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Functional interaction between nuclear inhibitor of protein phosphatase type 1 (NIPP1) and protein phosphatase type 1 (PP1) in *Drosophila*: consequences of over-expression of NIPP1 in flies and suppression by co-expression of PP1

Louise PARKER*[1], Sascha GROSS*, Monique BEULLENS†, Mathieu BOLLEN†, Daimark BENNETT* and Luke ALPHEY*[2]

*Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K., and †Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

The catalytic subunit of type 1 Ser/Thr protein phosphatases (PP1c) forms complexes with many proteins that target it to particular subcellular locations and regulate its activity towards specific substrates. We report the identification of a *Drosophila* orthologue of nuclear inhibitor of PP1 (NIPP1Dm) through interaction with PP1c in the yeast two-hybrid system. NIPP1Dm shares many properties with mammalian NIPP1 including inhibition of PP1c *in vitro*, binding to RNA and PP1c, and localization to nuclear speckles. However, the mechanism controlling interaction of PP1c with NIPP1 is not conserved in *Drosophila*. NIPP1 can function independently of PP1c as a splicing factor, but the relative importance of this function is unknown. Over-expression of NIPP1Dm in *Drosophila* is cell-lethal in a range of tissues and developmental stages. The effects of ectopic NIPP1Dm are suppressed by co-expression of PP1c, indicating that the only effect of ectopic NIPP1Dm is to affect PP1c function. Co-expression of NIPP1Dm and PP1c does not have any detectable physiological effect *in vivo*, suggesting that the NIPP1Dm–PP1c holoenzyme is not normally limiting in *Drosophila*. These data show that NIPP1Dm and PP1c interact *in vivo* and suggest that NIPP1's role as a phosphatase regulator is conserved in *Drosophila*.

Key words: inhibitor, protein phosphatase, regulatory subunit.

INTRODUCTION

Serine/threonine protein phosphatases type 1 (PP1) are a group of enzymes that share a highly conserved catalytic subunit [1,2]. PP1, in common with many protein kinases and phosphatases, is thought to be controlled *in vivo* by association with regulatory proteins which modify its subcellular location and catalytic activity against particular substrates [3–5]. This allows independent control over the different PP1-regulated processes, which include cell-cycle control, glycogen metabolism, RNA processing and muscle function [3,6,7]. Each regulatory subunit binds the catalytic subunit of PP1 (PP1c) and one or more other proteins or subcellular structures and so anchors a proportion of the cell's PP1c to a particular structure or substrate. Most PP1-binding proteins reported to date share a short RVXF motif that mediates binding to the catalytic subunit [6,8]. Although the regulators of PP1 have multiple interactions with the catalytic subunit [4,5], the existence of a common binding site may explain why binding of the regulatory subunits is usually mutually exclusive. The regulatory subunits may also modify the catalytic activity of PP1c against particular substrates. For example, the best-known family of regulatory subunits, the G-subunits, anchor PP1c to glycogen particles and increase the activity of the holoenzyme (PP1G) towards glycogen synthase. Different G-subunits are expressed in different tissues and respond differently to various signals, allowing tissue-specific control of PP1c at the glycogen particle (reviewed in [3]). Clearly, a full understanding of the regulation of different cellular processes by PP1 requires the identification and characterization of the various PP1 regulatory proteins and holoenzyme complexes.

PP1c is found in the cell nucleus as well as the cytosol and has been implicated in several nuclear functions including RNA splicing [9,10], interphase chromatin structure [11] and mitotic exit [12–15]. In mammals, the major nuclear targeting subunits identified so far are nuclear inhibitors of PP1 (NIPP1s) [16,17] and a protein variously known as R111 [16], p99 [18] or PNUTS (putative nuclear targeting subunit of PP1) [19]. NIPP1s were isolated originally from bovine thymus as 16–18 kDa peptides, which acted as extremely potent inhibitors of PP1 activity *in vitro* [20]. Cloning of the cDNA indicated that the NIPP1 gene actually encodes a 38 kDa protein from which the smaller peptides originally purified were derived by proteolytic degradation [21]. Recently a cDNA and corresponding genomic DNA have been cloned from humans encoding a homologue of NIPP1, differing from the bovine gene by only a single amino acid residue [22].

Biochemical analysis of NIPP1 indicates that it is composed of several functional domains. The basic N-terminal module contains a forkhead-associated domain (FHA domain) [23,24] that interacts with phosphorylated CDC5L, a regulator of pre-mRNA splicing and mitotic entry, and another splicing factor, SAP155 [25]. The acidic central third, expressed in bacteria, is a potent

Abbreviations used: Ard, activator of RNA decay; EGFP, enhanced GFP; FHA domain, forkhead-associated domain; FHALP, FHA-like protein; GFP, green fluorescent protein; GST, glutathione S-transferase; NIPP1, nuclear inhibitor of protein phosphatase type 1; NIPP1Dm, *Drosophila* orthologue of NIPP1; PKA, protein kinase A; PP1, protein phosphatase type 1; PP1c, catalytic subunit of protein phosphatase type 1; UAS, upstream activator sequence.

The NIPP1Dm sequences reported here were deposited in the EMBL Nucleotide Sequence Database under accession numbers AJ427611 (cDNA) and AJ427612 (genomic).

[1] Present address: Developmental Biology Center, University of California at Irvine, Irvine, CA 92697-2300, U.S.A.

[2] To whom correspondence should be addressed (e-mail Luke.Alphey@zoo.ox.ac.uk).

inhibitor of PP1c [21]. This central third contains a consensus RVXF motif for binding to PP1 [8], an additional inhibitory PP1-binding site [26] and several phosphorylation sites for protein kinase A (PKA) and protein kinase CK2. As with many PP1 regulatory subunits, the interaction of NIPP1 with PP1c is phosphorylation-dependent; treatment of the holoenzyme, consisting of NIPP1 complexed with PP1c ($PP1N_{NIPP1}$), with PKA or CK2 results in its activation [27,28]. Phosphorylation disrupts the interaction of the RVXF motif with PP1c and also reduces the inhibitory potency of the central domain of NIPP1 [26,28]. The basic C-terminal domain of NIPP1 is identical to the polypeptide Ard-1 (activator of RNA decay) [22,29]. Ard-1 was originally isolated due to its ability to rescue the *rne* mutant of *Escherichia coli*, which lacks RNase E activity. Subsequent evidence indicated that Ard-1 is a rare splice variant of NIPP1 [22]. NIPP1 can bind RNA [17,30] and this binding site has been mapped close to the C-terminus [31]. Ard-1, and some fragments of NIPP1, have endoribonuclease activity but full-length NIPP1 does not [29,31], suggesting that this activity is masked by another part of the protein. NIPP1 also has a role in spliceosome assembly that is unrelated to its ability to bind PP1 and RNA and this function maps to the C-terminal half of the protein [32].

