detection of proteins associated with it, but contrasted with a previous report of a failure to overexpress the NIPP1 protein in COS-1 cells, despite the overexpression of its mRNA (Wera et al., 1997). GST-NIPP1 was purified from nuclear extracts by affinity chromatography on glutathione-Sepharose and the isolated proteins separated by SDS-PAGE and electroblotted on to nitrocellulose membranes. Proteins copurifying with GST-NIPP1 were detected by immunoblotting. As expected, PP1 was specifically pulled down with GST-NIPP1 (Fig. 7A) and served as a positive control for this experiment, while protein phosphatase 2A (PP2A) did not copurify with GST-NIPP1 (data not shown). Interestingly, antibody staining showed that a fraction of the Sm proteins co-purified with

NIPP1 (Fig. 7B). In contrast, U1A (Fig. 7C) and U2AF65 (Fig. 7D) did not specifically associate with GST-NIPP1 under these conditions, suggesting that NIPP1 may interact with a subset of splicing components. C-myc-NIPP1 was immunoprecipitated from lysates using the 9E10 monoclonal antibody and treated as described above. Sm proteins but not U1A coprecipitated with c-myc-NIPP1 (Fig. 7E and F), supporting the results observed with the GST fusion protein.

Loss of *in vitro* splicing activity following immunodepletion of NIPP1

NIPP1 could be almost completely depleted from a nuclear extract by incubation of the extract with anti-NIPP1a antibody bound to Protein G-Sepharose (Fig. 8A, lanes 2-3). This depletion was blocked by preincubation of the antibody with a 10-fold molar excess of peptide immunogen (Fig. 8A, lanes 4-5). In comparison to a control extract (Fig. 8B, lane 2), nuclear extracts depleted of NIPP1 showed greatly decreased *in vitro* pre-mRNA splicing activity (Fig. 8B, lanes 3-4). The extract remained active when the depletion of NIPP1 was specifically prevented by preincubation of the antibody with its peptide immunogen (Fig. 8B, lanes 5-6). Similar results were obtained using the anti-ARD1 antibody to immunodeplete NIPP1 (data not shown).

Inhibition of *in vitro* splicing activity by a dominant negative NIPP1 mutant

All the NIPP1 in nuclear extracts copurifies with PP1 during chromatography on Mono Q (Jagiello et al., 1995) and all the PP1 in these fractions can be immunoprecipitated with an anti-NIPP1 antibody (Fig. 9), indicating that nuclear PP1

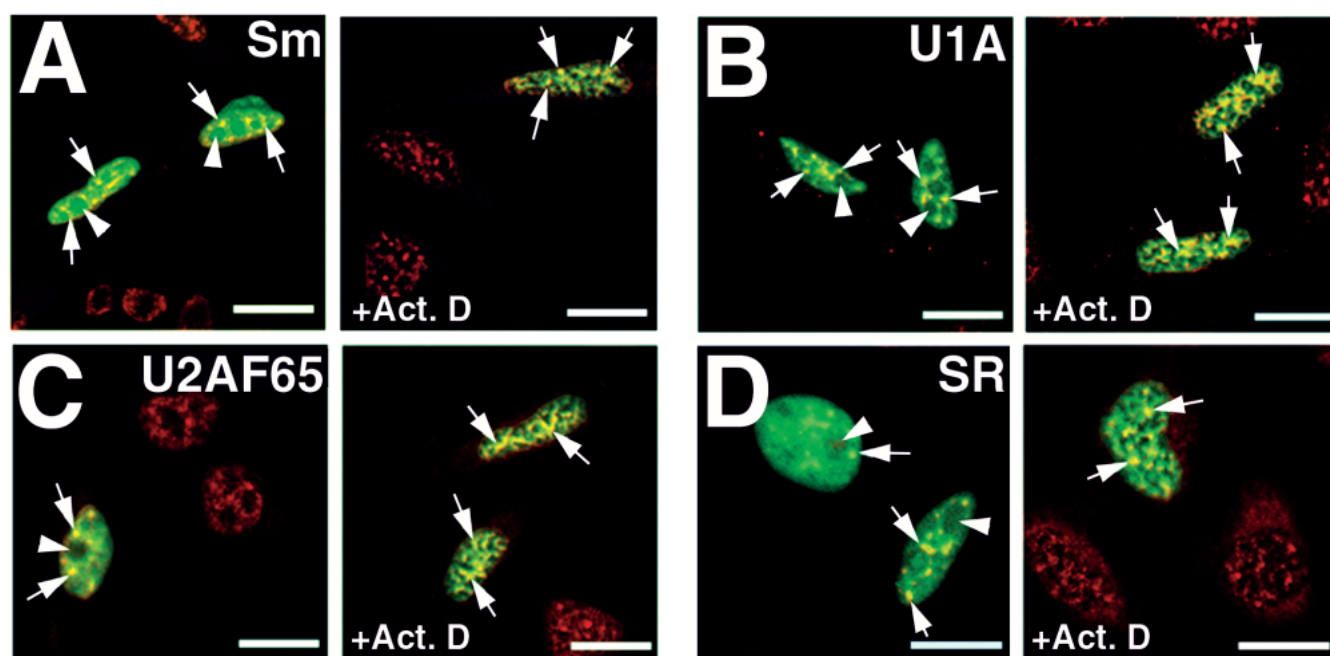


Fig. 5. Confocal images of methanol-fixed HeLa cells expressing EGFP-NIPP1 counter-stained with antibodies to a variety of splicing factors. Images on the right-hand side of each panel are from cells treated for 5 hours with actinomycin D to inhibit transcription (+ Act. D). Nucleoli are indicated by arrowheads, while yellow speckles, such as those marked by arrows, represent areas within the nucleus where the EGFP proteins and antibodies colocalise. (A) EGFP-NIPP1 counter-stained with anti-Y12 (recognises the Sm family of snRNP proteins). (B) EGFP-NIPP1 counter-stained with anti-856 (recognises U1A). (C) EGFP-NIPP1 counter-stained with anti-MC3 (recognises U2AF65). (D) EGFP-NIPP1 counter-stained with anti-3C5 (recognises the SR family of non-snRNP splicing proteins). Bar, 10 μ m.

