Purification of protein phosphatase 4 catalytic subunit: inhibition by the antitumour drug fostriecin and other tumour suppressors and promoters

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Abstract Protein phosphatase 4 (PP4) is a protein serine/threonine phosphatase that predominantly localises to centrosomes and plays a role in microtubule organisation at centrosomes. Here, PP4 catalytic subunit has been purified from porcine testis to near homogeneity and a specific activity of 680 mU/mg against phosphorylase *a*. The antitumour drug, fostriecin, inhibits PP4 catalytic subunit (IC_{50} 3 nM) with similar potency to PP2A catalytic subunit (IC_{50} 1.5 nM). PP4 is also inhibited in the nanomolar range by several naturally occurring tumour promoters and toxins, with similar IC_{50} values to those obtained for PP2A. The gene for human PP4 catalytic subunit localises to 16p11.2.

Key words: Protein phosphatase; Centrosome; Microtubule; Fostriecin; Tumor promoter; Chromosome 16

1. Introduction

Reversible phosphorylation of serine and threonine residues is a major mechanism for regulation of cellular processes in eukaryotes. Protein phosphatase 4 (PP4, formerly termed PPX) is a member of the PPP family of protein serine/threonine phosphatases, which includes PP1, PP2A, PP2B (calcineurin) and PP5 [1,2]. Although most closely related to PP2A with 65% amino acid sequence identity, PP4 does not bind the 65 kDa regulatory subunit [3] that forms a tight complex with PP2A catalytic subunit [4]. Furthermore PP4 and PP2A are immunocytologically and functionally distinct. Although PP4 is present in the nucleus and more weakly in the cytoplasm, antibodies to PP4 (but not those against PP2A) intensely stain centrosomes in mammalian and *Drosophila* cells and in *Drosophila* embryos [4,5]. A *Drosophila* mutant carrying a P element in the PP4 gene and possessing reduced levels of PP4 protein in the embryo shows a semi-lethal phenotype, in which microtubule organisation at centrosomes is disrupted, implicating PP4 in the initiation of microtubule growth [5]. In contrast *Drosophila* deficient in PP2A catalytic subunit exhibit a lethal phenotype in which long microtubules radiate from centrosomes but are unconnected to the chromosomes [6]. PP2A (or a closely related okadaic acid sensitive phosphatase) has also been shown to dephosphorylate stathmin/Op18 and thereby maintain a shorter steady-state length of microtubules in vitro [7].

Fostriecin, a novel antitumour antibiotic isolated from *Streptomyces pulveraceus* (subspecies *fostreus*) [8,9] has antineoplastic activity on a wide spectrum of tumour cells in vitro and against L1210 and P388 murine leukaemias in vivo [10,11]. Currently, fostriecin is being evaluated as an anticancer drug in human clinical trials [12]. Early studies showed that a possible target of fostriecin was topoisomerase II which is inhibited with an IC_{50} of 40 μM [13]. However, more recent studies suggest that the antitumour activity of fostriecin may reside in the more potent inhibition of the protein serine/threonine phosphatases PP2A and PP1. Enhanced histone 2A and 3 phosphorylation is associated with fostriecin induced chromosome condensation, and hyperphosphorylation of vimentin in response to fostriecin correlates with intermediate filament reorganisation [14–16]. While the IC_{50} of fostriecin for PP1 was found to be in the micromolar range, the antitumour agent inhibited PP2A with an IC_{50} in the nanomolar range [15,17]. These findings raise the question of whether fostriecin at nanomolar concentrations is specific for inhibition of PP2A or whether it also inhibits related phosphatases, such as PP4. Since PP4 cannot be assayed in cell extracts, we have purified the native PP4 catalytic subunit to near homogeneity from porcine testis and we show that PP4, like PP2A, is inhibited by nanomolar concentrations of fostriecin.

2. Materials and methods

2.1. Materials

The PP2A catalytic subunit and the trimeric $PP2A_1$ complex [18] were purified to homogeneity from bovine heart by Dr S. Hawley and Dr N. Morrice, University of Dundee. Human $PP1\gamma_1$ [19] was expressed in *Escherichia coli* and purified as described in [20]. Fostriecin was generously donated by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA. Okadaic acid was a gift from Dr Y. Tsukitani, Fugisawa Pharmaceutical Company, Tokyo and tautomycin was provided by Dr K. Isona, Institute of Physical and Chemical Research, Saitama, Japan. Microcystin-LR was prepared by Dr L. Lawton, Robert-Gordons University, Aberdeen, UK. Cantharidin was purchased from Calbiochem, Nottingham, UK and calyculin A from Life Technologies, Paisley, UK.

