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PPM1D430, a Novel Alternative Splicing Variant of the Human PPM1D, can Dephosphorylate p53 and Exhibits Specific Tissue Expression

Yoshiro Chuman, Wataru Kurihashi, Yohei Mizukami, Takehiro Nashimoto, Hiroaki Yagi and Kazuyasu Sakaguchi*

Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo, 060-0810, Japan

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PPM1D is a PPM1 type protein phosphatase and is induced in response to DNA damage. PPM1D-deficient mice show defects in spermatogenesis and lymphoid cell functions but the mechanisms underlying these phenotypes remain unknown. In our current study, we identify and characterize an alternative splicing variant (denoted PPM1D430) of human PPM1D at both the mRNA and protein level. PPM1D430 comprises the common 420 residues of the known PPM1D protein (PPM1D605) and contains a stretch of PPM1D430-specific 10 amino acids. Semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis revealed that PPM1D430 mRNA is also induced in response to the genotoxic stress in a p53-dependent manner. *In vitro* phosphatase analysis and PPM1D430-specific RNA interference analysis further indicated that PPM1D430 can dephosphorylate Ser15 of human p53 both *in vitro* and *in vivo*. On the other hand, expression profiling of this gene by RT–PCR analysis of a human tissue cDNA panel revealed that PPM1D430 is expressed exclusively in testes and in leucocytes whereas PPM1D605 is ubiquitous. In addition, PPM1D430 shows a different subcellular localization pattern and protein stability when compared with PPM1D605 under some conditions. Our current findings thus suggest that PPM1D430 may exert specific functions in immune response and/or spermatogenesis.

Key words: alternative splicing variant, p53, phosphatase, PPM1D, tissue-specific expression.

Abbreviations: ADR, adriamycin; ATM, ataxia-telangiectasia-mutated; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRG, Heregulin; hPPAR γ, human peroxisome proliferator-activated receptor γ; IR, ionizing radiation; MDM2, mouse double minute 2; NLS, nuclear localization signal; NMD, nonsense-mediated decay; PP2C, Protein Phosphatase type 2C; PPM, Mg^{2+}- or Mn^{2+}-dependent protein phosphatase; PPM1D, Protein Phosphatase Magnesium-dependent 1, delta; S(P), phosphorylated Serine; UNG2, uracil DNA glycosylase 2.

Protein phosphorylation is regulated by kinases and phosphatases and is central to widely divergent cellular processes including proliferation, differentiation, migration, survival and metabolism. PPM1D (Wip1, PP2Cδ) is a member of the PPM1 type (former as PP2C) protein phosphatases and was originally identified as a p53-inducible protein phosphatase in response to DNA damage by ionizing radiation (*1*). PPM1D has been shown to function as a negative regulator of the p38–p53 pathway through its ability to attenuate the phosphorylation of Thr180 in p38 and Ser15 in p53 (*2*, *3*). In addition, many cell cycle regulatory proteins, including Chk1, Chk2, UNG2, ATM and MDM2 are dephosphorylated and thus inactivated by PPM1D (*3–8*). One of the major functions of PPM1D is thought to be the reversal of the p53 and Chk1-induced DNA damage and cell cycle check point responses, which returns the cell to a homeostatic state following the completion of DNA repair (*3*).

The gene amplification of PPM1D has been observed in several human neoplasias, including breast cancer (*9–11*), neuroblastoma (*12*), medulloblastoma (*13–15*), ovarian clear cell adenocarcinoma (*16*), gastric carcinoma (*17*) and pancreatic adenocarcinoma (*18*). In addition, PPM1D-deficient mice are resistant to the emergence of spontaneous tumours, suggesting that this phosphatase is an oncoprotein (*19–21*). Moreover, the inhibition of PPM1D can reduce the viability of human tumour cells, and this effect is specific for PPM1D-overexpressing cells suggesting that PPM1D-specific inhibitors may successfully target cancer cells only and have minimal side effects (*22*). So far several such inhibitors, both chemical and peptide based, have now been reported (*23–26*).

The attention has recently focused on the role of PPM1D in the immune system and in spermatogenesis as mice deficient in this gene exhibit both immunological and spermatozoa defects (*20*). In particular, both B and T cells show a partially compromised function upon mitogenic stimulation in the PPM1D null mouse. Furthermore, PPM1D has been reported to play a role in the double negative to double positive transition during αβ T-cell

*To whom correspondence should be addressed. Tel: +81-11-706-2698, Fax: +81-11-706-4683, E-mail: kazuyasu@sci.hokudai.ac.jp

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development *via* the down-regulation of the p53 tumour suppressor (*27*). Shreeram *et al*. (*28*) have also reported that the onset of myc-induced B-cell lymphoma is dramatically delayed in PPM1D-null mice in an ATM- and p53-dependent manner. However, the precise roles of PPM1D in the B- and T-cell responses following mitogen stimulation and in spermatogenesis remain to be fully clarified.

Alternative splicing plays a central role in modulating gene function by expanding the diversity of expressed mRNA transcripts. Bioinformatic analyses have now indicated that 35–65% of human genes undergo some form of alternative splicing (*29*, *30*). Furthermore, the aberrant regulation of alternative splicing has been implicated in human diseases (*31*, *32*).

Thus far, 16 different human PPM1 family members have been identified of which some have been shown to have alternative splicing variants. These include two isoforms of PPM1A (PP2Cα) (*33*) and five of PPM1B (PP2Cβ) (*34–36*). During the cloning of PPM1D from breast cancer MCF7 cells, we had identified a longer cDNA than putative size and evaluated whether there are alternative splicing variants in human PPM1D. In our current study, we describe for the first time an alternative splicing variant of human PPM1D (PPM1D430). PPM1D430 retains its dephosphorylation capability but it shows different properties from the known PPM1D protein (PPM1D605) in terms of its subcellular localization and tissue distribution. Interestingly, the strong and specific expression of PPM1D430 transcripts in testes and in lymphocytes indicates an important role for this phosphatase isoform in spermatogenesis and in the immune response. Our current findings thus suggest that PPM1D430 may exert different biological functions from PPM1D605.

MATERIALS AND METHODS

Cell Lines—MCF7, H1299 cells were obtained from ATCC (Rockville, MD, USA) and T47D cells, MDA-MB-231 cells from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 nM glutamine and penicillin/streptomycin in a humidified atmosphere with 5% CO_2.