We have identified a *Drosophila* orthologue of NIPP1, NIPP1Dm, in a two-hybrid screen for PP1c-binding proteins. We show that NIPP1Dm shares many biochemical and cell-biological properties with mammalian NIPP1, including binding to RNA and PP1, and localization to nuclear speckles. Sequence conservation identifies functional domains of NIPP1 that correspond to and refine the regions identified by biochemical analysis. However, there are some important differences between NIPP1 and NIPP1Dm that suggest that the role and/or regulation of NIPP1 differ in *Drosophila* and humans. In addition, we have shown a functional interaction between NIPP1Dm and PP1, which suggests that NIPP1Dm can act as a phosphatase regulator *in vivo* in *Drosophila*.

EXPERIMENTAL

Isolation and sequencing of NIPP1Dm

The two-hybrid screen for binding proteins of PP1α87B and PP1β9C, two PP1 catalytic subunit isoforms in *Drosophila*, encoded by two different genes, was performed as described in [33,34]. A full-length NIPP1Dm cDNA was isolated by the method of Alphey [35] and sequenced. The Berkeley *Drosophila* Genome Project (BDGP) lists several genomic P1 clones in chromosome region 53F; four of them cross-hybridized with a NIPP1 cDNA probe (DS00964, DS03108, DS03728 and DS08568) and three did not (DS00078, DS01347 and DS06278). A 6.5 kb *Bgl*II–*Sal*I fragment was subcloned from DS08568 and sequenced.

Construction of glutathione S-transferase (GST)-NIPP1Dm and green fluorescent protein (GFP)-NIPP1Dm

An *Eco*RI site was engineered 5′ of the translation start of NIPP1Dm and the complete NIPP1Dm coding sequence was inserted as an *Eco*RI/*Not*I fragment into the *Eco*RI/*Not*I sites of pGEX-2TK and pUAS-EGFP [36].

Expression and purification of GST-NIPP1Dm

The NIPP1Dm cDNA was subcloned in the pGEX-2TK plasmid. Following transformation of *Escherichia coli* BL21(DE3) cells, expression of the fusion protein was induced with 1 mM isopropyl β-D-thiogalactoside for 2 h. The cells were lysed and the fusion protein was purified as described in [25].

Phosphatase and kinase reactions

Protein phosphatase assays using phosphorylase *a* as a substrate were performed as in [16]. The PP1 concentration used in each assay was 1 nM. Kinase reactions were performed as in [28].

Poly(U)-Sepharose

Poly(U)-Sepharose (Amersham Biosciences) was equilibrated in buffer A, consisting of 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 0.5 mM PMSF, 50 μM 1-chloro-3-tosylamino-7-amino-2-heptanone hydrochloride ('TLCK'), 50 μM L-1,4′-tosylamino-2-phenylethyl-chloromethyl ketone ('TPCK') and 5 μM leupeptin. Binding of NIPP1Dm and PP1c to poly(U)-Sepharose and washing of the column were performed as described in [31] for NIPP1. Protein remaining on the column was eluted as 10 fractions with a 0–1.5 M KSCN gradient. Fractions were collected and analysed by SDS/PAGE and Western blotting and by PP1 phosphatase assays after treatment with trypsin.

Fly strains and crosses

The GAL4 lines *Hsp70-GAL4*, *how24B-GAL4*, *MS1096-GAL4* and *arm-GAL4* were obtained from the Bloomington *Drosophila* Stock Center and are as described by Flybase (http://flybase.bio.indiana.edu). UAS-PP1c constructs (where UAS is upstream activator sequence) contain the coding region of the relevant PP1c in pUAS-HA [36]. Strains carrying UAS-EGFP-NIPP1Dm (where EGFP is enhanced GFP) or UAS-PP1c were created from injected embryos of a *y w* strain. UAS-EGFP is described in [37]. Flies carrying a GAL4 element were crossed with *UAS-EGFP-NIPP1Dm* flies. The F_1 progeny were screened for phenotypic abnormalities and reduced viability. For induction of *Hsp70-GAL4* a 30 min heat shock at 37 °C was applied daily. Otherwise all crosses were carried out at 25 °C.

Immunofluorescence and confocal microscopy

Antibodies to NIPP1Dm were raised against a peptide (AKEAWPGRKPMLGQL) corresponding to the C-terminal residues 368–383 of NIPP1Dm and affinity purified as described in [31]. EGFP-NIPP1Dm was visualized in *UAS-EGFP-NIPP1Dm*/+; *arm-GAL4*/+ embryos. Live 0–16 h embryos expressing EGFP-NIPP1Dm were examined by time-lapse confocal microscopy.

RESULTS

Isolation of NIPP1Dm

A yeast two-hybrid interaction trap (reviewed in [38]) was used to identify potential PP1c-binding proteins using the catalytic subunit encoded by *PP1α87B* as 'bait'. A cDNA library from *Drosophila* third-instar larval RNA was screened, and 25 independent cDNAs from 16 independent genes were isolated which interacted with PP1α87B but not with control baits [33]. One of these cDNAs showed strong sequence similarity to *NIPP1* and *Ard-1* [21,22,29]. A subsequent screen for PP1β9C-binding proteins [34] identified 236 positive clones, of which six were derived from *NIPP1Dm*. We sequenced the ends of all the *NIPP1Dm* two-hybrid positive clones; all share the region between residues 190 and 383, which also corresponds to the shortest positive clone. This region, therefore, contains all the sequences necessary for binding to PP1c in the two-hybrid system. Further two-hybrid analysis of a representative

A

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NIPP1Dm ---MANS------YD IPSWAGKPPTGLHLD VLKDDKLVQKLMVDE KRCYLFGRNSQMNDF CIDHASCSRVHSAFV YHKHLNIAYLVDLGS  81
NIPP1   MAAAANSGSSLPLFD CPTWAGKPPPGLHLD VVKGDKLIEKLIIDE KKYYLFGRNPDLCDF TIDHQSCSRVHAALV YHKHLKRVFLIDLNS  90
FHALP   MEEIDDK--SK--FK VPSWAAKPPNGAHLD VQKGDALIQKLLIDD KKAYYFGRNNKQVDF AVEHASCSRVHALLL YHGLLQRFALIDMDS  86
PinA    --------------- -PEWASKPISNVYLE IIKNGVNIDRVDISK DKFTVFGRSSEVASV LLDHPSVSRRHAALV YHGANNRFYLIDLQS  74