2.2. Purification of PP4 catalytic subunit from porcine testis

Freshly excised porcine testes were placed on ice at the slaughterhouse. All subsequent steps were performed at 0–4°C, except the fast protein liquid chromatography separations were at room temperature. Ten porcine testes (4 kg) were decapsulated after 20 min on ice, minced and homogenised in 1 volume buffer A (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM EGTA, 0.03% (v/v) Brij-35, 0.1% (v/v) 2-mercaptoethanol) containing 0.1 mM phenylmethylsulphonyl fluoride and 1 mM benzamidine then centrifuged at $4200 \times g$ for 30 min. The supernatant was filtered through glass wool and subjected to a 10–30% ammonium sulphate precipitation. The pellet was resuspended in 1 litre buffer A, dialysed against buffer A overnight and centrifuged for 20 min at $100\,000 \times g$. The supernatant was poured through a Buchner funnel containing 20 g of QAE-Sephadex equilibrated in buffer A. The column was washed with 1 litre of buffer A containing 0.1 M NaCl and PP4 eluted with 1.5 litres buffer A con-

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taining 0.4 M NaCl. Solid ammonium sulphate was added to the eluate to bring the final concentration to 40% saturation. After stirring for 30 min, the suspension was centrifuged for 30 min at 4200×*g*. The pellet resuspended in 100 ml buffer A containing 5% (v/v) glycerol (buffer B) and dialysed against buffer B overnight. The dialysate was centrifuged for 20 min at 100 000×*g* and the supernatant chromatographed on a HiLoad 16/10 Q-Sepharose column equilibrated in buffer B. The column was developed with a 300 ml linear gradient of NaCl from 0.1 M to 0.5 M at a flow rate of 5 ml/min and 5 ml fractions collected. Fractions eluting between 265 and 330 mM NaCl containing PP4 (detected here and at subsequent stages by immunoblotting) were pooled, dialysed into buffer B, applied to a 5 ml HiTrap Q-Sepharose column equilibrated in buffer B and concentrated by elution with 8 ml of 0.5 M NaCl. The eluate was dialysed against buffer C (50 mM MOPS (pH 7), 1 mM EDTA, 0.1 mM EGTA, 0.03% (v/v) Brij-35, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol) and applied to a 5 ml HiTrap heparin-Sepharose column equilibrated in buffer C. The flowthrough containing PP4 was applied to a 5 ml HiTrap SP-Sepharose column equilibrated in buffer C. Again, the flowthrough containing PP4 was dialysed against buffer B and chromatographed on a HR 5/5 Mono-Q column equilibrated in buffer B. The column was developed with a 25 ml 0–0.5 M NaCl linear gradient at a flow rate of 1 ml/min and 0.5 ml fractions collected. Fractions containing PP4 eluting at 250–300 mM NaCl were pooled, concentrated to 200 μl in a Centricon-30 and subjected to gel filtration on a HR 10/30 Superose 6 column equilibrated in buffer B containing 0.4 M NaCl. The peak PP4 fractions were concentrated to 200 μl in a Centricon-30 and subjected to gel filtration on a HR 10/30 Superose 12 column equilibrated in buffer B containing 0.4 M NaCl. Fractions containing PP4 were snap frozen in liquid nitrogen and stored at −70°C.