Reverse transcription–polymerase chain reaction—Reverse transcription–polymerase chain reaction (RT–PCR) was performed on obtained mRNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo dT (Amersham Pharmacia Biotech, Uppsala, Sweden). Below are indicated, respectively, the number of cycles, annealing temperature and the sequences of 5′ and 3′ primers used for each of the tested genes. For the detection of the expression of PPM1D605 in MCF7 cells: 30 cycles, 68°C, using 5′-GAGGCAGCGTATGCTCCGAGCAGATAACACTAGTGC CATAGT-3′ and 5′-GACTACACCTTGGACATTCTCTCGA GCTATCTCAGCTGAAACCTC-3′. For the detection of the expression of the PPM1D430: 30 cycles, 68°C, using 5′-GAGGCAGCGTATGCTCCGAGCAGATAACACTAGTGCC ATAGT-3′ and 5′-GCTGTACACATGGCTGGCATTCAGAT TAGTATTGATGTAAAGATGGCTCTCTG-3′. DNA fragments of full-covered open reading frame (ORF) for each PPM1D variants were amplified using following primers; 5′-GGAATTCCATATGGCGGGGCTGTACTCGCTG-3′ (as sense primer), 5′-TCGCGTCGACAAGTTCAACATCGGCA CC-3′ (as anti-sense primer for PPM1D605), 5′-TCGCGTC GACATTAGTGCTGTACAC-3′ (as anti-sense primer for PPM1D430). PPM1D605 and PPM1D430 were subcloned into the *Nde*I/*Sal*I site of pColdI vector (Takara, Otsu, Japan). The cloned cDNAs were confirmed by DNA sequence analysis.

Quantification of PPM1D Splicing Variant mRNA Levels by Semi-quantitative PCR—Total RNA obtained from MCF7 and T47D cells were reverse-transcribed by adaptor-conjugated primers specific for each splicing variant: 5′-GGCCACGCGTCGACTAGTACCTCCAGTGA CTTG-3′ for PPM1D605 and 5′-GGCCACGCGTCGACT AGTACCAAATCCAAAATCCTTG-3′ for PPM1D430. All obtained cDNAs were amplified by touch-down PCR method using following primers: 5′-GGCCACGCGTCGA CTAGTAC-3′ (adaptor primer as anti-sense primer) and isotope-labelled 5′-TCAATGTGCCAGGACCAAGAG GAG-3′ (as consensus sense primer). PCR products were resolved on an 8% acrylamide and expression level of PPM1D variants were quantified by BAS-1800II (Fujifilm, Tokyo, Japan). The amount of cDNAs were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression level using following primers; isotope-labelled 5′-GAAGGTGAAGGTCGGAGT C-3′ and 5′-GAAGATGGTGATGGGATTTC-3′.

Plasmid and DNA Transfection—ORFs of PPM1D605 and PPM1D430 were cloned into phCMV2 vector (Gene Therapy Systems, Inc., San Diego, CA, USA) for expression of HA-tagged protein in mammalian cells. A series of EGFP-PPM1D605 constructs were subcloned into *Hin*dIII/*Sal*I sites of pEGFP-C3 vector (Clontech, Palo Alto, CA, USA). The cloned cDNAs were confirmed by DNA sequence analysis.

Phosphatase Assay—Phosphatase activity was carried out as described (*24*). Phosphorylated p53 peptide analogues as substrates are as follows: p53(10–23)15P: Ac-VEPPLS(P)QETFSDLW-NH_2; p53(10–23)15P-3K: Ac-VK PPLS(P)QKTFSKLW-NH_2; p53(10–23)15P-TD: Ac-VEPPLS (P)QEDFSDLW-NH_2; p53(10–23)15P-3E: Ac-VEPPLS(P) QEEEEDLW-NH_2. All assays were carried out in Tris buffer (50 mM Tris–HCl pH 7.4, 30 mM $MgCl_2$, 0.1 mM EGTA, 0.02% 2-mercaptoethanol) by incubation with phosphopeptide and PPM1D430 derivatives (4 nM) for 10 min at 30°C.

Antibodies and Western Blotting—Mouse monoclonal antibody (MoAb) 6E2 for HA tag and anti-phospho p53(Ser15)(16G8) were purchased from Cell Signaling Technology Inc., Beverly, MA, USA. Mouse monoclonal anti-p53 (DO-1) was purchased from Santa Cruz Biotechnology, Santa Cruz, California, USA. Mouse monoclonal anti-actin (Ab-1) was from Calbiochem, San Diego, CA, USA. Rabbit polyclonal antibodies specific for PPM1D605 and PPM1D430 were raised against KLH-CVKSLEEDPWPRVNSKDHI-NH_2 and KLH-CVKDFGF ELDSRK-OH, respectively. Each obtained serum from immunized rabbits was purified by affinity column conjugated with PPM1D605 and PPM1D430 specific antigen. Depletion was also carried out for anti-PPM1D430 antibody using affinity column-conjugated VKDFGFELD

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SRKG-NH_2 peptide to recover the anti-PPM1D430 antibody, which can recognize only carboxyl terminal-free proteins.

Adriamycin (ADR; Sigma Chemical Co., St Louis, MO, USA) was added to cultures at a final concentration of 345 nM and remained in the cultures until they were harvested 12 h later. Cell lysate were prepared from cultured cells using Laemmli buffer. Normalized protein extracts were used for analysis by SDS–PAGE and immunoblotting with Immobilon-P membranes (Millipore, MA, USA). Immunoblots were probed with antibodies described above.

Subcellular Localization Analysis in Living Cells—Transfection of H1299 cells with 2 μg of each expression construct using Metafectene (Biontex, Munich, Germany) was performed according to the manufacturer's instructions. Forty eight hours after the transfection, subcellular localization of EGFP-PPM1D fusion proteins were analysed using LSM510 confocal laser microscopy (Carl Zeiss, Jena, Germany). Subcellular localization of endogenous PPM1D605 and PPM1D430 in MCF7 cells and T47D cells were carried out using ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's instructions. Subcellular extractions from cells were analysed by western blotting using anti-PPM1D605- and anti-PPM1D430-specific antibodies.

siRNA—The siRNA duplexes were synthesized by Invitrogen. The sequences of siRNA oligos specific for all PPM1D variants and PPM1D430 were 5′-GAAGUGGA CAAUCAGGGAAACUUUA-3′ and 5′-GAGAGCCAUCUU UACAUCAAUACUA-3′, respectively. Stealth^{TM} RNAi negative control medium GC Duplex #2 (Invitrogen) was used as control siRNA. Cells were transfected with siRNA duplexes using lipofectamine (Invitrogen) following the manufacturer's instructions 48 h prior to ADR stimulation. Twelve hours after stimulation, cells lysate were recovered as described above and used for western blotting analysis.