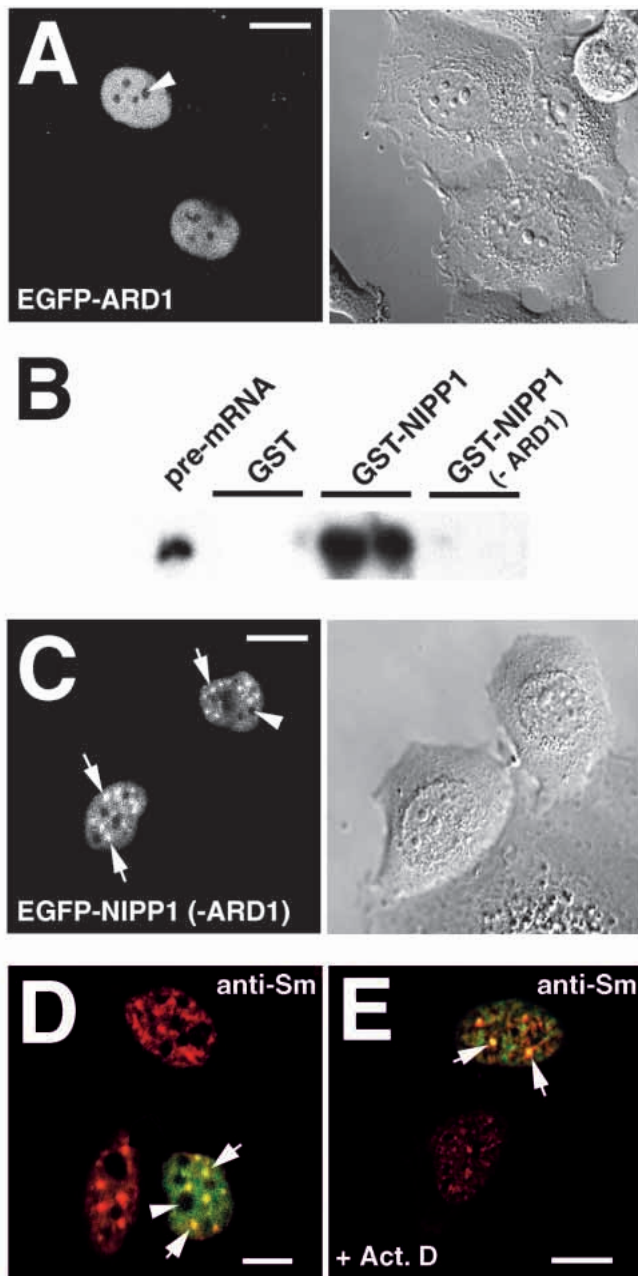


Fig. 6. Localisation and loss of RNA binding of a NIPP1 mutant lacking the ARD1 domain. (A) HeLa cells expressing EGFP-ARD1. A DIC image of the cell structure is shown to the right of the EGFP image. The arrowhead marks a nucleolus. Bar, 10 μ m. (B) Co-purification of pre-mRNA with full-length GST-NIPP1 (lanes 4-5) but not GST alone (lanes 1-2) or GST-NIPP1 lacking the ARD1 domain (lanes 6-7), as visualised by autoradiography of a polyacrylamide gel used to separate the RNA associated with the proteins following purification on glutathione-Sepharose. (C) HeLa cells expressing EGFP-NIPP1 lacking the ARD1 domain. Arrows indicate accumulation in nuclear speckles while the arrowhead indicates a nucleolus. Bar, 10 μ m. (D) EGFP-NIPP1 lacking the ARD1 domain, counter-stained with anti-Y12 (which recognises the Sm family of snRNP proteins). Nucleoli are indicated by arrowheads, while yellow speckles, such as those marked by arrows, represent areas within the nucleus where the EGFP proteins and antibodies colocalise. Bar, 5 μ m. (E) Same as D except that the cells were treated for 5 hours with actinomycin D to inhibit transcription (+ Act. D) Bar, 5 μ m.

associates tightly in a complex with NIPP1. The results presented above therefore indicate that the NIPP1-PP1 complex is associated with pre-mRNA splicing factors and that NIPP1 may target PP1 to a component of the spliceosome. In this case, it should be possible to displace the endogenous NIPP1-PP1 complex from spliceosomes by addition of an excess of a mutant NIPP1 that is still capable of targeting to splicing factors, but unable to bind to PP1. We therefore mutated (to alanine residues) the valine and phenylalanine of the putative PP1-binding motif (Arg-Val-Thr-Phe) located between residues 200-203 of NIPP1. In contrast to wild-type NIPP1, the V201A/F203A double mutant was unable to interact with PP1 in Far Western blotting experiments (Fig. 10A), was not associated with PP1 in nuclear extracts (Fig. 10B) and did not suppress the PP1-activity towards glycogen phosphorylase (Fig. 10C). However, the V201A/F203A mutant showed an identical localisation within the nucleus as the wild-type protein (Fig. 4B,D,F), demonstrating that the domain that targets NIPP1 to splicing factors is distinct from the PP1-binding site. In this dominant negative approach, addition of the GST-tagged V201A/F203A NIPP1 mutant to nuclear extracts partially inhibited pre-mRNA splicing, whereas GST alone had no effect (Fig. 11).

