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PPM1D regulates p21 expression via dephoshporylation at serine 123

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The cyclin-dependent kinase inhibitor p21 plays a critical role in regulating cell cycle and cell proliferation. We previously cloned the dog p21 gene and found that unlike human p21, dog p21 is expressed as 2 isoforms due to the proline-directed phosphorylation at serine 123 (S123). Here, we identified that PPM1D, also called Wip1 and a Mg^{2+}-dependent phosphatase, dephosphorylates dog p21 protein at serine 123. Specifically, we showed that the level of S123-phosphorylated dog p21 is increased by a PPM1D inhibitor in a dose-dependent manner. We also showed that over-expression of PPM1D decreases, whereas knockdown of PPM1D increases, the level of S123-phosphorylated dog p21 regardless of p53. Additionally, in vitro phosphatase assay was performed and showed that phosphorylated S123 in dog p21 is dephosphorylated by recombinant rPPM1D, which contains the catalytic domain of human PPM1D (residue 1–420), but not by the phosphatase dead rPPM1D (D314A). Furthermore, dephosphorylation of S123 by rPPM1D can be abrogated by PPM1D inhibitor or by withdrawal of Mg^{2+}. Finally, we showed that upon PPM1D inhibition, the level of S123-phosphorylated dog p21 was increased, concomitantly with decreased expression of cyclin A, cyclin B, Rb, and PCNA. Together, our results indicate that PPM1D functions as a phosphatase of dog p21 at serine 123 and plays a role in cell cycle control via p21.

Introduction

The cyclin-dependent kinase inhibitor p21 belongs to the Cip/Kip family of cyclin kinase inhibitors (CKIs) that includes p21, p27, and p57. p21 was discovered in 1993 by multiple groups. It was identified as a target of wild-type p53 and thus named WAF1 (wild-type p53 activated factor).[1] In addition, due to its ability to bind and interact with Cdk, it was also named Cip1 (Cdk interacting protein 1).[2] Since then, p21 is found to play a key role in multiple biological processes, including cell cycle control, DNA replication/repair, apoptosis, cell differentiation, and senescence.[3] Indeed, the biological functions of p21 are mainly mediated by its ability to induce growth suppression. First, p21 inhibits the kinase activity of the cyclin-dependent kinases (CDKs) via protein interaction and consequently, results in cell cycle arrest.[5,6] Second, p21 inhibits DNA replication by disrupting the interaction of proliferating cell nuclear antigen (PCNA) with DNA polymerase δ and several other proteins involved in DNA synthesis.[7-9] Interestingly, recent studies also showed that cytoplasmic localization of p21 promotes cell proliferation and thus, has oncogenic activity. Consistent with this, accumulation of cytoplasmic p21 is found in several types of cancers and associated with tumor progression and poor prognosis.[10]

Together, these studies suggest that depending on its cellular context, p21 may inhibit or promote tumorigenesis and thus, understanding the mechanism by which p21 is regulated may help us to explore its potential as an anticancer therapeutic target.

Since its discovery, much effort has been made to understand how p21 expression is regulated at both the transcriptional and protein levels. For example, p21 transcription is regulated by the p53 family proteins, including p53, p63, and p73, and several other transcriptional factors.[11] In addition, p21 expression is regulated by several RNA-binding proteins or micro RNAs via mRNA stability or protein translation.[4] Furthermore, p21 expression is regulated by posttranslational modifications, including phosphorylation and ubiquitylation.[4] Interestingly, phosphorylation of p21 has been found to impact its cellular localization, the interaction with PCNA, and protein stability.[12] As a result, several protein kinases are identified to modulate p21 phosphorylation at various sites.[12] However, very little is known about the dephosphorylation of p21 by a phosphatase.

The PPM1D protein phosphatase, also called wild-type p53-induced phosphatase 1 (Wip1) or PP2Cδ, belongs to the type 2C family of protein phosphatases (PP2C). PPM1D was originally identified as a target of p53 in response to ionizing radiation.[13] Subsequent studies showed that PPM1D exerts inhibitory

function on several tumor suppressor pathways, including the ATM-CHK2-p53 pathway[14,15] and the p38-p53 pathway.[16] In line with this, PPM1D is found to be over-expressed in several types of human cancers and mice deficient in PPM1D are resistant to cancer.[17,18] Together, these data suggest that PPM1D has oncogenic activities and thus, is a promising target for cancer management.