RESULTS

Identification of Alternatively Spliced Transcripts of PPM1D—The human *PPM1D* gene comprises six exons encoding 605 amino acids and although its amplification has been detected in >15% of breast cancers, no mutations that alter its phosphatase activity or affect its transcriptional regulation have yet been identified (*9*). In our present study, we sequenced *PPM1D* transcripts by first generating cDNAs of this gene (PPM1D605) from two types of breast cancer cells, MCF7 (p53 wild-type) and T47D (p53 mutant) cells by RT–PCR. Sequence analysis of these PPM1D605 cDNA fragments revealed that there was no mutation in the ORF of this gene in these cells. However, we recovered cDNA fragments that were longer than PPM1D605 from both cell types using PPM1D-specific primers. Nucleotide sequence analyses subsequently showed the existence of a novel PPM1D cDNA fragment containing a 111 bp insertion with a stop codon corresponding to a region between exon 5 and exon 6 (exon 5′) in the *PPM1D* gene (Fig. 1A). Comparison of this 111 bp insertion sequence with the human *PPM1D* genomic region revealed the presence of a consensus GT/AG sequence and a similar sequence with an adjacent polypyrimidine tract in the anterior portion, which are characteristic intronic motifs. Furthermore, the insertion sequence terminates with an AAG which is a consensus 5′ splicing domain (Fig. 1B). These findings suggested that this variant form of the *PPM1D* gene had been generated by alternative splicing.

Further analysis of the ORF of the alternatively spliced *PPM1D* cDNA demonstrated that this variant encodes a putative 430 amino acid protein, PPM1D430 (Fig. 1C). The PPM1D430 protein sequence is identical to PPM1D605 between residues 1 and 420 but contains an additional 10 PPM1D430-specific residues. Residues 1–420 of both PPMID proteins contain the catalytic domain, which suggested that PPM1D430 would retain phosphatase activity.

To examine whether PPM1D430 expressed in other cell lines, we carried out RT–PCR analysis and found both PPM1D605 and PPM1D430 transcripts in cell lines we analysed, with both variants showing the highest expression in MCF7 cells in which gene amplification of *PPM1D* has occurred (Fig. 1D) (*9*). These data suggested that PPM1D430 is indeed commonly expressed in different cancer cell lines.

We further performed semi-quantitative RT–PCR using radioisotope-labelled primers to compare the transcription levels of PPM1D605 and PPM1D430 in MCF7 and T47D cells. The expression levels of PPM1D430 were found to be ~15% of PPM1D605 in MCF7 cells (data not shown). T47D cells showed a similar expression profile for these two variants by RT–PCR, suggesting that the ratio of PPM1D430 and PPM1D605 transcript is not affected with gene amplification of *PPM1D* gene.

Alternative splicing has been reported to be a fundamental mechanism for generating protein diversity in normal tissues (*31*). To thus examine whether the PPM1D430 transcript is expressed in normal human cells, we performed RT–PCR analysis using a human tissue cDNA panel. The result showed that PPM1D605 mRNA was found to be ubiquitously expressed in all of the tissues represented on the panel (Fig. 1E). High levels of PPM1D605 expression were observed in the placenta, spleen pancreas, ovary and leucocytes but maximal expression was detected in testis. This expression pattern is similar to that previously described for PPM1D in mouse tissues (*37*). Interestingly, PPM1D430 was found to be expressed specifically in testis and leucocyte, although slight expression of this variant could be detected in other tissues. The quantitative analysis showed that relative amount of PPM1D430 mRNA to PPM1D605 mRNA was $21 \pm 1.4\%$ in leucocyte and $8.7 \pm 3.9\%$ in testis, respectively (Table 1). These data suggested that the regulation of PPM1D splicing is tissue-specific and that PPM1D430 may play an important role in testis and in leucocyte.

Identification of PPM1D Isoforms in Protein Level—PPM1D430 mRNA contains a 111 bp insertion with a stop codon corresponding to a region between exon 5 and exon 6 (exon 5′) in the *PPM1D* gene. It is known that mRNAs that contain premature stop codons can be commonly degraded by nonsense-mediated decay (NMD), however, it is reported that NMD-targeted nonsense transcripts can escape NMD under some conditions (*38*, *39*).

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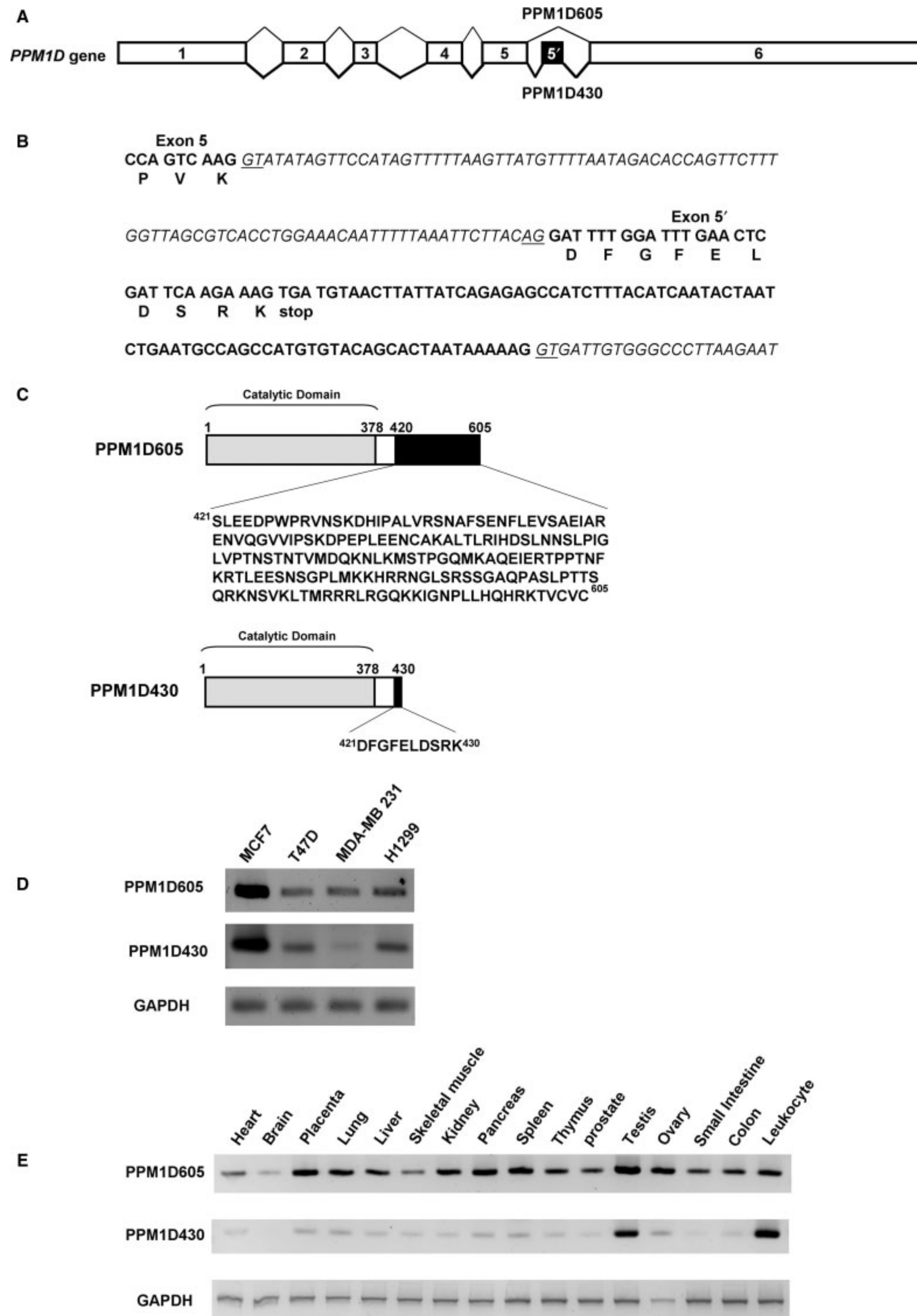