DISCUSSION

In this paper we identified the subnuclear distribution of NIPP1 by studying the location of the endogenous protein and transiently overexpressed fusion proteins. A consistent localisation pattern was observed using several different approaches. We found that NIPP1 has a widespread nucleoplasmic distribution, with accumulations in snRNP speckles where it colocalises with factors involved in pre-mRNA splicing, including Sm proteins, U1A, U2AF65 and SR proteins. EGFP-NIPP1 also colocalised with these proteins after inhibition of transcription with actinomycin D, which causes the redistribution of splicing factors to larger structures (Fig. 5). In addition, Sm proteins were shown to coimmunoprecipitate with NIPP1 in nuclear extracts (Fig. 7). In contrast, p99, the other major nuclear PP1-binding protein (see Introduction), shows a quite different subnuclear localisation (C. E. Lyon and A. I. Lamond, unpublished experiments).

Splicing snRNPs associate in the nucleus in several distinct structures, including interchromatin granules and coiled bodies (for a review, see Lamond and Earnshaw, 1998). However, NIPP1 only associates with factors found in the interchromatin granules. This again indicates that NIPP1 most likely interacts with a subset of the total splicing snRNPs. The different functions of splicing factors within the interchromatin granules and coiled bodies have not yet been clearly defined. Coiled bodies have been shown to contain splicing snRNPs, but not the essential splicing factor SC35, a member of the SR protein family (Carmo-Fonseca et al., 1991, 1992; Raska et al., 1991; Spector et al., 1991; Huang and Spector, 1992). This and other evidence indicates that coiled bodies are unlikely to be major sites of pre-mRNA splicing, but may instead be involved in some aspect of snRNP maturation, transport or recycling. The interchromatin granules, on the other hand, contain the snRNP and non-snRNP splicing factors, including the SR proteins. It

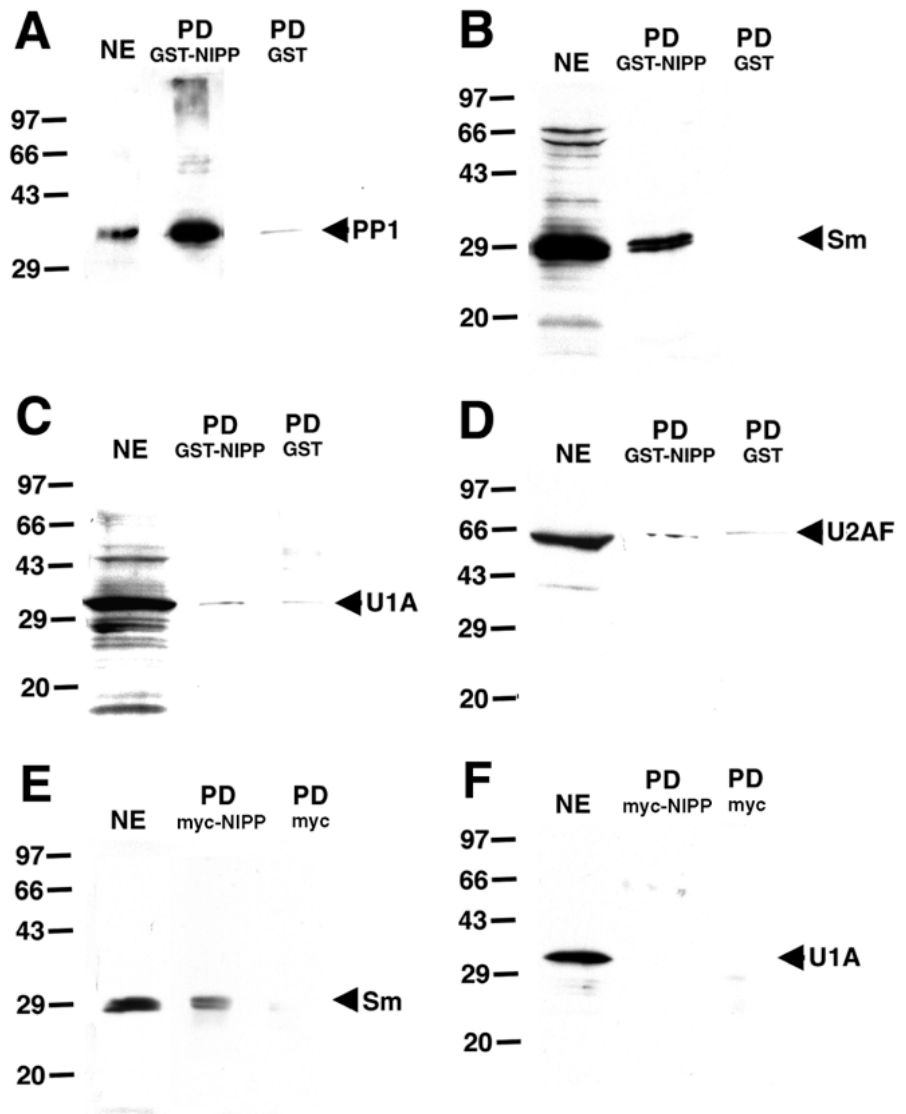


Fig. 7. Copurification of proteins with GST- or c-myc-tagged NIPP1 in mammalian cells. 293 cells were transiently transfected with either the pCMV5 vector expressing GST-NIPP1 or the pSVG8M vector expressing c-myc-NIPP1. The nuclear extracts (NE) were incubated with glutathione-Sepharose to deplete GST-NIPP1 or anti-c-myc coupled to Protein G-Sepharose to deplete c-myc-NIPP1. After washing the beads the NIPP1 fusion proteins and associated proteins were eluted with SDS (PD). The nuclear extracts and eluates were separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with antibodies and visualized using the enhanced chemiluminescence (ECL) detection system (Amersham). The figure shows that a fraction of the PP1 (A) and Sm proteins (B,E) present in nuclear extracts are associated with GST- and c-myc-NIPP1, while U1A (C,F) and U2AF (D) are not.