2.3. Immunoblotting

Protein extracts were fractionated by 10% polyacrylamide/sodium dodecyl sulphate gel electrophoresis and stained for protein using silver stain [21] or transferred to nitrocellulose and probed with 0.1 μg/ml affinity purified anti-PP4 antibodies. Antibody binding was detected using anti-sheep IgG antibodies conjugated to horseradish peroxidase, followed by enhanced chemiluminescence (Amersham, UK). Rabbit PP4 expressed in *E. coli* [4] was used as a control on immunoblots. Anti-PP4 antibodies were raised against bacterially expressed glutathione *S*-transferase (GST) fused to the N-terminal 57 amino acids of human PP4 and affinity purified against bacterially expressed PP4 by Dr N. Helps. Anti-PP6 antibodies were produced against bacterially expressed GST fused to the N-terminal 55 amino acids of *Drosophila* PPV 6A (homologue of mammalian PP6) and affinity purified against the N-terminal 55 amino acids by Dr H. Snaith. Anti-PP1 antibodies were raised and affinity purified against PP1γ. Antibodies were raised in sheep at the Scottish Antibody Production Unit, Carluke, Lanarkshire, UK. After affinity purification they detected ≤1 ng of their respective antigen and the appropriate phosphatase band in mammalian tissue extracts. Anti-PP2A antibodies were from Promega Corp., Madison, WI, USA, and detected ≤0.1 ng PP2A catalytic subunit.

2.4. Protein phosphatase assays

Rabbit skeletal muscle glycogen phosphorylase (prepared by Ms F. Douglas) was ^{32}P-labelled by phosphorylase kinase to a stoichiometry of 1 mol phosphate per mol subunit as in [18] by Dr L. Tung and was used at 10 μM in the assays. Partially hydrolysed bovine casein was phosphorylated by cyclic AMP-dependent protein kinase (PKA) to 3.5 nmol phosphate per mg protein [22] and was present at 6 μM in the assays. Protein phosphatase assays were performed in the absence of divalent cations [18], except for PP1γ assays which contained 1 mM Mn^{2+}. One unit of activity is the amount of enzyme which catalyses the release of 1 μmol of [^{32}P]phosphate per minute. Inhibitors were incubated with the phosphatase diluted in the assay buffer for 10 min prior to the addition of substrate. Fostriecin was dissolved in phosphate buffered saline containing 0.1 mM ascorbic acid. Okadaic acid, cantharidin, calyculin A and tautomycin were dissolved in dimethyl sulphoxide and microcystin in methanol. All inhibitors were diluted in assay buffer immediately before use. At these dilutions, none of the solvents or the phosphate buffered saline containing 0.1 mM ascorbic acid inhibited any of the phosphatases.

2.5. Human gene mapping

DNA from human-rodent hybrid somatic cell lines with chromosome contents described in [23,24] was analysed for the presence of the PP4 gene by the polymerase chain reaction (PCR). Human PP4-specific oligonucleotides encoding amino acids 177–184 and complementary to nucleotides encoding amino acids 224–233 were used to amplify a section of the PP4 coding region, which contained an intron [25]. The PCR reactions were analysed by gel electrophoresis and ethidium bromide staining, followed by Southern blotting and hybridisation with the ^{32}P-labelled oligonucleotide complementary to nucleotides 642–665 encoding amino acids 216–222.

^{32}P-Labelled human PP4 cDNA [25] was used to screen a yeast artificial chromosome (YAC) library constructed from human lymphoblastoid cell line DNA [26,27]. Three positive YAC clones were analysed further by PCR with PP4-specific oligonucleotides which confirmed that all three contained the human PP4 gene. Genomic DNA from one PP4 positive YAC clone was used for fluorescent in situ hybridisation to human metaphase chromosomes.

3. Results

3.1. Isolation of a native active protein phosphatase 4 catalytic subunit from porcine testis

There is no specific assay for PP4 that can distinguish it from many other members of the PPP family, and therefore PP4 was detected throughout the purification with anti-PP4-specific antibodies. Table 1 shows the 3100-fold purification of PP4 catalytic subunit from porcine testes, the amounts of PP4 at each step being estimated semi-quantitatively by immunoblotting using known quantities of bacterially expressed PP4 as standards. The final purification step was gel filtration on Superose 12 which showed that PP4 eluted essentially as a single silver stained band in fractions 71 and 72 (Fig. 1 shows fraction 72) with an apparent molecular mass of 35 kDa, indicating that the free catalytic subunit was a monomer. A symmetrical peak of phosphorylase phosphatase activity was observed (Fig. 1, top panel). Digestion of the 35 kDa band with trypsin and examination of the peptides by matrix-assisted laser desorption/ionisation mass spectrometry (performed by Dr N. Morrice) confirmed that it was indeed PP4. The specific activities of porcine PP4 in fractions 70, 71 and 72

Table 1
Purification of protein phosphatase 4 catalytic subunit from porcine testis