Fig. 1. **Identification of an alternative splicing variant of human *PPM1D*.** (A) Schematic representation of the genomic organization of *PPM1D* leading to two alternatively spliced products; full length PPM1D605 and the splicing variant PPM1D430. Exons are shown as boxes and the variant transcript was generated by the inclusion of exon 5′ incorporating 111 bp and a stop codon. (B) The partial nucleotide sequence of the human *PPM1D* gene and the predicted amino acid sequence. The nucleotide sequences representing the introns are shown in italics and the exons are in bold. The underlined regions indicate the GT/AG sequence in the splicing sites. (C) Schematic representation of the PPM1D605 and PPM1D430 proteins. The grey box represents the catalytic domain of PPM1D and the black box indicates variant-specific C-terminal sequences. Residues 1–420 are completely conserved between PPM1D605 and PPM1D430, but these proteins contain 185 and 10 amino acids, respectively. (D) mRNA expression of alternative splicing variants of PPM1D in cultured cells. RT–PCR was performed using specific primers for each PPM1D variant and a GAPDH control. (E) Expression profile of human PPM1D alternative splicing variants in normal tissues. RT–PCR was performed using a multiple-tissue cDNA panel. GAPDH was amplified as a loading control.

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In order to identify PPM1D605 and PPM1D430 proteins independently, we generated polyclonal antibodies by immunizing rabbits with specific peptides for each variant. Western blotting analysis of cells that had been transfected with the corresponding HA-tagged PPM1D validated the specificity of these antibodies and showed no cross-reactivity (Fig. 2A). To analyse the expression of endogenous PPM1D605 and PPM1D430 proteins, immunoblotting analysis using the above variant-specific antibodies was performed for different cell types. PPM1D gene located on chromosomal region 17q23 and overexpression of PPM1D605 protein was reported in MCF7 cells detecting genomic amplification of PPM1D (*9*). We also detected overexpression of the PPM1D605 protein in MCF7 cells with our generated polyclonal antibody for PPM1D605, whereas a weak or no signal was detected in all other cells tested (Fig. 2B). Interestingly, strong expression of PPM1D430 was detected in both MCF7 and T47D cells even though the mRNA levels for this variant are considerably lower in the latter cell line (Fig. 1D). These data suggested that the regulation of the PPM1D430 protein may differ from PPM1D605, possibly through post-translational modifications or proteolysis.

Table 1. **Relative amount of PPM1D430 mRNA to PPM1D605 mRNA in leucocyte and testis.**

	Leucocyte	Testis
PPM1D605	100	100
PPM1D430	21 ± 1.4	8.7 ± 3.9

Semi-quantitative RT–PCR analysis was carried out using common primers designed in exon 5 and exon 6 to amplify cDNA fragments of both PPM1D605 and PPM1D430 coincidentally. Relative amount of PPM1D430 mRNA to PPM1D605 mRNA were quantified by LAS-3000 (Fujifilm).

Subcellular Localization Analysis of PPM1D Splicing Variants—We have previously reported that PPM1D605 has two putative nuclear localization signals (NLSs): ^{247}KRPR^{250} in the basic rich loop (B-loop) of the catalytic domain and ^{535}KRTLEESNSGPLMKKHRR^{552} in the C-terminal domain (*24*). However, PPM1D430 lacks this C-terminal domain NLS. In order to clarify whether this had implications for the subcellular localization of PPM1D430, we designed a series of EGFP-PPM1D deletion mutants and analysed their expression in H1299 cells (Fig. 3A). This analysis revealed that both EGFP-PPM1D430 and EGFP-PPM1D605 are localized in the nucleus (Fig. 3B). Moreover, both EGFP-PPM1D(1–378) and EGFP-PPM1D605(375–605) also showed the nuclear localization, suggesting that a functional NLS is present in both the catalytic domain and C-terminal domain of PPM1D605. To then determine whether the basic sequence in the B-loop acts as NLS in PPM1D variants, we designed a mutant protein in which the B-loop was substituted with the corresponding sequences in the

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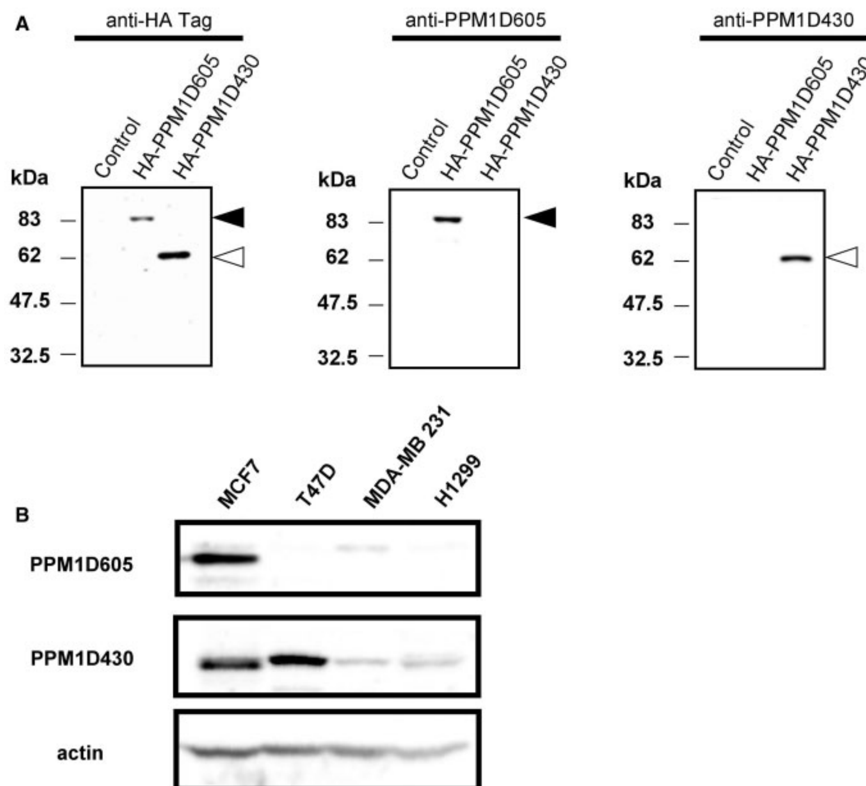

Fig. 2. **Identification of endogenous PPM1D605 and PPM1D430 proteins.** (A) Human PPM1D variant-specific antibodies were prepared and validated using lysates from H1299 cells transfected with or without the corresponding HA-tagged proteins. (B) Immunoblot detection of PPM1D605 and PPM1D430 in breast cancer cell lines (MCF7, T47D and MDA-MB-231) and lung cancer cells (H1299). The detection of actin was used to control for protein loading.