is unlikely that the speckled pattern represents major splicing sites, however, since there is little or no transcriptional activity in these structures and splicing is likely to occur co-transcriptionally (reviewed by Lamond and Earnshaw, 1998). In addition, splicing factors are still detected in these speckles even when transcription, and hence splicing, have been blocked by treatment with actinomycin D. The speckles may instead be implicated in storage of splicing factors and/or preassembly of the splicing machinery (Misteli et al., 1997). Although NIPP1 is associated in vivo with various splicing factors, it is not detected in purified spliceosomes (Neubauer et al., 1998). This indicates a weak and/or transient interaction with these structures, like the SR proteins that also bind weakly to the spliceosome and hence are dissociated during spliceosome purification.

The second catalytic step of pre-mRNA splicing can be selectively inhibited by the addition of low levels of okadaic acid to nuclear extracts, a potent inhibitor of PP2A, while the first and second catalytic steps of pre-mRNA splicing are blocked by the addition of tautomycin to the extracts, a specific inhibitor of PP1, or by microcystin, a potent inhibitor of both PP1 and PP2A (Mermoud et al., 1992). These

observations indicate that pre-mRNA splicing activity is regulated by at least two different phosphorylation events. The inactivating event prior to the first catalytic step appears to be reversed by a PP1-like phosphatase activity, and the inactivating step prior to the second catalytic step by a PP2A-like activity. These regulatory steps may be more complicated, however, involving regulation of the phosphatases themselves by phosphorylation (see below). Neither tautomycin nor microcystin prevent spliceosome assembly, indicating that PP1 acts after assembly but before catalysis (Mermoud et al., 1992). However, spliceosome assembly can be inhibited by adding a large excess of the PP1 catalytic subunit to nuclear extracts (Mermoud et al., 1994), indicating that one or more phosphorylation events are essential for spliceosome assembly.

In the present study, the depletion of NIPP1 from a HeLa nuclear extract led to loss of *in vitro* pre-mRNA splicing activity (Fig. 8) without affecting spliceosome assembly (data not shown), but activity was retained when the depletion of NIPP1 was blocked by preabsorption of the antibody with the antigen (Fig. 8). This is similar to the effects of tautomycin and microcystin and suggests that it may be the NIPP1-PP1

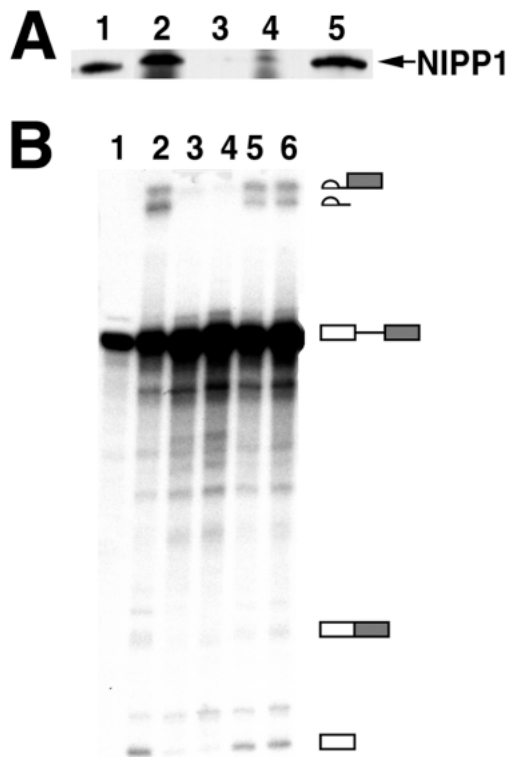


Fig. 8. Depletion of NIPP1 from nuclear extracts abolishes pre-mRNA splicing activity. (A) Immunodepletion of NIPP1 from nuclear extracts using affinity purified anti-NIPP1a conjugated to Protein G-Sepharose, as visualised by immunoblotting with the same antibody. Lane 1, nuclear extract before depletion; lanes 2 and 3, pellet and supernatant obtained after immunoprecipitation of NIPP1; lanes 4 and 5, same as lanes 2 and 3 except that the anti-NIPP1a antibody was preincubated with a 10-fold molar excess of peptide immunogen prior to immunoprecipitation. (B) Autoradiograph of a polyacrylamide gel showing the ^{32}P -labelled pre-mRNA substrate for splicing (lane 1), the products of splicing for a control nuclear extract (lane 2), a NIPP1 depleted nuclear extract (lanes 3 and 4) and nuclear extracts in which the depletion of NIPP1 was blocked by preadsorption of the antibody with its peptide immunogen (lanes 5-6). The structures of the intermediates and products of splicing are represented by cartoons to the right of the autoradiograph.