Step	Total protein (mg)	Total PP4 (μg)	Purification (fold)	Yield (%)
Extract	110 000	28 000	1	100
10–30% ammonium sulphate fractionation	30 000	10 000	2	36
QAE-Sepharose	3 000	4 000	5	15
Q-Sepharose	550	1 300	10	4.6
Heparin and SP-Sepharose	155	1 200	31	4.3
Mono-Q	70	900	52	3.2
Superose 6 (fr. 89–93)	1.2	300	1000	1.1
Superose 12 (fr. 70–72)	0.11	85	3100	0.3

were 400, 680 mU/mg and 500 mU/mg towards phosphorylase respectively, and the average specific activity of porcine PP4 in fractions 70–72 was 530 mU/mg using phosphorylase and 420 mU/mg using casein as substrate. Immunoblotting of PP4 fractions from the Superose 6 column showed that they were free of PP1, PP2A and PP6 (data not shown).

3.2. Inhibition of PP4 by tumour suppressors and tumour promoters

Native porcine PP4 catalytic subunit, assayed using phosphorylase as substrate, was inhibited by the antitumour drug fostriecin with an IC_{50} of 3 nM, while the IC_{50} for the native PP2A catalytic subunit (Fig. 2) and the $PP2A_1$ complex was 1.5 nM (data not shown). Similar results were obtained with casein as substrate, the IC_{50} of PP4 for fostriecin being 3 nM (data not shown). In contrast the IC_{50} of PP1γ for fostriecin was 45 μM with phosphorylase as substrate. Inhibition of PP4 by several other tumour suppressors, promoters or toxins was also virtually identical to that observed with PP2A (Fig. 2). PP4 was inhibited by okadaic acid, microcystin-LR, calyculin A tautomycin, and cantharidin with an IC_{50} of 0.1 nM, 0.15 nM, 0.2 nM, 0.4 nM and 50 nM, respectively.

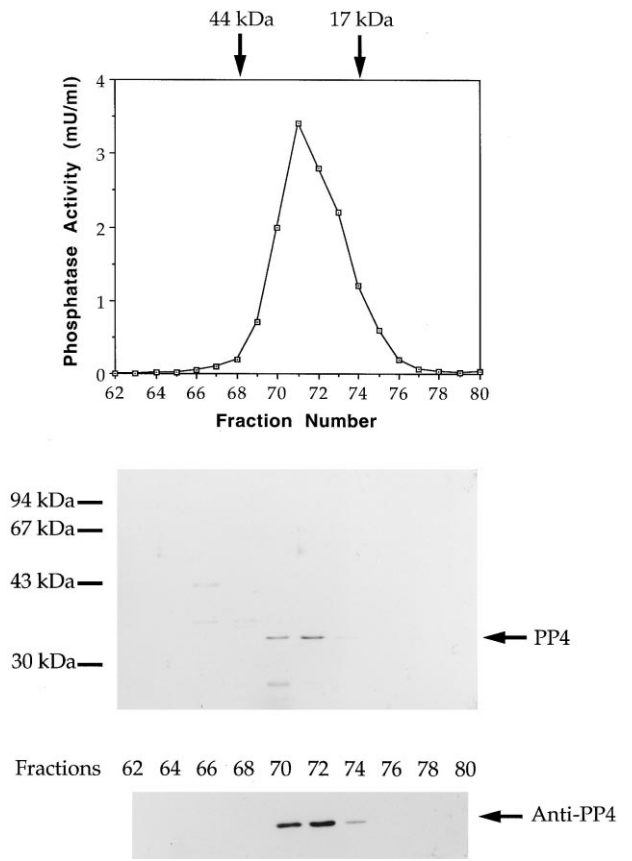

Fig. 1. Purification of PP4 by gel filtration on Superose 12. A: Phosphorylase phosphatase activity detected in the Superose 12 fractions. The molecular mass markers were ovalbumin (44 kDa) and myoblobin (17 kDa). B: Polyacrylamide/sodium dodecyl sulphate gel electrophoresis of the Superose 12 fractions. Protein bands were detected using silver stain. The position of PP4 is indicated by an arrow. Molecular size markers are given in kDa. C: Immunoblot of the Superose 12 fractions probed with antibodies to PP4.