We previously cloned the dog *CDKN1A* gene and found that unlike human p21, dog p21 is expressed as 2 isoforms due to proline-directed phosphorylation at serine 123.[19] Interestingly, phosphorylation of dog p21 at serine 123 can be easily visualized as a slower migrating band compared to the underphosphorylated dog p21. This unique feature of dog p21 prompted us to identify the modulator that regulates serine 123 phosphorylation of dog p21. Here, we found that PPM1D is a phosphatase of dog p21 via serine 123.

Results

The level of S123-phosphorylated dog p21 is increased by a PPM1D inhibitor independent of p53

To screen for potential phosphatase of dog p21, madin-darby canine kidney (MDCK) cells, which contains a wild-type p53, were treated with various phosphastase inhibitors and the level of dog p21 was determined by Western blot analysis. Interestingly, we found that upon inhibition of PPM1D with CCT007093, the level of S123-phosphorylated dog p21 was markedly increased, whereas the level of underphosphorylated dog p21 was only slightly increased (**Fig. 1A**). PPM1D phoshpatase is known to inhibit p53 expression by dephosphorylating several modulators of p53 such as Mdm2.[18] Thus, to rule out the potential effect of p53, MDCK cells with stable p53 knockdown were used and treated with various amounts of PPM1D inhibitor CCT007093. We found that PPM1D inhibitor significantly increased the expression of S123-phosphorylated dog p21 and to a much less extent, the underphosphorylated dog p21 in MDCK-p53KD cells (**Fig. 1B**). To further verify that PPM1D regulates dog p21 expression in the absence of p53, Cf2Th cells,

which express an ectopic dog p21, were used. We would like to mention that Cf2Th cells harbors a mutant p53 (C226F) and thus, the basal level of p21 is very low in these cells.[20] Interestingly, we found that the level of S123-phosphorylated dog p21, but not the underphosphorylated dog p21 was increased upon treatment with PPM1D inhibitor in Cf2Th cells (**Fig. 1C**). Together, these data suggest that PPM1D inhibitor increases the level of S123-phosphorylated dog p21 regardless of p53.

Knockdown of PPM1D increases, whereas ectopic PPM1D decreases, S123-phosphorylated dog p21

To determine whether endogenous PPM1D directly regulates dog p21 expression, siRNA against dog PPM1D was synthesized and transfected into MDCK cells along with a scrambled siRNA. As expected, the level of PPM1D mRNA was decreased by PPM1D siRNA but not control siRNA (**Fig. 2A**). Importantly, we found that level of S123-phosphorylated dog p21 was increased by PPM1D knockdown (**Fig. 2B**). To verify this, we generated an antibody specifically recognizing S123-phosphorylated dog p21, designated as α-p-dog p21. We showed that α-p-dog p21 recognized the S123-phosphorylated dog p21, the upper band, but not the undersphosphorylated dog p21, the lower band (**Fig. 2C**). Importantly, we found that the level of S123-phosphorylated dog p21 was markedly increased upon knockdown of PPM1D (**Fig. 2C**). Similarly, we showed that knockdown of PPM1D increased the level of S123-phosphorylated dog p21 in MDCK-p53KD cells (**Figs. 2D-F**) and Cf2Th cells with ectopic dog p21 expression (**Figs. 2G-I**), suggesting that PPM1D inhibits S123-phosphorylated dog p21 independent of p53.