complex that reverses the inactivating phosphorylation prior to the first catalytic step of splicing. This was supported by the finding that splicing was partially inhibited by adding an excess of a NIPP1 'dominant negative' mutant that does not interact with PP1 (Fig. 11) and which presumably exerts its effects by displacing the endogenous NIPP1-PP1 complex. Although both catalytic steps of splicing were inhibited by the addition of the NIPP1 mutant, the second step appeared to be more sensitive to inhibition, as demonstrated by an initial accumulation of the first intermediate at low levels of the mutant NIPP1. The reason for this is unclear, but it is possible that the effects observed with okadaic acid reflected the control of PP1, and hence the second catalytic step of splicing, by a mechanism involving PP2A, rather than a direct involvement of PP2A in the regulation of the second catalytic step of splicing. This would be consistent with the observation that the PP1-NIPP1 complex is likely to be subject to regulation by

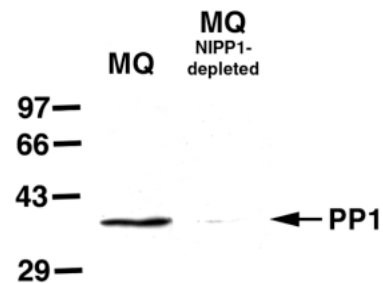


Fig. 9. Immunodepletion of PP1 from the Mono Q fraction containing the NIPP1-PP1 complex using anti-NIPP1. The Mono Q fraction containing the NIPP1-PP1 complex was incubated with anti-NIPP1a coupled to Protein G-Sepharose to deplete NIPP1. An aliquot of the fraction (40 μg) was separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-PP1 both before (MQ) and after depletion of NIPP1 (MQ, NIPP1-depleted). The figure shows that virtually all of the PP1 in this fraction is associated with NIPP1.

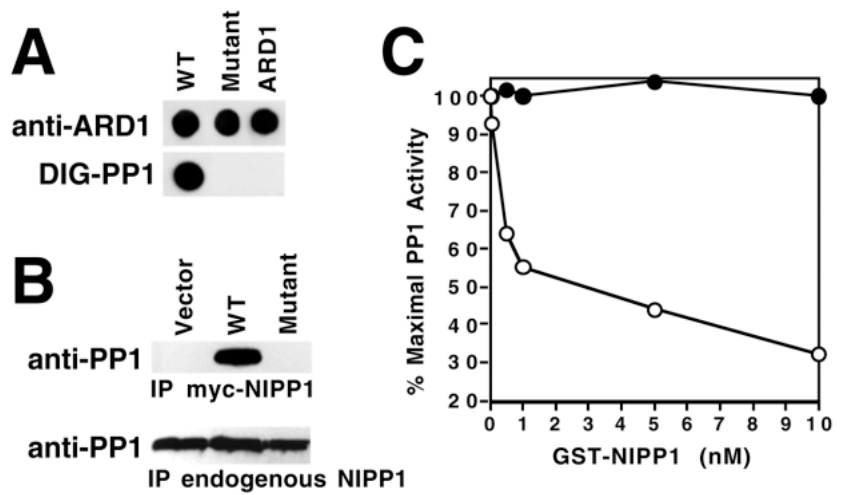
phosphorylation mechanisms (see Introduction). A clear answer to this question requires a much more detailed analysis, however, and identification of the splicing factors that are substrates for the phosphatase(s).

The NIPP1 mutant that did not bind to PP1 showed the same nuclear localisation as the wild-type protein, indicating that it is NIPP1, and not the PP1 catalytic subunit, that targets the NIPP1-PP1 complex to splicing factors. However, it was recently reported that the three different isoforms of the PP1 catalytic subunit (α , β/δ and γ) localise to distinct nuclear compartments, including nuclear speckles that may represent either interchromatin granules and/or coiled bodies (Andreassan et al., 1998). It would therefore be interesting to know whether NIPP1 is associated with a particular PP1 isoform *in vivo*, although *in vitro* it is capable of interacting with at least two isoforms (α and γ ; L. Trinkle-Mulcahy, unpublished experiments).

We have been unable, so far, to restore splicing activity to nuclear extracts by the addition of bacterially expressed NIPP1 with or without bacterially expressed PP1 γ , or by the addition of the native NIPP1-PP1 complex (inactive towards glycogen phosphorylase) that had been partially purified by chromatography of HeLa nuclear extracts on Mono Q (L. Trinkle-Mulcahy, unpublished experiments). The reason for this is unclear, but one possible explanation is that immunodepletion of NIPP1 removes one or more essential splicing factors in addition to PP1 and the Sm proteins (Fig. 7). It is also possible that the phosphorylation state of NIPP1 may be critical in order to restore splicing or that the bacterially expressed NIPP1 is not fully active or correctly folded.

Most of the PP1-targeting subunits described to date contain an Arg/Lys-Val/Ile-Xaa-Phe/Trp motif and small peptides containing these motifs have been shown to bind to PP1 (Johnson et al., 1996; Egloff et al., 1997; Zhao and Lee, 1997; Kreivi et al., 1997). Moreover, mutation of the Val/Ile and Phe/Trp residues in these peptides has been shown to prevent them from interacting with PP1. However, the importance of this motif for PP1-binding has not been established previously by mutagenesis of a full length targeting subunit. In this paper we have shown that the