NIPP1Dm THGTFIGTLRLEAHK PTQLQINSTFHFGAS TRNYILRERPS---- GHHSNIMEDLPLSET SD---------- ---------GALLGL  145
NIPP1   THGTFLGHIRLEPHK PQQIPIDSTVSFGAS TRAYTLREKPQ---- TLPSAVKGDEKMGGE DDEL---------- ---------KGLLGL  156
FHALP   SHGTFLGNVRLRPLE VVFMDPGTQFHLGAS TRKYSVRLKTEHHVE DDPTVAASDEQLDPK SQNFQTFRSHICWKL TFISLSFFRHGTLGK  176
PinA    ATGTQVNDEQVKPLT PTTVKENFTFKFGSS SKHFILKGTVNTNPS SSSSSSSSSSEPKT VT-CRHLLVKHQGSR NP-SWRESKITRTKE  162

NIPP1Dm PES-QTELDNL---- ----------TEY NTAHNRRISMLGIDD DTNMRKQNALKQGRR TRNVTFNDEEIVINP EDVDPNVGRFRNLVQ  218
NIPP1   PEE-ETELDNL---- ----------TEF NTAHNKRISTLTIEE G-NLDIQRPKRK-RK NSRVTFSEDDEIINP EDVDPSVGRFRNMVQ  227
FHALP   IQDFWLKLANFKFFE QLLRSFIIKLHQTSY NTAQNRRIPQLPISV E------EARRKKRP RGNVAFLEEEEVINP EDVDPSVGRFRNLVT  260
PinA    RAIAKLNEYRATIIS GSATFEDLAHKNSDC SSAKRGGYLDPFKRGQ MQRPFEDCAFSLKV GEVSGIVDTDSGVHI IERLA----------  207

NIPP1Dm TTVVPAKRARCDVNH MGIHSGNSSLSSANA AHVHQMFQQSLVDMK QQHREMPPPNAVLHS PTNSLYQGLPAEMHG KGDLEPISPLSIGSK  308
NIPP1   TAVVPVKKKRVEG-- ----PG--------- ---------SLVLEE SGSRRMQN-----FA FSGGLYGGLPPTHSE AG-SQPHG--IHGTA  285
FHALP   TAIISTNPNKRPGD- ----PKRS------- -------EPPRKIVR PG-RELMS------A PLSSTFG--PMALNA APDLELYG-------  315
PinA    --------------- --------------- --------------- --------------- ---------------

NIPP1Dm LGLLLPNPAPEVLPV YDEAVETSTLAQKLA VANANVRRFGEDPHD SSGEGDSLCPQKKKY AKEAWPGRKPMLGQL -  383
NIPP1   LIGGLPMPYPNLAPD VDLTPVVPSAVNMNP APNPAVY--N--PEA VN-E-----PKKKKY AKEAWPGKKPTPSLL I  351
FHALP   --KLLPEPG------ -HHQYVTSTAT---- -ED----------EA DD-------HHKKRY AKEAWPGRKPGAGIF -  359
PinA    --------------- --------------- --------------- --------------- ---------------  -
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B

NIPP1Dm domains	% Identity / Similarity over domain		
	NIPP1	FHALP	PinA
8 -118	69 / 85	52 / 74	35 / 66
141 - 168	78 / 89	41 / 58	N/A
182 - 228	57 / 77	51 / 70	N/A
363 - 383	71 / 81	66 / 76	N/A

C

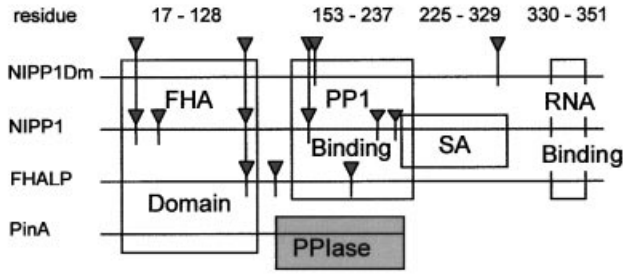

Figure 1 Sequence alignment of NIPP1Dm and related sequences

(**A**) Alignment of NIPP1Dm, NIPP1, FHALP (FHA-like protein from *C. elegans*, accession number Z92839) and PinA (accession number U78757). Residues comprising the core FHA consensus sequence [24] are in bold; the putative PP1-binding region is in bold italics. The regulatory phosphorylation sites flanking the PP1-binding region are boxed in NIPP1 (Ser-199 and Ser-204). Phosphorylation sites for PKA and Src kinases (Ser-178 and Tyr-335 in NIPP1) are also boxed. (**B**) Percentage similarity and identity of NIPP1, FHALP and PinA, over the FHA domain, PP1-binding domain and RNA-binding domain relative to NIPP1Dm. N/A, not applicable. (**C**) A diagrammatic representation of the domains in NIPP1Dm and related proteins. Residue numbers indicate the position of domains in NIPP1. SA is the region of NIPP1 involved in spliceosome assembly [32]. Only a small sequence of 17 residues of NIPP1 within this region (residues 286–302) shows significant homology to NIPP1Dm. Intron positions for NIPP1Dm, NIPP1 and FHALP are shown by arrowheads, NIPP1Dm and NIPP1 share three intron positions (shown by vertical lines), which are located in highly conserved regions of the FHA and PP1-binding domains. FHALP also shares a single intron position with NIPP1Dm and NIPP1, located in one of the highly conserved motifs (SGT motif) of the FHA domain.

NIPP1Dm clone showed that it was capable of binding all four *Drosophila* PP1c isoforms (results not shown). A full-length *NIPP1Dm* cDNA was isolated from an embryonic cDNA library. *NIPP1Dm* was mapped to 53F4-5 on chromosome 2 by *in situ* hybridization to polytene chromosomes. Analysis of the genomic DNA sequence around *NIPP1Dm* revealed that *NIPP1Dm* and *NIPP1* share three intron/exon boundaries, indicating that the genes are derived from a common ancestor.

Sequence comparison reveals important differences between NIPP1Dm and mammalian NIPP1

NIPP1Dm is closely related to mammalian NIPP1 but there are key differences that suggest that NIPP1Dm and NIPP1 may have distinctive functional properties (Figure 1). Sequence comparison of NIPP1Dm with NIPP1 shows high identity in three specific regions: the N-terminal region, the largely acidic central region and the C-terminal domain. The N-terminal region of NIPP1Dm (residues 6–126) is well conserved, sharing 64% identity and 80% similarity with mammalian NIPP1, and contains an FHA domain [23], which has been identified as a phosphopeptide-interacting domain in NIPP1 that interacts with CDC5L and SAP155 in a phosphorylation-dependent manner [24,25]. The PP1 regulatory region of NIPP1Dm, located in the central third, contains two blocks of identity with the mammalian homologue, separated by divergent sequence. This contains a putative RVXF PP1c-binding motif [8]. In NIPP1 the PP1c-binding motif is flanked by two regulatory phosphorylation sites (Ser-199 and Ser-204), which are substrates for PKA and CK2. However, these are both absent in NIPP1Dm and also in FHA-like protein (FHALP), which probably represents a *Caenorhabditis elegans* NIPP1 homologue (Figure 1).