Next, to determine whether ectopic PPM1D has an effect on S123-phosphorylated dog p21, a control or dog PPM1D expression vector was transfected into Cf2Th cells with ectopic dog p21 expression. We found that ectopic dog PPM1D greatly reduced the level of S123-phosphorylated dog p21 as compared to that in control cells (**Fig. 3A**). To verify this, p53-null melanoma 36 cells were transiently transfected with a fixed amount of dog p21 expression vector together with various amounts of dog PPM1D expression vector. Again, we found that ectopic PPM1D decreased the level of S123-phosphorylated dog p21
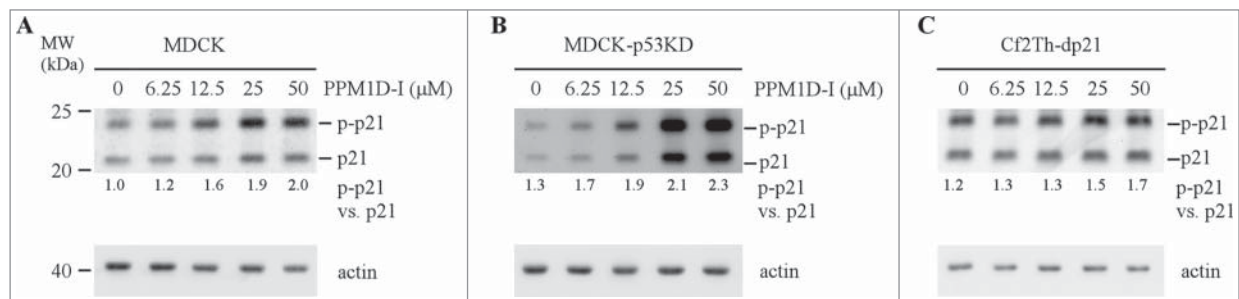