Fig. 10. Mutagenesis of conserved hydrophobic residues in the canonical PP1-binding motif disrupts the interaction of NIPP1 with PP1. (A) GST fusion proteins of wild-type NIPP1 and mutant NIPP1(V201A/F203A) and His-tagged ARD-1 were spotted on to nitrocellulose filters and probed with anti-ARD1 or digoxigenin-labelled PP1. (B) c-myc-tagged wild-type NIPP1 and the mutant NIPP1(V201A/F203A) were transfected into 293 cells from the vector pSVG8M and immunoprecipitated from the lysates with an anti-c-myc antibody. The immunoprecipitates were washed, solubilised in SDS, subjected to electrophoresis on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and probed with anti-PP1 (upper panel). In the lower panel, the endogenous NIPP1 was immunoprecipitated with anti-NIPP1a and then analysed in the same way as the c-myc-immunoprecipitates shown in the upper panel. (C) The catalytic subunit of PP1 γ was assayed using glycogen phosphorylase as a substrate (Cohen et al., 1988) in the presence of the indicated concentrations of GST-NIPP1 (open circles) or GST-NIPP1(V201A/F203A) (closed circles). PP1 activity is plotted as a percentage of the activity observed in the absence of NIPP1. The experiment was repeated three times with similar results.



V201A/F203A double mutant of NIPP1 is unable to interact with PP1, yet shows an identical subnuclear distribution to the wild-type protein. This demonstrates that the Arg-Val-Thr-Phe sequence located between residues 200 and 203 is indeed a PP1-binding site of NIPP1, and that the subnuclear localisation of NIPP1 is not determined by the PP1 catalytic subunit, but by a region of NIPP1 distinct from the PP1-

binding site. Similar amino acid substitutions in other PP1-targeting subunits that prevent interaction with PP1 without affecting subcellular localisation may provide useful dominant negative mutants for identifying the functions of the different forms of PP1 in vivo. Such mutants may also facilitate the identification of proteins other than PP1 that interact with PP1-targeting subunits. We have also shown that deletion of the ARD1 domain of NIPP1, resulting in a loss of pre-mRNA binding, has no effect on the subcellular localisation of NIPP1 (Fig. 6). Taken together, these data suggest that targeting of NIPP1 to areas of the nucleus containing high concentrations of splicing factors is independent of both PP1 and RNA binding.

In summary, our data suggest that one or more protein factors, whose activity is required for the first catalytic step of splicing, is/are inactivated by phosphorylation and reactivated by dephosphorylation catalysed by the NIPP1-PP1 complex. NIPP1 may therefore play an important role in targeting PP1 to this protein factor(s). However, NIPP1 may not be the only protein that targets PP1 to spliceosomes, because Hirano et al. (1996) have shown that protein splicing factor (PSF) interacts with PP1 in a 'two hybrid' assay. This protein possesses an Arg-Val-Xaa-Phe motif close to its N terminus that is presumably critical for interaction with PP1. Future experiments will continue to address the role of NIPP1 in the splicing mechanism, including identification of the domains critical for localisation of the protein and those involved in the interaction with specific splicing factors.

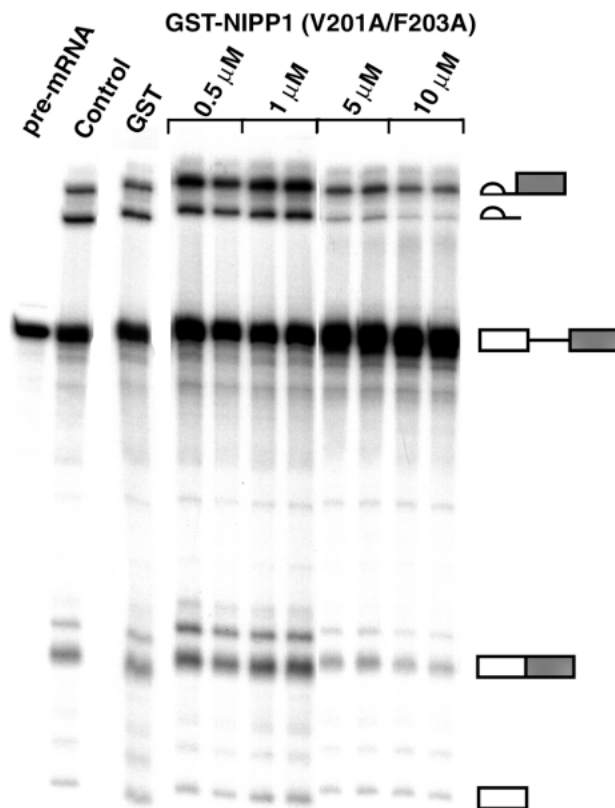


Fig. 11. GST-NIPP1(Val201Ala/Phe203Ala) inhibits pre-mRNA splicing in nuclear extracts. The figure shows an autoradiograph of a polyacrylamide gel used to separate the products of pre-mRNA splicing. The splicing reactions were carried out in the presence or absence of the indicated concentrations of GST-NIPP1(V201A/F203A), or in the presence of 10 μM GST or an equivalent volume of buffer alone (control). The structures of the intermediates and products of splicing are represented by cartoons to the right of the autoradiograph.