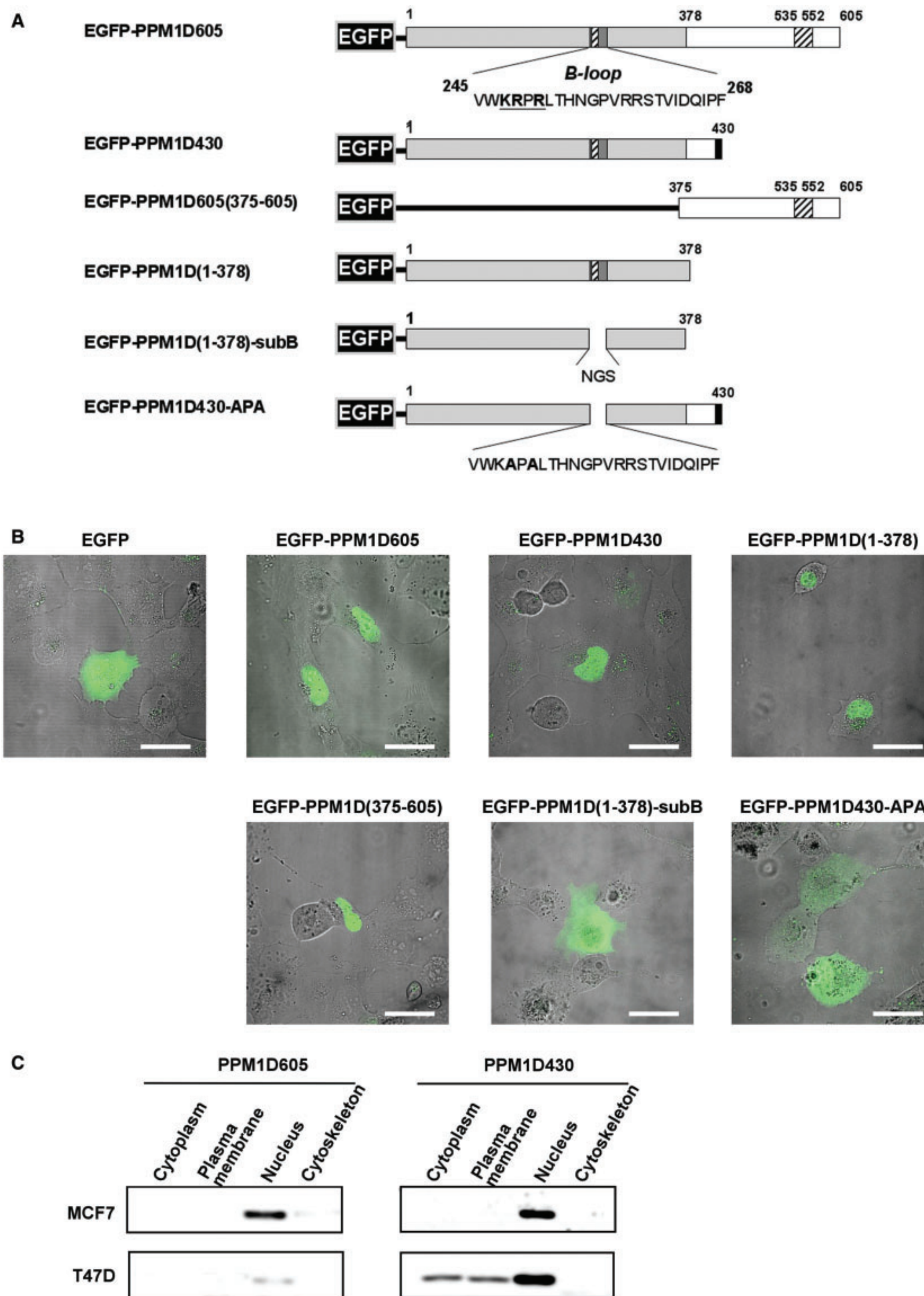

Fig. 3. **Subcellular localization of PPM1D605 and PPM1D430.** (A) Schematic representation of PPM1D605, PPM1D430 and a series of PPM1D mutants N-terminally fused to EGFP. Grey box indicates the catalytic domain and diagonal line box is a putative NLS containing sequence. The basic rich loop (B-loop) is indicated in dark grey and located within the catalytic domain of the PPM1D variants. The putative NLS is indicated by the diagonal line box and putative NLS sequence residues in the B-loop are underlined. The PPM1D430-specific sequence at the C-terminus is represented by a black box. Substituted Ala residues in EGFP-PPM1D430-APA are highlighted in bold. (B) Intracellular distribution of the EGFP-PPM1D605, EGFP-PPM1D430 and EGFP-PPM1D mutants. H1299 cells were transiently transfected with constructs expressing these fusion proteins and analysed 48 h post-transfection. The subcellular localization of each fusion product was determined by confocal fluorescence microscopy. Scale bar, 20 μm. (C) Subcellular localization of endogenous PPM1D605 and PPM1D430 in MCF7 and T47D cells. Cell lysates recovered by subcellular fractionation were analysed by immunoblotting using PPM1D605- and PPM1D430-specific antibodies.