It has been shown that NIPP1 has RNA-binding ability, and that this function is localized to the extreme C-terminal region (residues 330–351) [26]. This corresponds to a highly conserved block at the C-terminus of NIPP1Dm (residues 363–383), which shares 68% identity and 77% similarity with NIPP1. This suggests that mammalian NIPP1 and NIPP1Dm share an RNA-binding function mediated by this motif. The C-terminus of NIPP1 has also recently been implicated in the assembly of C-

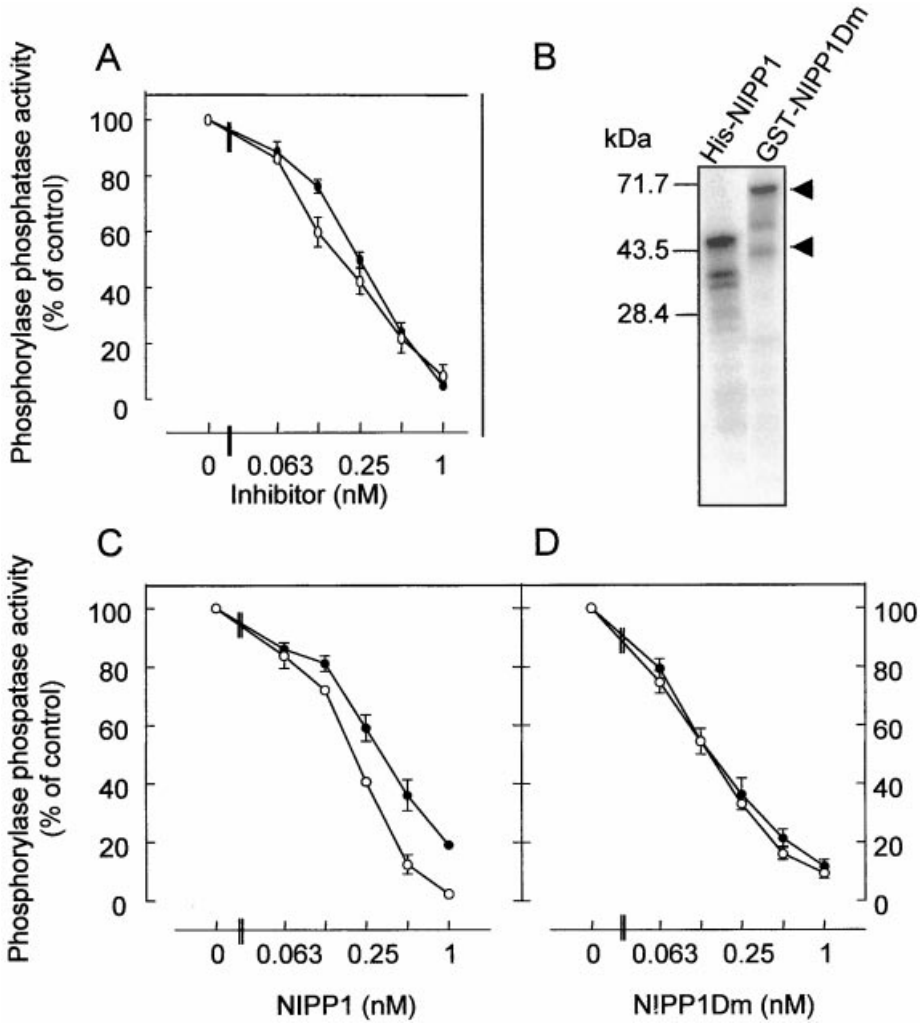

Figure 2 NIPP1Dm is a potent inhibitor of PP1 *in vitro*

(**A**) NIPP1Dm (○) inhibits PP1c activity against phosphorylase *a* as a substrate with an IC_{50} of 0.1–0.2 nM, similar to mammalian NIPP1 (●). Phosphatase activity is plotted as the means ± S.E.M. from four experiments. (**B**) Phosphorylation of purified His-tagged NIPP1 and GST-tagged NIPP1Dm (indicated by arrowheads) by PKA in the presence of 0.1 mM ATP and 1 mM magnesium acetate for 60 min at 30 °C as [27]. (**C** and **D**) NIPP1 and NIPP1Dm were incubated with (●) or without (○) PKA and assayed at various dilutions for their ability to inhibit 1 nM PP1c. In each case the results represent the means ± S.E.M. from three experiments. NIPP1 is inactivated by phosphorylation with PKA (**C**), whereas phosphorylation of NIPP1Dm (**D**) has no effect on NIPP1Dm's inhibitory activity.

type spliceosome complexes. The domain responsible for this function maps to residues 225–329 of NIPP1 (SA domain; Figure 1C) and may constitute an interaction site for a spliceosomal component [32]. NIPP1Dm has 29 extra residues in this region (residues 216–353) that are not present in mammalian NIPP1, and is only 22 % identical with NIPP1 over this domain, which is at the borderline of significance [39]. The only significant homology with NIPP1 in this domain is over a stretch of 17 amino acids (residues 286–302 of NIPP1), of which seven residues are identical in NIPP1Dm and three residues are identical in FHALP.

NIPP1Dm is a potent inhibitor of PP1c

Mammalian NIPP1 was isolated as a potent inhibitor of PP1, with an IC_{50} value of 0.2 nM [26–28,40]. NIPP1Dm, expressed in bacteria as a GST fusion protein, was tested for PP1 inhibitory ability by assaying PP1 activity against glycogen phosphorylase in the presence of various concentrations of NIPP1Dm. NIPP1Dm shows a similar inhibitory effect to that of its mammalian homologue, with an IC_{50} of around 0.2 nM (Figure 2A). GST alone was not inhibitory towards PP1 until 5 μM. Like NIPP1, NIPP1Dm was not inhibitory against PP2A until 5 μM. Addition of an RVXF peptide of NIPP1 [26] in the phosphorylase phosphatase assay with NIPP1Dm abolished the inhibition of PP1 by NIPP1Dm. Inhibition of PP1 by recombinant NIPP1Dm was also abolished by treatment with trypsin (results not shown). Therefore, it is clear that NIPP1Dm protein, and not anything else in our preparation, is a potent and specific inhibitor of PP1.