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REFERENCES

- Alessi, D., MacDougall, L. K., Sola, M. M., Ikebe, M. and Cohen, P. (1992). The control of protein phosphatase-1 by targeting subunits. The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. *Eur. J. Biochem.* **210**, 1023-1035.
- Alessi, D. R., Street, A. J., Cohen, P. and Cohen, P. T. (1993). Inhibitor-2 functions like a chaperone to fold three expressed isoforms of mammalian protein phosphatase-1 into a conformation with the specificity and regulatory properties of the native enzyme. *Eur. J. Biochem.* **213**, 1055-1066.
- Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15**, 6541-6551.
- Allen P. B., Kwon Y. G., Nairn A. C. and Greengard P. (1998). Isolation and characterization of PNUTS, a putative protein phosphatase 1 nuclear targeting subunit. *J. Biol. Chem.* **273**, 4089-4095.
- Andreassen, P. R., Lacroix, F. B., Villa-Moruzzi, E. and Margolis, R. (1998). Differential subcellular localization of protein phosphatase-1 α , γ 1, and δ isoforms during both interphase and mitosis in mammalian cells. *J. Cell Biol.* **141**, 1207-1215.
- Barker, H., Craig, S. P., Spurr, N. K. and Cohen, P. T. (1993). Sequence of human protein serine/threonine phosphatase 1 gamma and localization of the gene (PPP1C) encoding it to chromosome bands 12q24.1-q24.2. *Biochim. Biophys. Acta* **1178**, 228-233.
- Bohmann, K., Ferreira, J. and Lamond, A. I. (1995). Mutational analysis of p80 coilin indicates a functional interaction between coiled bodies and the nucleolus. *J. Cell Biol.* **131**, 1-15.
- Bohmann, K. (1996). Klonierung und Charakterisierung des Coiled Body Proteins p80 Coilin. PhD thesis, Justus-Liebig-Universität, Giessen, Germany.
- Cao, W., Jamison, S. F. and Garcia-Blanco, M. A. (1997). Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro. *RNA* **3**, 1456-1467.
- Carmo-Fonseca, M., Pepperkok, R., Sproat, B. S., Ansgore, W., Swanson, M. S. and Lamond, A. I. (1991). In vivo detection of snRNP-organelles in the nuclei of mammalian cells. *EMBO J.* **10**, 1863-1873.
- Carmo-Fonseca, M., Pepperkok, R., Carvalho, M. T. and Lamond, A. I. (1992). Transcription-dependent colocalization of the U1, U2, U4/U6 and U5 snRNPs in coiled bodies. *J. Cell Biol.* **117**, 1-14.
- Chen, C. A. and Okayama, H. (1988). Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* **6**, 632-638.
- Claverie-Martin F., Wang, M. and Cohen, S. N. (1997). ARD-1 cDNA from human cells encodes a site-specific single-strand endoribonuclease that functionally resembles Escherichia coli RNase E. *J. Biol. Chem.* **272**, 13823-13828.
- Cohen, P., Alemany, S., Hemmings, B. A., Resink, T. J., Stralfors, P. and Tung, H. Y. L. (1988). Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. *Meth. Enzymol.* **159**, 390-408.
- Duncan, P. I., Stojdl, D. F., Marius, R. M. and Bell, J. C. (1997). In vivo regulation of alternative pre-mRNA splicing by the Clk1 protein kinase. *Mol. Cell Biol.* **17**, 5996-6001.
- Egloff, M.-P., Johnson, D. F., Moorhead, G., Cohen, P. T. W., Cohen, P. and Barford, D. (1997). Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J.* **16**, 1876-1887.
- Fu, X. D. and Maniatis, T. (1990). Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature* **343**, 437-441.
- Gama-Carvalho, M., Krauss, R. D., Chiang, L., Valcarcel, J., Green, M. R. and Carmo-Fonseca, M. (1997). Targeting of U2AF65 to sites of active splicing in the nucleus. *J. Cell Biol.* **137**, 975-987.
- Helps, N. R., Barker, H. M., Elledge, S. J. and Cohen, P. T. W. (1995). Protein phosphatase 1 interacts with p53BP2, a protein which binds to the tumour suppressor p53. *FEBS Lett.* **377**, 295-300.
- Hirano, K., Erdodi, F., Patton, J. G. and Hartshorne, D. J. (1996). Interaction of protein phosphatase type 1 with a splicing factor. *FEBS Lett.* **389**, 191-194.
- Huang, S. and Spector, D. L. (1992). U1 and U2 small nuclear RNAs are present in nuclear speckles. *Proc. Nat. Acad. Sci. USA* **77**, 1311-1315.
- Hubbard, M. J. and Cohen, P. (1993). On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem. Sci.* **18**, 172-177.
- Jagiello, I., Beullens, M., Stalmans, W. and Bollen, M. (1995). Subunit structure and regulation of protein phosphatase-1 in rat liver nuclei. *J. Biol. Chem.* **270**, 17257-17263.
- Jagiello, I., Beullens, M., Vulsteke, V., Wera, S., Sohlberg, B., Stalmans, W., von Gabain, A. and Bollen, M. (1997). NIPP-1, a nuclear inhibitory subunit of protein phosphatase-1, has RNA-binding properties. *J. Biol. Chem.* **272**, 22067-22071.
- Jakes, S., Mellgren, R. L. and Schlander, K. K. (1986). Isolation and characterization of an inhibitor-sensitive and a polycation-stimulated protein phosphatase from rat liver nuclei. *Biochim. Biophys. Acta* **888**, 135-142.
- Johnson, D. F., Moorhead, G., Caudwell, F. B., Cohen, P., Chen, Y. H., Chen, M. X. and Cohen, P. T. (1996). Identification of protein-phosphatase-1-binding domains on the glycogen and myofibrillar targeting subunits. *Eur. J. Biochem.* **239**, 317-325.
- Kambach, C. and Mattaj, I. W. (1992). Intracellular distribution of the U1A protein depends on active transport and nuclear binding to U1 snRNA. *J. Cell Biol.* **118**, 11-21.
- Konarska, M. M. and Sharp, P. A. (1987). Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. *Cell* **49**, 763-774.
- Kanopka, A., Muhlemann, O., Petersen-Mahrt, S., Estmer, C., Ohmalm, C. and Akusjarvi, G. (1998). Regulation of adenovirus alternative RNA splicing by dephosphorylation of SR proteins. *Nature* **393**, 185-187.
- Kreivi, J.-P., Trinkle-Mulcahy, L., Lyon, C. E., Morrice, N. A., Cohen, P. and Lamond, A. I. (1997). Purification and characterisation of p99, a nuclear modulator of protein phosphatase 1 activity. *FEBS Lett.* **420**, 57-62.
- Kuret, J., Bell, H. and Cohen, P. (1986). Identification of high levels of protein phosphatase-1 in rat liver nuclei. *FEBS Lett.* **203**, 197-202.
- Lamond, A. I., Konarska, M. M. and Sharp, P. A. (1987). A mutational analysis of spliceosome assembly: evidence for splice site collaboration during spliceosome formation. *Genes Dev.* **1**, 532-543.
- Lamond, A. I. and Carmo-Fonseca, M. (1993). Localisation of splicing snRNPs in mammalian cells. *Mol. Biol. Rep.* **18**, 127-133.
- Lamond, A. I. and Earnshaw, W. C. (1998). Structure and function in the nucleus. *Science* **280**, 547-553.
- Liu, Q. and Dreyfuss, G. (1996). A novel nuclear structure containing the survival of motor neurons protein. *EMBO J.* **15**, 3555-3565.
- MacKintosh R. W., Dalby, K. N., Campbell, D. G., Cohen, P. T. W., Cohen, P. and Mackintosh, C. (1995). The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. *FEBS Lett.* **371**, 236-240.
- Mermoud, J. E., Cohen, P. and Lamond, A. I. (1992). Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing. *Nucl. Acids Res.* **20**, 5263-5269.
- Mermoud, J. E., Cohen, P. T. and Lamond, A. I. (1994). Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism. *EMBO J.* **13**, 5679-5688.
- Misteli, T. and Spector, D. L. (1996). Serine/threonine phosphatase 1 modulates the subnuclear distribution of pre-mRNA splicing factors. *Mol. Biol. Cell* **7**, 1559-1572.
- Misteli, T., Caceres, J. F. and Spector, D. L. (1997). The dynamics of a pre-mRNA splicing factor in living cells. *Nature* **387**, 523-527.
- Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A. I. and Mann, M. (1998). Combined mass spectrometry and EST-database searching allows rapid characterisation of the multi-protein spliceosome complex. *Nature Genet.* **20**, 46-50.
- Peterson, I., Hinterberger, M., Mimori, T., Gottlieb, E. and Steitz, J. A. (1984). The structure of mammalian small nuclear ribonucleoproteins. Identification of multiple protein components reactive with anti-(U1) ribonucleoprotein and anti-Sm autoantibodies. *J. Biol. Chem.* **259**, 5907-5914.