Figure 1. The level of S123-phosphorylated dog p21 is increased by PPM1D inhibitor independent of p53. (A-B) MDCK (**A**) and MDCK-p53KD (**B**) cells were mock-treated or treated with various amounts of PPM1D inhibitor for 12 h, and the level of p21 was determined by Western blot analysis with antibodies against p21 and actin. The relative level of underphosphorylated p21 (p21) and S123-phohosphorylated p21 (p-p21) was measured by densitometry, and the ratio of p-p21 versus p21 is shown *below*. (**C**) The experiment was performed as in (**A**) except that Cf2Th cells were induced to express dog p21 for 12 h prior to the treatment.
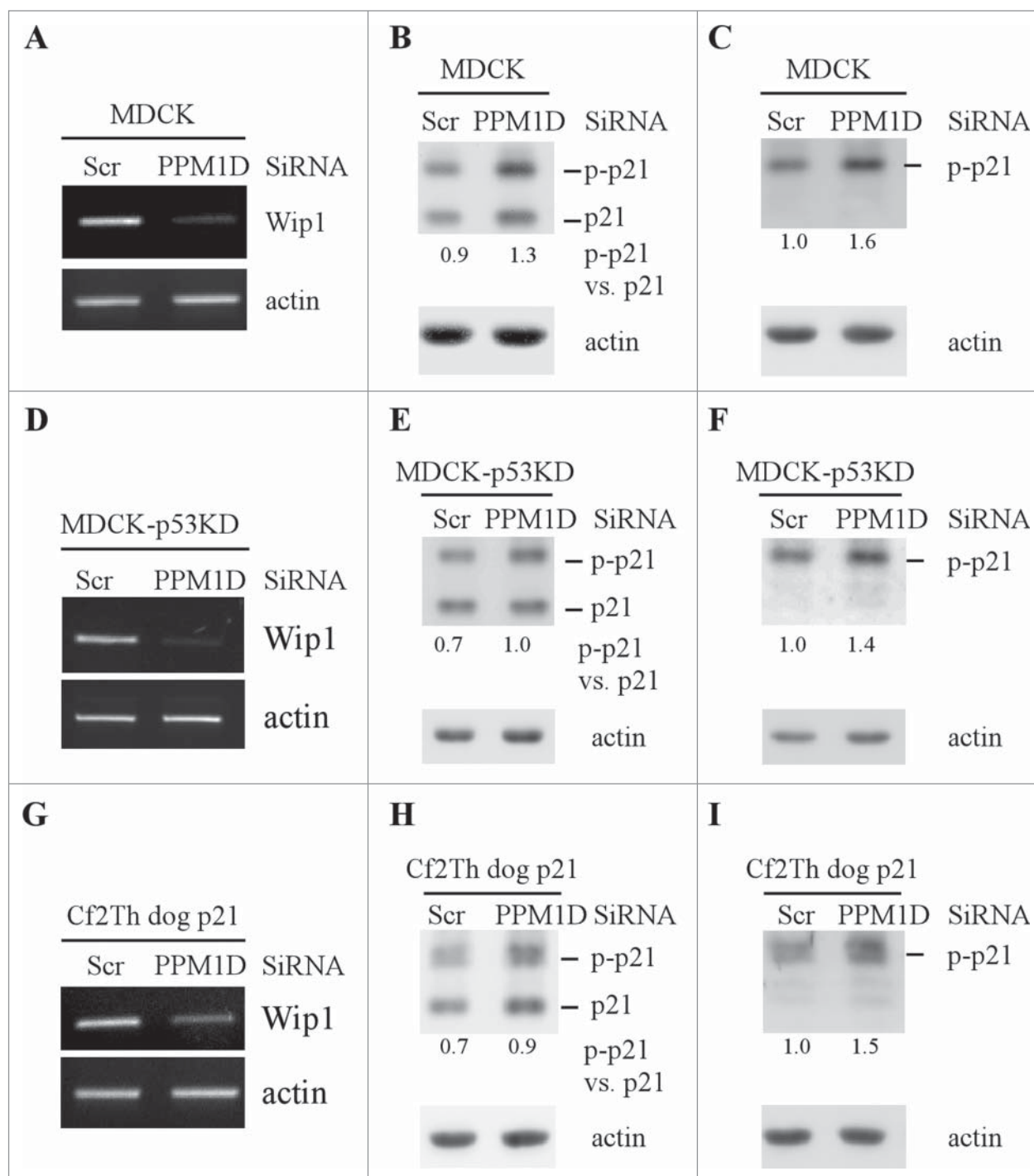
Figure 2. Knockdown of PPM1D increases the level of S123-phosphorylated dog p21. (**A**) MDCK cells were transiently transfected with a control or PPM1D SiRNA for 60 h, followed by RT-PCR analysis to measure the level of dog PPM1D and actin transcript. (**B-C**) MDCK cells were treated as described in (**A**) and the level of actin and total dog p21 (**B**) or S123-phosphorylated dog p21 (**C**) was determined by Western blot analysis with actin, total p21, and p-dog p21 antibodies. (**D**) The experiment was performed as in (**A**) except MDCK-p53KD cells were used. (**E-F**) The level of actin, total dog p21 (**E**), and S123-phosphorylated dog p21 (**F**) was determined by Western blot analysis using MDCK-p53KD cells treated as in (**D**). (**G**) The experiment was performed as in (A) except that Cf2Th cells were induced to express dog p21 for 12 h prior to SiRNA transfection. (**H-I**) The level of actin, total dog p21 (**H**), and S123-phosphorylated dog p21 (**I**) was determined by Western blot analysis using dog p21-expressing Cf2Th cells treated as in (**G**).