Phosphorylation of NIPP1 by PKA causes an 8-fold increase in the concentration of NIPP1 required for half-maximal inhibition of PP1c [27]. We found that NIPP1Dm is also a substrate for PKA (Figure 2B). However, unlike the situation for mammalian NIPP1, phosphorylation did not affect NIPP1Dm's activity towards PP1 (Figures 2C and 2D). This clearly indicates

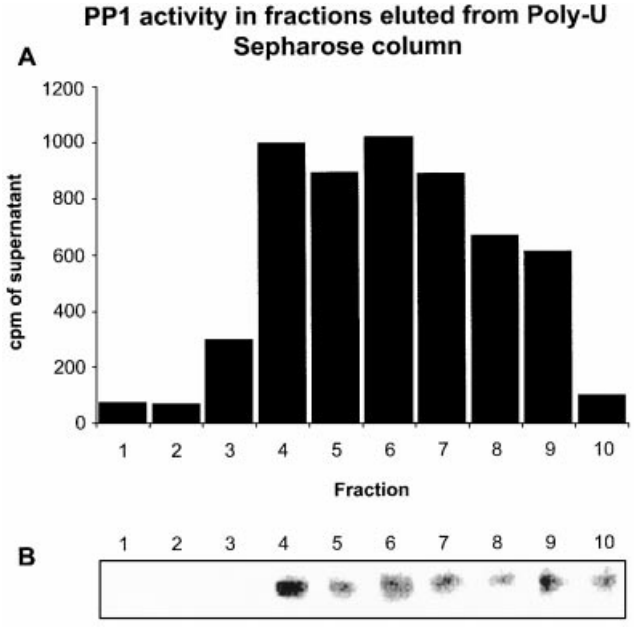

Figure 3 NIPP1Dm can bind simultaneously to PP1c and to RNA

NIPP1Dm and PP1c were applied to a poly(U)-Sepharose column. Bound protein was eluted using a KSCN gradient as 10 fractions. (**A**) PP1 activity of eluate after trypsin treatment, which destroys NIPP1Dm. Fractions 1–10 are successive eluate fractions. PP1 activity (assayed as described in [20]) was highest in fractions 4–7. (**B**) Western blot of fractions 1–10 with anti-NIPP1Dm antibody. NIPP1Dm is only present from fraction 4 onwards, corresponding to the PP1 activity of the fractions. NIPP1Dm is not present in the flow-through and wash fraction, whereas unbound PP1c is present in this fraction (results not shown). Thus NIPP1Dm binds to poly(U)-Sepharose strongly, as it is not found in the flow-through or wash fractions, and also binds simultaneously to PP1c, as high PP1 activity is only found in eluted fractions containing NIPP1Dm.

that the regulation of NIPP1Dm differs from that of mammalian NIPP1 in this respect.

NIPP1Dm binds RNA

To test whether NIPP1Dm has RNA-binding ability, like NIPP1, and if it will bind PP1c and RNA at the same time, recombinant NIPP1Dm and PP1c were incubated for 10 min to allow binding,

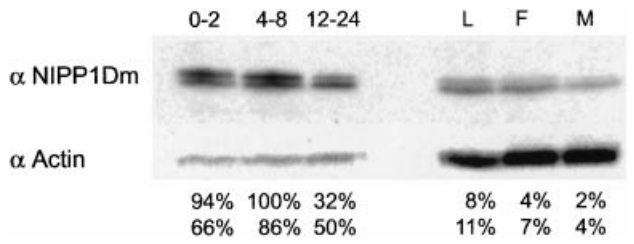

Figure 4 NIPP1Dm developmental expression pattern

NIPP1Dm is expressed in all developmental stages examined. Western blots of protein extracts from 0–2 h (0–2), 4–8 h (4–8) and 12–24 h (12–24) embryos, third-instar larvae (L), adult females (F) and adult males (M) were probed with antibodies against NIPP1Dm or actin as a loading control. Lanes 4–6 were loaded with half of a larva or adult fly. Two immunoreactive proteins of approx. 47–48 kDa were detected in all extracts. The larger band was more prominent in early embryos than third-instar larvae or adults, especially males. The relative intensities of the two bands in each lane are given as percentages below, normalized for loading based on the intensity of the actin signal.

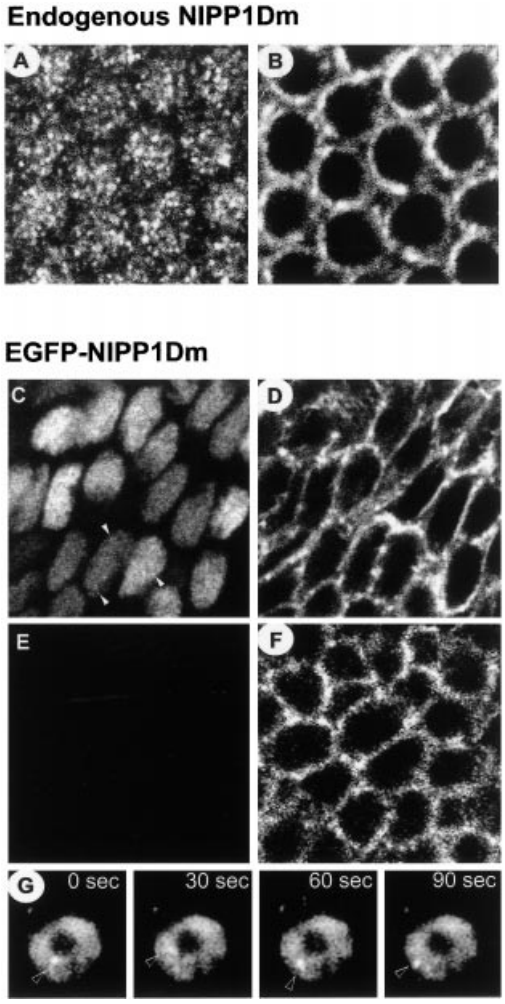

Figure 5 Endogenous NIPP1Dm and EGFP-NIPP1Dm are localized to nuclear speckles

y w embryos at the cellular blastoderm stage were double-stained with anti-tubulin (**B**, **D**, **F**) and anti-NIPP1Dm (**A**) or for EGFP (**C**, **E**). At this stage in development, the nuclei are very close to neighbouring nuclei. Anti-tubulin staining (**B**, **D**, **F**) marks the cytoplasm. Anti-NIPP1Dm staining shows that endogenous NIPP1Dm is predominantly nuclear in a distinct speckled pattern (**A**). EGFP-NIPP1Dm, which was expressed under the control of the *armadillo* promoter (*arm-GAL4*, *UAS-EGFP-NIPP1Dm*), is also localized to nuclear speckles (arrowheads), but in a background of diffuse nuclear localization (**C**). *arm-GAL4* control flies lacking *UAS-EGFP-NIPP1Dm* showed no fluorescence under the same conditions (**E**), demonstrating that the pattern of ectopic NIPP1Dm staining was specific. (**G**) Time-lapse pictures of EGFP-NIPP1Dm in 0–16 h embryos taken at 30 s intervals after photo-bleaching. EGFP-NIPP1Dm speckles move dynamically throughout the nucleus, but not the nucleolus.