- Raska, I., Andrade, L. E. C., Ochs, R. L., Chan, E. K. L., Chang, C.-M., Roos, G. and Tan, E. M.** (1991). Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. *Exp. Cell Res.* **195**, 27-37.
- Spector, D. L., Fu, X.-D. and Maniatis, T.** (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO J.* **10**, 3467-3481.
- Stralfors, P., Hiraga, A. and Cohen, P.** (1985). The protein phosphatases involved in cellular regulation. Purification and characterisation of the glycogen-bound form of protein phosphatase-1 from rabbit skeletal muscle. *Eur. J. Biochem.* **149**, 295-303.
- Tazi, J., Dageron, M. D., Cathala, G., Brunel, C. and Jeanteur, P.** (1992). Adenosine phosphorothioates (ATP alpha S and ATP tau S) differentially affect the two steps of mammalian pre-mRNA splicing. *J. Biol. Chem.* **267**, 4322-4326.
- Turner, B. M. and Franchi, L.** (1987). Identification of Protein Antigens associated with the nuclear matrix and with clusters of interchromatin granules in both interphase and mitotic cells. *J. Cell Sci.* **2**, 269-282.
- Van Eynde, A., Wera, S., Beullens, M., Torrekens, S., Van Leuven, F., Stalmans, W. and Bollen, M.** (1995). Molecular cloning of NIPP-1, a nuclear inhibitor of protein phosphatase-1, reveals homology with polypeptides involved in RNA processing. *J. Biol. Chem.* **270**, 28068-28074.
- Vulsteke, V., Beullens, M., Waelkens, E., Stalmans, W. and Bollen, M.** (1998). Properties and phosphorylation sites of baculovirus-expressed nuclear inhibitor of protein phosphatase-1 (NIPP-1). *J. Biol. Chem.* **272**, 32972-32978.
- Wang, M. and Cohen, S. N.** (1994). Ard-1: A human gene that reverses the effects of temperature-sensitive and deletion mutations in the *Escherichia coli* rne gene encodes an activity producing RNase E-like cleavages. *Proc. Nat. Acad. Sci. USA* **91**, 10591-10595.
- Wera, S., Van Eynde, A., Stalmans, W. and Bollen, M.** (1997). Inhibition of translation by mRNA encoding NIPP-1, a nuclear inhibitor of protein phosphatase-1. *Eur. J. Biochem.* **247**, 411-415.
- Xiao, S. H. and Manley, J. L.** (1997). Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. *Genes Dev.* **11**, 334-344.
- Zhao, S. and Lee, E. Y. C.** (1997). A protein phosphatase-1-binding motif identified by the panning of a random peptide display library. *J. Biol. Chem.* **272**, 28368-28372.