in a dose-dependent manner (**Fig. 3B**). Similarly, we also showed that the level of S123-phosphorylated dog p21 was reduced by ectopic expression of human rPPM1D in Cf2Th or Melanoma 36 cells (**Fig. 3C and D**). rPPM1D, which contains catalytic domain (residue 1–420) of human PPM1D, has similar catalytic activities as compared to full-length human
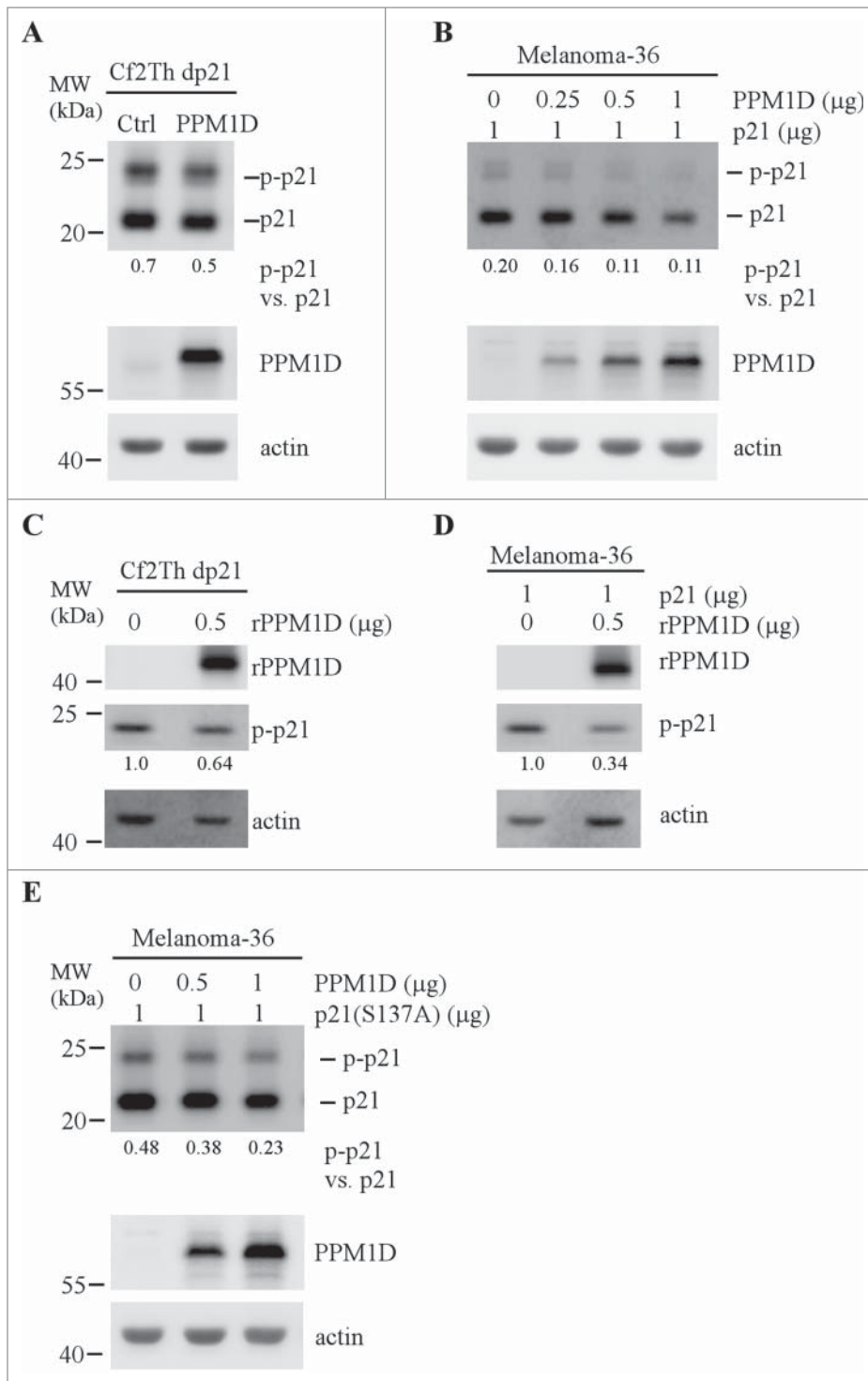
Figure 3. Over-expression of dog PPM1D decreases S123-phosphorylated dog p21. (**A**) Cf2Th cells were induced to express dog p21 for 12 h, and then transiently transfected with a control or dog PPM1D expression vector. The level of dog p21, PPM1D, and actin was determined by Western blot analysis. (**B**) Melanoma-36 cells were transiently transfected with 1 μg of dog p21 expression vector together with various amounts (0–1 μg) of dog PPM1D expression vector. Twenty-four h post transfection, the level of p21, PPM1D, and actin was determined by Western blot analysis. (**C**) Cf2Th cells were induced to express dog p21 for 12 h, and then transiently transfected with a control or human rPPM1D expression vector. The level of p-p21, rPPM1D, and actin was determined by Western blot analysis. (**D**) Melanoma-36 cells were transiently transfected with 1 μg of dog p21 expression vector together with a control or rPPM1D expression vector. Twenty-four h post transfection, the level of p-p21, PPM1D, and actin was determined by Western blot analysis. (**E**) The experiment was performed as in (**B**) except that dog p21(S137A) expression vector was used.

the expression of S123-phosphorylated dog p21 independent of p53.