and then separated on a poly(U)-Sepharose column. The column was washed, and then the bound components were eluted with a KSCN gradient as 10 fractions. These fractions were then trypsinized to remove NIPP1Dm (this also removes a C-terminal fragment of PP1c, but does not affect its phosphatase activity), and then assayed for phosphorylase *a* phosphatase activity. The fractions were also Western blotted and probed with an anti-NIPP1Dm antibody. As shown in Figure 3, NIPP1Dm bound to the poly(U)-Sepharose column and was not removed by extensive washing. NIPP1Dm also bound PP1c in combination with poly(U)-Sepharose as PP1 activity is only found in those fractions that also contain eluted NIPP1Dm, whereas free PP1c was found

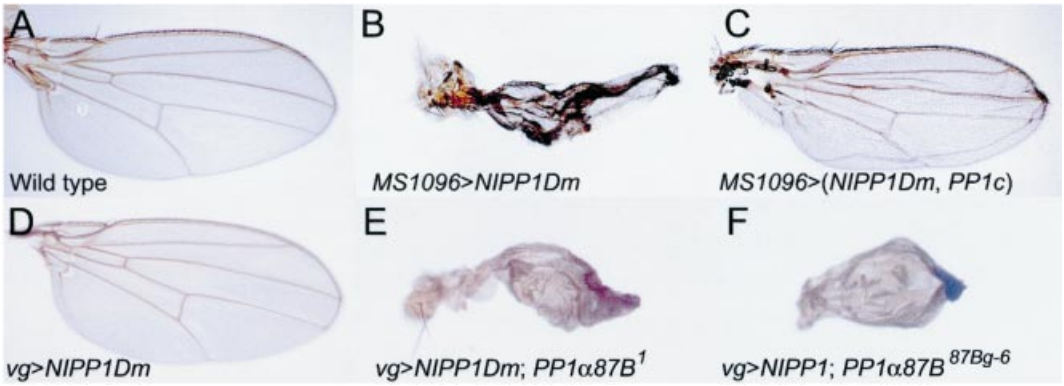

Figure 6 Effect of NIPP1Dm *in vivo*

(**A**) Wild-type adult wing. (**B**) Over-expression of *UAS-EGFP-NIPP1Dm* under the control of the strong wing-specific driver *MS1096-GAL4* leads to severe loss of wing tissue. (**C**) The phenotype is almost completely suppressed by simultaneous over-expression of *UAS-PP1α87B* (or the other PP1c isoforms *UAS-PP1α13C*, *UAS-PP1α96A* and *UAS-PP1β9C*; results not shown), suggesting that the phenotypes observed in (**B**) are due to the titration of endogenous PP1c by EGFP-NIPP1Dm. (**D**) Wings from flies over-expressing *UAS-EGFP-NIPP1Dm* under the control of the weak wing-specific driver *vg-GAL4* (*vg > NIPP1Dm*) resemble the wild type. The effect of *vg > NIPP1Dm* is dominantly enhanced by (**E**) a hypomorphic point mutation in *PP1α87B* and (**F**) a *PP1α87B* protein-null allele, indicating that the effects of NIPP1Dm over-expression are a consequence of a reduction in PP1c function rather than the result of a PP1-independent effect of free NIPP1Dm.

in the flow-through and wash fractions. NIPP1Dm therefore binds RNA and PP1c simultaneously.

Tissue distribution of NIPP1Dm

Human NIPP1 is expressed in all tissues examined [21]. We analysed the expression pattern of NIPP1Dm in embryos, larval brains and imaginal discs, and adult testes by *in situ* hybridization to RNA. NIPP1Dm RNA was expressed in all these tissues (results not shown). In view of the apparent post-transcriptional regulation of mammalian NIPP1 protein levels [41], we analysed the expression of NIPP1Dm protein during *Drosophila* development (Figure 4). NIPP1Dm protein is present in all developmental stages. However, it runs on SDS/PAGE as two bands, the ratio between the two varying at different developmental stages. Since NIPP1 can be phosphorylated both *in vitro* and *in vivo* [16,21], it is possible that these two bands represent different phosphorylation states of NIPP1Dm. Unfortunately we were unable to test this hypothesis directly by treatment with a phosphatase as NIPP1Dm, like NIPP1 [20], is very unstable and invariably degraded during phosphatase treatment.

NIPP1Dm is localized to nuclear speckles

NIPP1 has a speckled nucleoplasmic distribution in mammalian cells where it is co-localized with pre-mRNA splicing factors such as Sm proteins and CDC5L [42,43]. We examined the subcellular localization of NIPP1Dm in *Drosophila* embryos. NIPP1Dm is also found almost exclusively in the nucleus, where it is localized to nuclear speckles (Figure 5). To analyse NIPP1Dm localization in living animals we constructed an EGFP-NIPP1Dm fusion protein and expressed it in embryos. EGFP-NIPP1 was also localized to nuclear speckles, although the pattern was somewhat masked due to the accumulation of EGFP-NIPP1Dm. Nuclear speckles, which correspond to splicing-factor storage sites, have been shown to move throughout the nucleus in response to activation of nearby genes [44]. To assess the movement of nuclear speckles marked by EGFP-NIPP1Dm within the cell nucleus of living *Drosophila* cells, we used time-lapse fluorescence microscopy. Images were taken over 30 min at intervals of 30 s after photobleaching and analysed in video format. EGFP-NIPP1Dm is first localized to speckles but is later found throughout the nucleus, presumably as NIPP1Dm-binding sites become saturated. Tracking single speckles, we found them to move dynamically within the nucleus (Figure 5G, and see http://www.BiochemJ.org/bj/368/bj3680789add.htm). These data show that, like NIPP1, NIPP1Dm is localized to nuclear speckles.