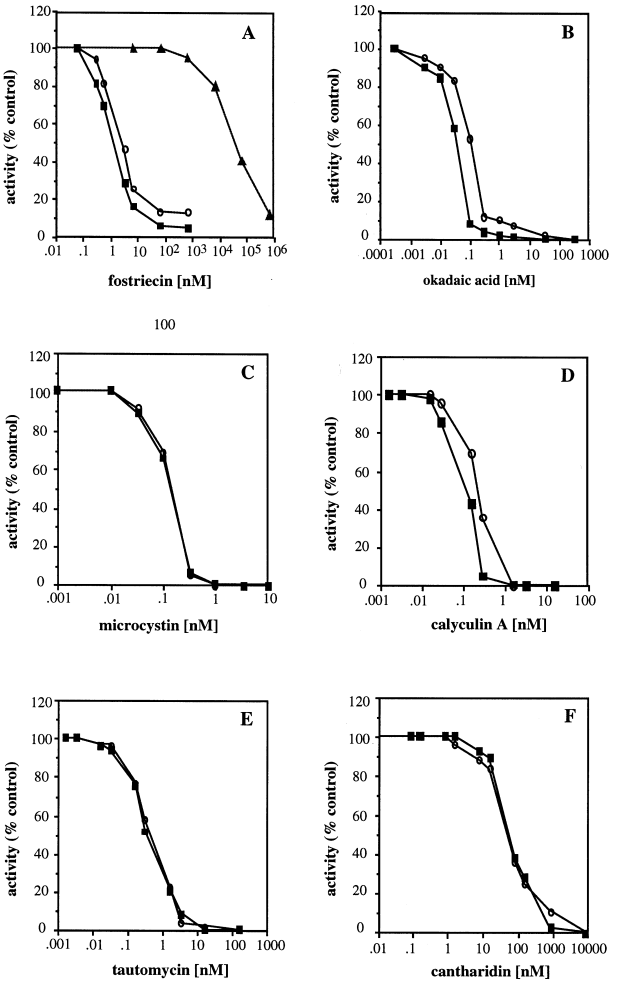

Fig. 2. Effect of tumour suppressors/promoters and toxins on protein phosphatases. Panel A shows the inhibition of PP4, PP2A and PP1γ by fostriecin. Panels B–F show the inhibition of PP4 and PP2A by okadaic acid, microcystin-LR, calyculin A, tautomycin and cantharadin, respectively. PP4 catalytic subunit from Superose 12 fraction 71 (○), PP2A catalytic subunit (■) and PP1γ (△) were assayed at 0.2 mU/ml using phosphorylase as substrate.

3.3. Mapping of the PP4 gene in the human genome

An approximately 0.7 kb PP4-specific band was detected in human but not in rodent (rat, hamster or mouse) DNA by PCR using human PP4-specific primers followed by hybridisation with a PP4-specific probe. Rodent-human somatic cell hybrid DNA from cell lines Dur4R3, Dur4.3, Horp 27RCL4, SIRii, FG10, 1αA9, HM76Dd, Fst9/10, MCP6, 5647Cl22, SIF4A31, TWIN19D12, Mog3.4.A4, LSR8, 2860H7 and Mog13/22 was analysed similarly by PCR and showed that the presence of the 0.7 kb human PP4-specific product was concordant with the presence of chromosome 16. Since the somatic cell hybrid Mog13/22 contained only the short arm of chromosome 16 (in addition to chromosomes 1, 22 and X), this localised the PP4 gene to 16p [28]. A YAC clone containing PP4 genomic DNA was used to localise the PP4 gene by fluorescent in situ hybridisation to position 16p11.2 (performed by Ms S. Marsh and Dr N. Carter, The Sanger Centre, Wellcome Trust Genome Campus, Cambridge). Although a second weaker signal was seen at 16p12 in some chromosome spreads, this is probably artifactual since Southern blotting of human genomic DNA only revealed a single

band hybridising to the PP4 human cDNA in *Eco*RI, *Bam*HI and *Hin*dII digests. In addition, a single PP4 gene has been partially sequenced [29] and the sequence corresponds to the cDNA sequence described in [25].