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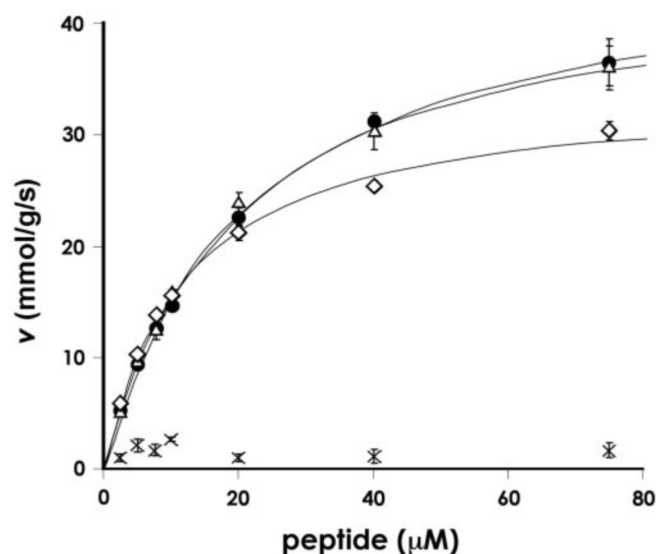

Fig. 4. **Phosphatase activity assay of PPM1D430.** PPM1D430 was incubated with p53(10-23)15P (filled circle), p53(10-23)15P-TD (open triangle), p53(10-23)15P-3K (cross) or p53(10-23)15P-3E (open diamond). Phosphatase analysis using recombinant PPM1D430 was performed with different concentrations of substrate for 10 min at 30°C. The resulting phosphatase activities were then analysed by measurement of the released free phosphate using malachite green.

related phosphatase PPM1A. Our results showed that this PPM1D(1–378)-subB mutant had a diffuse expression pattern throughout the cells (Fig. 3B). Furthermore, an Ala substitution mutant in the putative NLS of the B-loop, EGFP-PPM1D430-APA, also showed both nuclear and cytoplasmic localization. These results indicated that the basic residues of the PPM1D catalytic domain B-loop indeed function as an NLS and that the PPM1D430 variant can translocate to the nucleus in the H1299 cells.

To next determine the subcellular localization of endogenous PPM1D605 and PPM1D430, we performed western blotting analysis of subcellular fractions from MCF7 and T47D cells. PPM1D605 was found to localize in the nucleus of both cell lines. Interestingly, PPM1D430 was clearly detectable not only in the nucleus but also in the cytoplasmic and organelle/plasma membrane fractions in T47D cells, although it was located only in the nucleus in MCF7 cells (Fig. 3C). This different localization of PPM1D430 from PPM1D605 in T47D cells suggested that endogenous PPM1D430 may have novel functions via its interactions with proteins outside of the nucleus.

PPM1D430 Shows In Vitro Phosphatase Activity—Amino acids sequence of 1–420 in PPM1D605 and PPM1D430 is common and these regions contain catalytic domain. To examine whether the PPM1D430 thus possessed phosphatase activity towards a known PPM1D substrate, this variant was purified from *E. coli* as a recombinant protein and subjected to *in vitro* phosphatase analysis using phosphorylated peptide analogues of p53. Direct curve fitting of PPM1D430 over a p53(10–23)15P concentration range revealed K_m and V_{max} values of $20.8 \pm 0.7\,\mu M$ and $46.8 \pm 0.7\,mmol\,g^{-1}\,s^{-1}$, respectively

Table 2. **Kinetic parameters for recombinant PPM1D430.**

Substrates	K_m (μM)	V_{max} (mmol $g^{-1}\,s^{-1}$)	V_{max}/K_m ($\times 10^3\,l\,g^{-1}\,s^{-1}$)
p53(10-23)15P	20.8 ± 0.7	46.8 ± 0.7	2.3
p53(10-23)15P-3K	NA	NA	NA
p53(10-23)15P-TD	18.7 ± 0.7	44.8 ± 0.6	2.4
p53(10-23)15P-3E	11.8 ± 0.7	34.2 ± 0.7	2.9

All assays were performed in 50 mM Tris–HCl pH 7.5, 30 mM $MgCl_2$, 0.1 mM EGTA, 0.02% 2-mercaptoethanol for 10 min at 30°C. All data were fitted to the Michaelis–Menten equation (KaleidaGraph, Synergy Software, PA, USA) and are the averages from at least three independent experiments. NA, no activity.

Table 3. **Relative induction of PPM1D variants in response to ADR stimulation.**

	PPM1D605	PPM1D430
MCF7	3.2 ± 0.4	3.4 ± 0.3
T47D	1.3 ± 0.1	1.2 ± 0.3

The human breast cell lines MCF7 (p53 wild-type) and T47D (p53 mutant) were treated with ADR (345 nM). Relative values for samples extracted 8 h after treatment, compared with untreated controls, were determined using BAS-1800II. mRNA amounts from treated and untreated cells were normalized using GAPDH. All data are the average from at least three independent experiments.

(Fig. 4 and Table 2). The more acidic phosphorylated p53 analogues p53(10–23)15P-TD and p53(10–23)15P-3E showed lower K_m values than p53(10–23)15P ($18.7 \pm 0.7\,\mu M$ and $11.8 \pm 0.7\,\mu M$, respectively). On the other hand, recombinant PPM1D430 showed no phosphatase activity against the acidic residue-deficient analogue, p53(10–23)15P-3K. These substrate preferences and phosphatase activity levels of PPM1D430 are therefore comparable with PPM1D(1–420), which has been reported to show similar *in vitro* phosphatase properties to PPM1D605 against p38 phosphorylated peptides (*26*). Our current findings thus indicate that PPM1D430 has a phosphatase capability that is equivalent to PPM1D605 against phosphorylated peptides as substrate even though it differs in terms of its localization in some cellular contexts.

Effects of PPM1D Alternative Splicing Variants upon DNA Damage—PPM1D has been reported to be induced at the mRNA level after genotoxic stimulation in a p53-dependent manner (*1*). To determine whether PPM1D430 is also induced in response to genotoxic stress in a similar manner, we assayed its transcript levels after treatment of breast cancer cells with ADR by semi-quantitative PCR. Both PPM1D430 and PPM1D605 mRNA recovered from ADR-stimulated MCF7 cells showed a 3-fold induction compared with untreated cells (Table 3). In contrast, however, T47D cells expressing mutant p53 failed to show any induction of either isoform under these conditions. The induction levels of PPM1D605 mRNA in MCF7 cells we observed in our current experiments are consistent with the reported results of Fiscella *et al*. (*1*) although the cells were stimulated with IR in this previous study. Our present results thus indicate that both PPM1D605 and PPM1D430 transcripts are indeed induced in response to genotoxic stress in a p53-dependent manner. We further