Effect of ectopic NIPP1Dm in flies

Over-expression of NIPP1 has been reported to re-target exogenous PP1 from other sites in the nucleus to nuclear speckles [45]. However, the physiological effect of over-expressing NIPP1 has not been determined. NIPP1 has a PP1-independent role in spliceosome assembly [32], which raises the question of whether NIPP1 acts primarily as a splicing factor or phosphatase regulatory subunit *in vivo*. We examined flies expressing ectopic EGFP-NIPP1Dm using various promoters in the GAL4-UAS system [46]. Expression of EGFP-NIPP1Dm under the control of either the ubiquitous *arm-GAL4* or *Hsp70-GAL4* drivers, or the mesodermal *how24B-GAL4* driver, led to a complete loss of viability in F_1 flies. Expression of NIPP1Dm in non-essential tissues also led to cell lethality. Expression of NIPP1Dm in the wing using *MS1096-GAL4* led to severe loss of wing tissue and the wings appeared severely crumpled (Figure 6). Hence over-expression of NIPP1Dm is cell-lethal in a range of tissues and developmental stages. One possibility is that ectopic NIPP1Dm has an effect on pre-mRNA splicing by affecting the assembly of spliceosome complexes. However, the phenotypes we observed do not suggest an obvious effect on splicing. A more likely explanation is that PP1 is bound and inhibited by ectopic NIPP1Dm.

NIPP1Dm is a potent inhibitor of PP1 *in vitro* (above). Therefore we tested whether over-expression of PP1 could suppress the effect of ectopic NIPP1Dm. Co-expression of NIPP1Dm and PP1c completely rescued the lethality of ectopic NIPP1Dm (Table 1, and results not shown) and strongly suppressed the effect of NIPP1Dm over-expression in the wing (Figure 6). All of the *Drosophila* PP1 isoforms were capable of rescuing the effect of ectopic NIPP1Dm. When we examined flies

Table 1 Lethality of over-expression of NIPP1Dm and suppression by ectopic PP1c

Ubiquitous expression of NIPP1Dm results in lethality (**a**). Female flies carrying *UAS-NIPP1Dm* were crossed with males carrying *arm-GAL4*. Progeny of the genotype *UAS-NIPP1Dm/+*; *arm-GAL4/+*, which express NIPP1Dm at modest levels under the control of *arm-GAL4*, have reduced viability (< 2% surviving to adulthood) compared with their siblings. The effect of ectopic expression of NIPP1Dm is suppressed by co-expression of *Drosophila* PP1c isoforms: PP1α13C, PP1α87B, PP1α96A and PP1β9C (**b**). Females carrying *UAS-NIPP1Dm* were crossed with males containing *arm-GAL4* and *UAS-PP1α13C*, *UAS-PP1α87B*, *UAS-PP1α96A* or *UAS-PP1β9C*. The number of progeny with the genotype *UAS-NIPP1Dm/+*; *arm-GAL4/+*, *UAS-PP1c/+*, expressing both NIPP1Dm and PP1c, did not differ significantly from the number expected from Mendelian segregation.

(a)

♀ *w/w*; *UAS NIPP1Dm/CyO*; +/+ × ♂ *w/Y*; +/+; *arm-GAL4/TM3 Ser*

F_1 genotype	Number of progeny ($\Sigma = 173$)
w/w; *UAS NIPP1Dm/+*; *arm-GAL4/+*	1
w/Y; *UAS NIPP1Dm/+*; *arm-GAL4/+*	1
w/w; *UAS NIPP1Dm/+*; *TM3 Ser/+*	25
w/Y; *UAS NIPP1Dm/+*; *TM3 Ser/+*	23
w/w; *CyO/+*; *arm-GAL4/+*	34
w/Y; *CyO/+*; *arm-GAL4/+*	25
w/w; *CyO/+*; *TM3 Ser/+*	35
w/Y; *CyO/+*; *TM3 Ser/+*	29

(b)

♀ *w/w*; *UAS-NIPP1Dm/CyO*; +/+ × ♂ *w/Y*; +/+; *arm-GAL4*, *UAS-PP1c/TM6B*

F_1 genotype	Number of progeny			
	UAS-PP1β9C ($\Sigma = 398$)	*UAS-PP1α13C* ($\Sigma = 219$)	*UAS-PP1α87B* ($\Sigma = 419$)	*UAS-PP1α96A* ($\Sigma = 206$)
w/w; *UAS NIPP1Dm/+*; *arm-GAL4*, *UAS-PP1c/+*	41	30	42	11
w/Y; *UAS NIPP1Dm/+*; *arm-GAL4*, *UAS-PP1c/+*	32	21	22	14
w/w; *UAS NIPP1Dm/+*; *TM6B/+*	52	17	66	30
w/Y; *UAS NIPP1Dm/+*; *TM6B/+*	46	21	55	18
w/w; *CyO/+*; *arm-GAL4*, *UAS-PP1c/+*	62	37	55	46
w/Y; *CyO/+*; *arm-GAL4*, *UAS-PP1c/+*	72	48	66	36
w/w; *CyO/+*; *TM6B/+*	42	26	50	23
w/Y; *CyO/+*; *TM6B/+*	51	19	63	28

expressing NIPP1Dm and PP1c for other defects, we saw none, indicating that elevating the levels of NIPP1Dm-PP1c in either nuclear speckles or the nucleus as a whole has no deleterious effect. Unlike PP1c, co-expression of EGFP (*UAS-EGFP*) did not modify the phenotype of ectopic NIPP1Dm (results not shown), so this suppression is PP1c-specific and not, for example, due to titration of GAL4 by UAS. These data suggest that the major effect of ectopic NIPP1Dm is to sequester PP1 away from its other essential functions. Another possibility, however, is that non-PP1-dependent functions of NIPP1Dm are responsible for lethality of NIPP1Dm over-expression. Co-expression of PP1 may simply sequester NIPP1Dm and inhibit its other (non-PP1-dependent) functions. To address this, we examined whether different PP1 mutants can enhance the effect of low levels of ectopic NIPP1Dm. Use of two mutants with similar PP1c activity but different protein levels (point mutant and deletion) allows us to distinguish whether the effect of ectopic NIPP1Dm is due to inhibition of PP1 activity or a non-PP1-dependent effect of free NIPP1Dm. These mutants both reduce the level of PP1 catalytic activity, but only the deletion reduces the amount of PP1c protein. In combination with ectopic NIPP1Dm, only the deletion would increase the level of free NIPP1Dm, while both would reduce the level of PP1c catalytic activity. Flies weakly expressing NIPP1Dm using the wing-specific driver *vg-GAL4* (*vg > NIPP1Dm*) in a $PP1^{+/+}$ background are essentially wild type in appearance. Flies heterozygous for *PP1α87B* also have normal wings. We found that both a protein-null *PP1α87B* mutation ($PP1\alpha87B^{87Bg-6}$) and a point mutant with reduced PP1 activity ($PP1\alpha87B^{1}$) enhanced the effect of *vg > NIPP1Dm* (Figure 6), resulting in severely crumpled wings. Therefore, the effect of NIPP1Dm over-expression is to inhibit PP1-dependent functions.