4. Discussion

The data presented here show that the antitumour drug fostriecin is a potent inhibitor of the native PP4 catalytic subunit with an IC_{50} of 3 nM. The inhibition is virtually identical to that observed with PP2A (Fig. 2 and [17]). In contrast the IC_{50} of fostriecin for PP1γ was 45 μM (Fig. 2) and fostriecin was reported to have no effect on PP2B activity [17]. The mechanism of action of fostriecin as an antitumour drug could therefore be by inhibition of either PP4, PP2A or both. No specific in vivo substrates of PP4 are known, but the fact the fostriecin inhibits PP4 similarly with phosphorylase or casein (data not shown) indicates that the inhibition is not likely to be substrate-specific. The results raise the possibility that fostriecin might act by inhibition of microtubule organisation and dynamics, since both PP4 and PP2A are involved in the regulation of these processes (see Section 1). Other antitumour drugs are known to interfere with microtubule growth or stability [30].

The high affinity binding site of fostriecin would be expected to lie in the regions of PP2A and PP4 that are identical to each other but different from PP1 and PP2B. This may also be close to the active site, since fostriecin is a phosphate ester (containing an unsaturated lactone and conjugated triene), which may occupy the active site as would a phosphorylated substrate.

Okadaic acid, microcystin, calyculin A and cantharidin have been reported to promote or suppress tumour formation depending on the duration of the toxin application or the cell type (reviewed in [31]). All these toxins and tautomycin are found to inhibit native PP4 and PP2A at very similar concentrations. With the recognition that several members of the PPP family are inhibited by these toxins, it is possible that some of the variability of action depends on the relative levels of the phosphatases in each cell type together with the fact that each enzyme may regulate several functions. Although several of these toxins have proved useful in identifying physiological processes that are regulated by reversible protein serine/threonine phosphorylation and distinguishing which processes are likely to be controlled by PP1 [32], it is clear that none of them distinguish PP2A from PP4 (and probably PP6 as well). Caution must therefore be taken in ascribing a function to PP2A (or PP4) solely on the basis of inhibition by these toxins.

Attempts to express PP4 catalytic subunit in *E. coli* in an active form have been unsuccessful and the level of expression of soluble active PP4 catalytic subunit from baculovirus in insect cells is extremely low [4] making complete separation from the endogenous insect cell protein serine/threonine phosphatases difficult. For these reasons, we decided to purify PP4 catalytic subunit from testis, where the highest levels of PP4 mRNA and protein are found [4,33]. The native PP4 catalytic subunit purified here to near homogeneity has a specific activity of 680 mU/mg against phosphorylase, which is higher than that obtained with baculovirus expressed PP4 (150 mU/mg). From the level of PP4 (7 mg/kg tissue), it can be estimated that the intracellular concentration of PP4 is 0.4 μM in testis, which compares to about 1 μM for PP2A in skeletal muscle [18]. However, although immunoblotting shows that PP2A levels are fairly similar in most tissues, PP4 is approximately five-fold less abundant in liver and 20-fold less abundant in skeletal muscle than in testis (data not shown) indicating that the ratio of PP4/PP2A may be approximately 1/10 in liver and 1/50 in skeletal muscle.

Lack of expression of soluble active PP4 in *E. coli* may be either because a chaperone is required to fold the nascent protein as observed for PP1 [20] or because post-translational modification is essential. PP4, like PP2A, terminates in the sequence DYFL. The C-terminal leucine is carboxymethylated in PP2A [34,35] and PP4 [33]. Although this modification does not appear to be essential for activity in PP2A, it is possible that it is required for protein folding. Alternatively, carboxymethylation may influence interactions with regulatory subunits. Partial purification shows that, like PP2A, most of the PP4 in tissue extracts is found in higher molecular mass complexes (Hastie et al., unpublished data). However, fostriecin inhibits the trimeric form of PP2A (these studies and [17]) similarly to the catalytic subunit of PP2A, and therefore it is extremely likely that the complexed forms of PP4 will be inhibited similarly to the free PP4 catalytic subunit.

Since several tumour suppressors and promoters inhibit PP4, it is relevant to consider whether any neoplasms show alterations that could involve the PP4 gene at 16p11.2 (this paper and [28,36]). Liposarcomas mapping to 16p11 have been shown to result from translocations involving the FUS transcription factor gene, mapping to this location [37]. Other less common rearrangements at 16p11 have only been reported in sporadic neoplasms [38].

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