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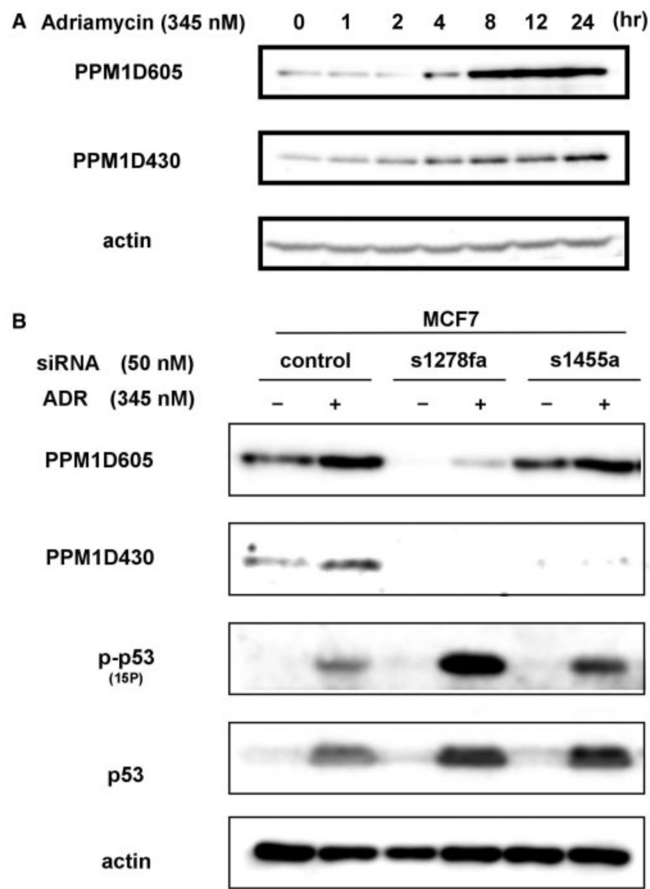

Fig. 5. **Regulation and functions of PPM1D430 in cells following genotoxic stress.** (A) Western blotting of PPM1D605 and PPM1D430 was performed using cells after stimulation by ADR for the indicated times. (B) Effects of the knockdown of PPM1D605/PPM1D430 and PPM1D430 in MCF7 cells undergoing genotoxic stress. MCF7 cells were transfected with a PPM1D605/PPM1D430 consensus siRNA (s1287fa) or PPM1D430-specific siRNA (s1455a) 2 days prior to stimulation with ADR. Cells were harvested 12 h after stimulation and analysed by immunoblotting with PPM1D605, PPM1D430, p53, phospho-p53(15P) and actin antibodies.

analysed the induction of these PPM1D variants at the protein level after ADR treatment of MCF7 cells. An increase in the protein levels was detectable in both cases and peaked at 12–24 h (Fig. 5A). Interestingly, the magnitude of the induction of PPM1D605 protein was much higher than PPM1D430 despite the fact that these isoforms showed comparable levels of mRNA up-regulation (Table 3). This suggested the involvement of a post-translational regulatory mechanism or differences in protein turnover for the PPM1D variants in DNA-damaged MCF7 cells.

It has been reported that the phosphatase activity of PPM1D605 is essential for its oncogenic effects which require the dephosphorylation of target proteins, such as p38 and p53 (*40*). To therefore analyse whether endogenous PPM1D430 exerts any such influence upon cell cycle regulation, we tested whether the down-regulation of PPM1D430 would have any impact upon the phosphorylation of p53 at serine 15 and p53 protein level, which correlate with transcriptional activity of p53 (*41*, *42*). MCF7 cells were transfected with PPM1D-specific siRNAs and this was followed by ADR stimulation for 12 h. The siRNAs used included s1278fa, which can down-regulate both PPM1D605 and PPM1D430, and s1455a, which is specific for PPM1D430. Treatment of MCF7 cells with s1455a resulted in the effective suppression of PPM1D430 and showed no effects against PPM1D605 (Fig. 5B). As expected, the transfection with s1278fa decreased the expression of both PPM1D605 and PPM1D430 in MCF7 cells and this resulted in a remarkable increase in the phosphorylation and protein levels of p53 in the response to ADR stimulation. Interestingly, the knockdown of PPM1D430 mRNA by s1455a also enhanced the phosphorylation and protein levels of p53 compared with untreated cells but to a lesser extent than s1278fa. These data suggested that both PPM1D605 and PPM1D430 function in the regulation of p53 through the dephosphorylation of serine 15 that stabilizes the protein. Hence, PPM1D430 may also have a role in cell cycle control following genotoxic stress.

DISCUSSION

In our present study, we have identified the human PPM1D phosphatase splicing variant, PPM1D430, at both the mRNA and protein levels. It has been shown that the oncogenic functions of PPM1D are associated with inhibition of cell cycle regulatory proteins such as p53, p38 and ATM (*43*). Moreover, *PPM1D* gene amplification has been observed in multiple human cancer types, including breast carcinomas (*43*). Here we demonstrated that PPM1D430 dephosphorylates phosphorylated p53 peptides and that the knockdown of endogenous PPM1D430 increased the phosphorylation and stability of p53 after genotoxic stress. These data indicate that PPM1D430 may also function as a cell cycle regulator by controlling the phosphorylation of p53 at serine 15. PPM1D430 mRNA is produced by an exon 5′ insertion between exons 5 and 6 and codes 430 amino acids. Residues 1–420 of PPM1D430 and PPM1D605 are common and include catalytic domain. The characteristic 10 amino acid residues exist at C-terminus of the PPM1D430 protein. The antibody specific for PPM1D430 can recognize carboxyl terminal-free proteins that end with ^{419}VKDFGFELDSRK^{430} sequence and PPM1D430 protein in MCF7 cells was detected at the putative size. Furthermore, PPM1D430 protein was decreased by treatment of two kinds of PPM1D-specific siRNAs. These results definitely indicated that PPM1D430 protein is translated from the PPM1D mRNA containing exon 5′. It is known that transcripts containing premature stop codons can be degraded by NMD. However, it is also reported that several mRNAs which contain the NMD motif, such as hPPARγ and diacylglycerol kinase τ were translated (*44*, *45*). The results of identification of PPM1D430 at protein level and knockdown analysis clearly indicated that the PPM1D430 mRNA was not involved in the NMD pathway.

In addition, similar exon 5′ sequences have been found in *PPM1D* genes derived from other species. The boundary sequences of their putative exons are also highly

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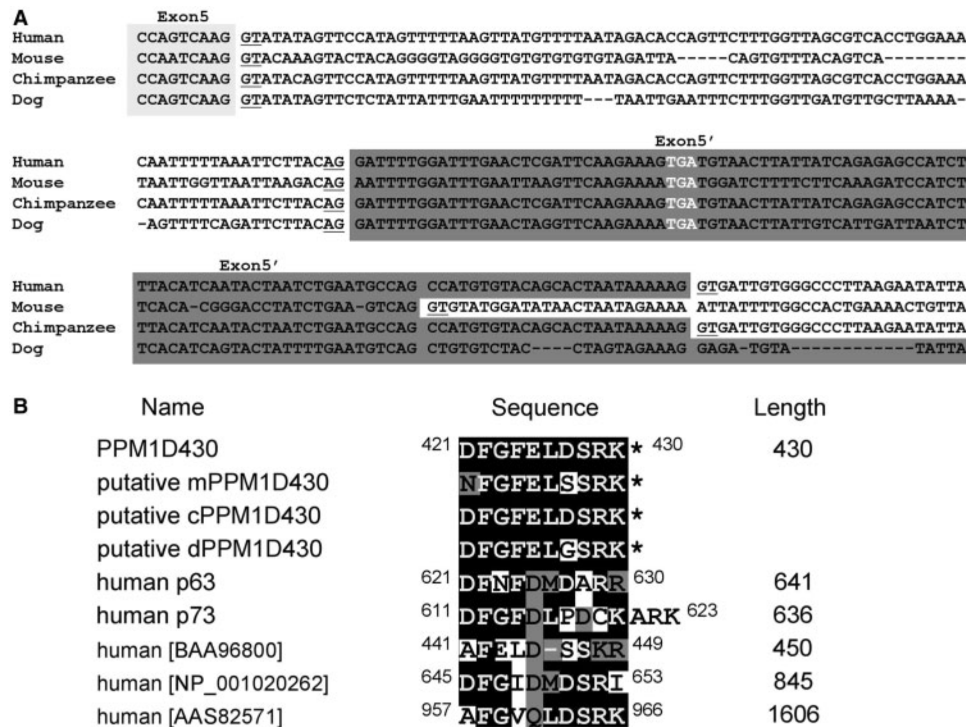