DISCUSSION

In vivo role of NIPP1 and PP1

NIPP1 belongs to an ancient family of PP1 regulatory proteins [47] but also has an essential role in spliceosome assembly that is unrelated to its ability to bind PP1 [32]. We have examined the biological activity of NIPP1Dm in *Drosophila* by over-expressing it. Over-expression of NIPP1Dm resulted in cell lethality in a range of tissues and stages of development. The effect of weak NIPP1Dm over-expression is enhanced by loss of PP1c activity, rather than reduction in levels of PP1c protein, indicating that the effects of ectopic NIPP1Dm are not due to a PP1c-independent role of free NIPP1Dm. Instead, these experiments indicate that the effect of ectopic NIPP1Dm can be attributed to its inhibitory properties on PP1. We found that the cell-lethal effects of ectopic NIPP1Dm can be suppressed by co-expression of PP1c, indicating that NIPP1Dm probably sequesters PP1c away from its other essential functions within the nucleus (and probably throughout the cell) and that exogenous PP1c can restore PP1c levels by titrating additional NIPP1Dm. Co-overexpression of NIPP1Dm and PP1 does not have a detectable physiological effect *in vivo*. Assuming that ectopic PP1 binds ectopic NIPP1, this would suggest that the NIPP1Dm–PP1 holoenzyme is normally in excess. This also implies that there are

mechanisms in place to regulate the activity of the NIPP1Dm–PP1c complex. However, the mechanisms (if any) to control the association of NIPP1Dm with PP1 are clearly unable to compensate for increased levels of NIPP1Dm. Ectopic NIPP1Dm appeared to have no phenotypic effect when co-expressed with PP1c, indicating that it has no PP1-independent effect when over-expressed, for example on splicing. Like mammalian NIPP1, NIPP1Dm localizes to nuclear speckles, suggesting that it probably interacts with splicing factors via its highly conserved FHA domain. The failure to see an effect of ectopic NIPP1Dm on splicing might be because other splicing factors are present at limiting concentrations and not all complexes containing NIPP1Dm are converted into mature spliceosomes. Alternatively it may reflect a functional difference between NIPP1 and NIPP1Dm: the domain of NIPP1 responsible for its role as a splicing factor shows little similarity to the equivalent region of either the *Drosophila* or *C. elegans* proteins, although there is one block of 17 residues in NIPP1 that shows partial homology with NIPP1Dm. Further mapping of the domain in NIPP1 will help to resolve whether this sequence has any functional significance.

Interaction between NIPP1 and PP1

Almost all known PP1c regulatory proteins are thought to bind to PP1c via an RVXF PP1c-binding motif, which may anchor PP1 and enable other sites to stabilize the interaction [4,5]. Analysis of the mammalian NIPP1–PP1c holoenzyme has identified multiple sites of contact between NIPP1 and PP1c [26,40]. The central domain of NIPP1 contains two sites for binding and inhibition of PP1c: a non-inhibitory RVXF-binding motif and an inhibitory sequence, residues 191–200 [26]. Conservation with NIPP1Dm over this short inhibitory region (residues 182–191 in NIPP1Dm) is surprisingly low: only one residue is identical and two are similar (Figure 1). However these residues correspond to three of five key basic residues (residues 193–197 of NIPP1), which are thought to be involved in PP1c inhibition [26,47], suggesting that inhibition relies more on the basic nature of this sequence than amino acid identity. Analysis of the minimal PP1-binding fragments of NIPP1Dm indicates that residues 182–191 are not essential for binding, at least in the yeast two-hybrid system. There are two additional blocks of sequence similarity (residues 167–182 and 200–231 in the human sequence; Figure 1) between fly, worm and mammalian NIPP1 in this central domain that do not correspond to known functions. Residues 200–210 contain the RVXF motif, but the function of the rest of the conserved block is not clear. Both NIPP1$^{191–200}$ and NIPP1$^{191–210}$ peptides have much higher IC_{50} values than full-length NIPP1 or the central domain, which have an IC_{50} of 0.2 nM [20,26], suggesting that these other conserved regions of the central domain are required for optimal inhibition, while not being capable of independent inhibition.

Regulation of NIPP1–PP1 interaction by phosphorylation

Efficient inhibition of the central domain requires the RVXF motif, since mutation of the motif decreases the inhibitory potency more than 1000-fold [40] and disruption of binding to RVXF by phosphorylation of the flanking Ser residues activates $PP1N_{NIPP1}$. Phosphorylation of Ser-199 or Ser-204 prevents binding of the NIPP1 peptide, NIPP1$^{191–210}$, to PP1c in far-Western assays and reduces its inhibitory activity towards PP1c relative to unphosphorylated NIPP1$^{191–210}$ [26]. Phosphorylation at these sites is thought to disrupt interactions with PP1c as a result of electrostatic interaction between a phosphate group and basic residues in the upstream inhibitory binding site (residues 193–197 of NIPP1). Neither of these key phosphorylation sites (Ser-199 or Ser-204) are conserved in NIPP1Dm, although there are other phosphorylation sites for both PKA and CK2. Like NIPP1, NIPP1Dm is subject to phosphorylation *in vitro*. However, phosphorylation of NIPP1Dm does not affect its ability to inhibit PP1c. This indicates that the mechanisms controlling activity of the NIPP1–PP1c holoenzyme differ in *Drosophila* and humans. NIPP1Dm may nevertheless be regulated by phosphorylation *in vivo*. We have observed two forms of NIPP1Dm from *Drosophila* extracts. These could represent phospho- and dephospho- forms of NIPP1Dm, although we cannot rule out the possibility that they are the result of alternative splicing, some other post-translational modification or proteolysis. The two forms differ in their relative abundance during different stages of development: the slower-migrating form being more abundant during early embryogenesis and the faster-migrating form being more abundant later. This raises the possibility that, despite a failure to see an effect of phosphorylation *in vitro*, there might be dynamic regulation of NIPP1Dm–PP1c activity by phosphorylation of NIPP1Dm *in vivo*.

In summary, we have identified a *Drosophila* orthologue of NIPP1, which shares many properties of the mammalian protein. However, the mechanism controlling the interaction between NIPP1 and PP1c is not conserved in *Drosophila*. NIPP1 has a role in spliceosome assembly that is independent from its role as a phosphatase regulator. This function may not be conserved in *Drosophila*, as NIPP1Dm shows very poor homology with NIPP1 over the region responsible for NIPP1's spliceosomal function. Moreover, ectopic PP1 can suppress the lethal effects of NIPP1Dm, which suggests that the only effect of NIPP1Dm over-expression is to inhibit PP1. This interaction demonstrates for the first time that NIPP1Dm and PP1 can functionally interact in *Drosophila*.

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