Fig. 6. **Genomic organization of mammalian *PPM1D* containing the putative 5′ exon.** (A) Sequence of the 5′ exon derived from the human *PPM1D* gene was compared with that from other mammalian *PPM1D* genes. The grey box indicates exon 5 and dark grey box indicates putative exon 5′. The first and last two bases in the intronic sequences (GT and AG for donor and acceptor) are underlined. Stop codons (TGA) in exon 5′ are shown using white characters. (B) Comparison of the human PPM1D430-specific C-terminal sequence with putative mammalian PPM1D430 (mPPM1D430 from mouse, cPPM1D430 from chimpanzee, dPPM1D from dog) and areas of homology in other human proteins including p73 and p63. Parenthetic numbers show accession numbers in the protein databases. Asterisks indicate stop codons. Conserved residues and residues similar to those in human PPM1D430 are indicated by black and grey boxes, respectively.

conserved (Fig. 6A) and also encode 10 amino acids of a similar sequence to human (Fig. 6B). This suggests that similar splicing may occur across species to produce PPM1D430.

Homology searches of the PPM1D430-specific C-terminal residues against human protein database resulted in the discovery of several proteins, including p73 and p63. The high homology residues in these p53 family members also located at their C-terminal region. The region in p73 is reported to interact with c-Myc-binding protein MM1 and a Trp–Asp repeat protein RACK1 (*46*, *47*). Furthermore Liu and Chen (*48*) reported that the region of p73 has the inhibitory activity by preventing the accessibility of p300/CBP to the activation domain. These suggested that PPM1D430 may also exert different functions from PPM1D605 through the specific residues.

One of the PPM1 family, PPM1B, has also been reported to generate alternative splicing variants in human, mouse and rat (*34–36*). Kusuda *et al*. (*36*) reported that five splicing variants of PPM1B exist in mouse and show different substrate specificities, while these variants have common catalytic domain and different C-terminal region. In this study, we demonstrate that the PPM1D430 variant maintains its phosphatase activity against Ser15 of p53 *in vitro* using synthetic phosphopeptide and *in vivo*. On the other hand, thus far, most *in vitro* phosphatase analyses of PPM1D605 have been carried out using a recombinant catalytic domain but not a PPM1D605 against phosphorylated peptide as substrate (*24*, *26*). The functions of their individual C-terminal region in target recognition have remained unknown and further studies of the phosphatase activity and substrate specificity using PPM1D605 against non-phosphorylated peptides but phosphorylated proteins will be essential to properly elucidate the differences in the functions of PPM1D605 and PPM1D430.

One of the most significant differences we uncovered herein between PPM1D605 and PPM1D430 was in their tissue distribution as PPM1D430 transcripts were found to be expressed specifically in testes and leucocytes, whereas PPM1D605 mRNA was ubiquitously detected (Fig. 1E). These results are interesting in light of the findings of Choi *et al*. (*20*) who reported that mice deficient for PPM1D exhibited immunological defects in their peripheral lymphoid organs and in spermatogenesis defects. More recently, Schito *et al*. (*27*) analysed PPM1D-deficient thymocyte and showed that down-regulation of p53 through the protein level and phosphorylation level at Ser15 by PPM1D is required for normal αβ T-cell development. These data indicate that PPM1D plays important roles in regulation of immunocyte. However, it is unclear whether either or both PPM1D605 and PPM1D430 are responsible for the immunological defects observed in PPM1D-deficient mice because the PPM1D-deficient mouse lacks exon 4 and exon 5 of the *PPM1D*

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gene. Hence, PPM1D-deficient mice express neither PPM1D605 nor PPM1D430 proteins. In this study, expression profile analysis using cDNA panel have shown that PPM1D430 is expressed specifically in leucocytes. Furthermore, down-regulation of PPM1D430 resulted in increase of the phosphorylation of Ser15 in human p53 and the stabilization of the p53 protein. This suggests that PPM1D430 itself may control immune system by regulating p53 phosphorylation and stabilization.

We also exhibited the different subcellular localization of PPM1D430 and PPM1D605. Endogenous PPM1D430 protein localizes not only in the nucleus but also in the cytoplasm and at the plasma membrane in T47D cells, while PPM1D605 is exclusively nuclear in the cells. On the other hand, both PPM1D430 and PPM1D605 located in the nucleus in MCF7 cells. Similar phenomenon was detected in Heregulins (HRG). HRG1α, one of the growth factors, localizes in both the nucleus and cytoplasm and the relative expression levels differed between MCF7 and T47D cells (*49*). Consist with the subcellular localization of PPM1D430 in T47D cells, Yoda *et al*. also reported that an EGFP fusion protein containing residues 1–375 of PPM1D605 has both a nuclear and cytoplasmic distribution in HEK293T cells, whereas the full-length protein located only in the nucleus (*50*). These data suggested that the basic residues within the B-loop are not sufficient to retain the nuclear localization of the PPM1D430 in HEK293T and T47D cells. Most of the PPM1D-interacting proteins so far reported are nuclear localization proteins, such as p38, p53, Chk1, Chk2, UNG2 and MDM2. The mechanism of the difference in subcellular localization of PPM1D430 between T47D and MCF7 cells remained unknown, however, the cytoplasmic localization of PPM1D430 may be responsible for its novel functions through the interaction with cytoplasmic proteins unlike PPM1D605.

In summary, we herein described an alternative splicing variant PPM1D430 in protein level and the mRNA detected specifically in testis and leucocyte. Identification of target proteins specific for PPM1D430 will support to clarify the novel functions of the protein and it may help to uncover the regulation of spermatogenesis and the immune system by PPM1D430 or/and PPM1D605.

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CONFLICT OF INTEREST

None declared.

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