PPM1D dephosphorylates dog p21 at serine 123

To examine whether PPM1D directly dephosphorylates dog p21 at serine 123, an in vitro phosphatase assay was performed. Briefly, dog p21 was immunoprecipitated from Cf2Th cells expressing ectopic dog p21, followed by incubation with recombinant GST or GST-tagged rPPM1D or phosphatase dead rPPM1D (D314A). rPPM1D (D314A) contains a point mutation at codon 314 with aspartic acid to alanine and is catalytically inactive.[22] Interestingly, we found that the level of under-phosphorylated dog p21 was not affected by recombinant rPPM1D or GST (**Fig. 4A**, lanes 1–4). By contrast, the level of S123-phoshporylated dog p21 was decreased by recombinant rPPM1D but not by recombinant GST in a dose-dependent manner (**Fig. 4A**, compare lanes 1–2 with 3–4). Furthermore, we showed that S123-phosphorylated dog p21 was not altered by the phosphatase-dead rPPM1D (D314A) or when the phosphatase activity of rPPM1D was inhibited by using Mg^{2+}-free buffer or by adding PPM1D inhibitor (**Fig. 4A**, lanes 5–7). Finally, we determined whether PPM1D mediated dephosphorylation of dog p21 has any effect of cell cycle regulation. We found that upon

PPM1D.[21] Next, to further verify that serine 123 is the primary site targeted by PPM1D, we generated a dog p21 mutant, dog p21(S137A), with serine 137 substituted to alanine. We showed that dog p21(S137A) was still expressed as 2 bands, indicating that serine 123 phosphorylation is intact in this mutant (**Fig. 3E**). Importantly, we showed that ectopic PPM1D inhibited phosphorylation of serine 123 in dog p21 (S137A). Together, these data suggest that PPM1D inhibits

treatment with PPM1D inhibitor, the level of S123-phosphorylated dog p21 was increased, which was accompanied with decreased expression of cyclin A, cyclin B, Rb, and PCNA (**Fig. 4B**). Together, these data suggest that PPM1D dephosphorylates dog p21 at serine 123, which may impact the activity of p21.

Discussion

Recent studies showed that phosphorylation of p21 plays an instrumental role in controlling its activity, localization, and stability.[12] However, despite that p21 phosphorylation by protein kinases is extensively studied, very little is known about the dephosphorylation of p21 by phosphatases. In the current study, we sought to identify potential phosphatase of dog p21. We found that S123-phosphorylated dog p21 is increased by PPM1D inhibitor or by PPM1D knockdown (**Figs. 1–2**). Conversely, overexpression of PPM1D dephosphorylates dog p21 at serine 123 (**Figs. 3–4**). Together, these data suggest that PPM1D dephosphorylates dog p21 at serine 123. Interestingly, the level of under-phosphorylated dog p21 was slightly increased upon treatment with PPM1D inhibitor (**Fig. 1**). We postulate that this alteration is likely through PPM1D-mediated regulation of other p21 modulators, such as p53 and Mdm2,[16,23] both of which are known to modulate p21 activity. Additionally, it is possible that PPM1D may target other sites of dog p21, which merits further investigation.

Phosphorylation of serine 123 was found to prolong the half-life of dog p21 and consequently, enhance the ability of dog p21 to suppress cell proliferation.[19] Consistent with this, we showed that upon PPM1D inhibition, the level of S123-phosphorylated dog p21 is increased, concomitantly with decreased expression of several cell cycle regulators including cyclin A, cyclin B, Rb, and PCNA (**Fig. 4B**). It is likely that dephosphorylation of dog p21 by PPM1D inhibits, whereas PPM1D inhibitor enhances, the ability of dog p21 to induce growth suppression. Thus, further studies are needed to determine the biological consequence of PPM1D-mediated dog p21 dephosphorylation, which may be explored for cancer management.
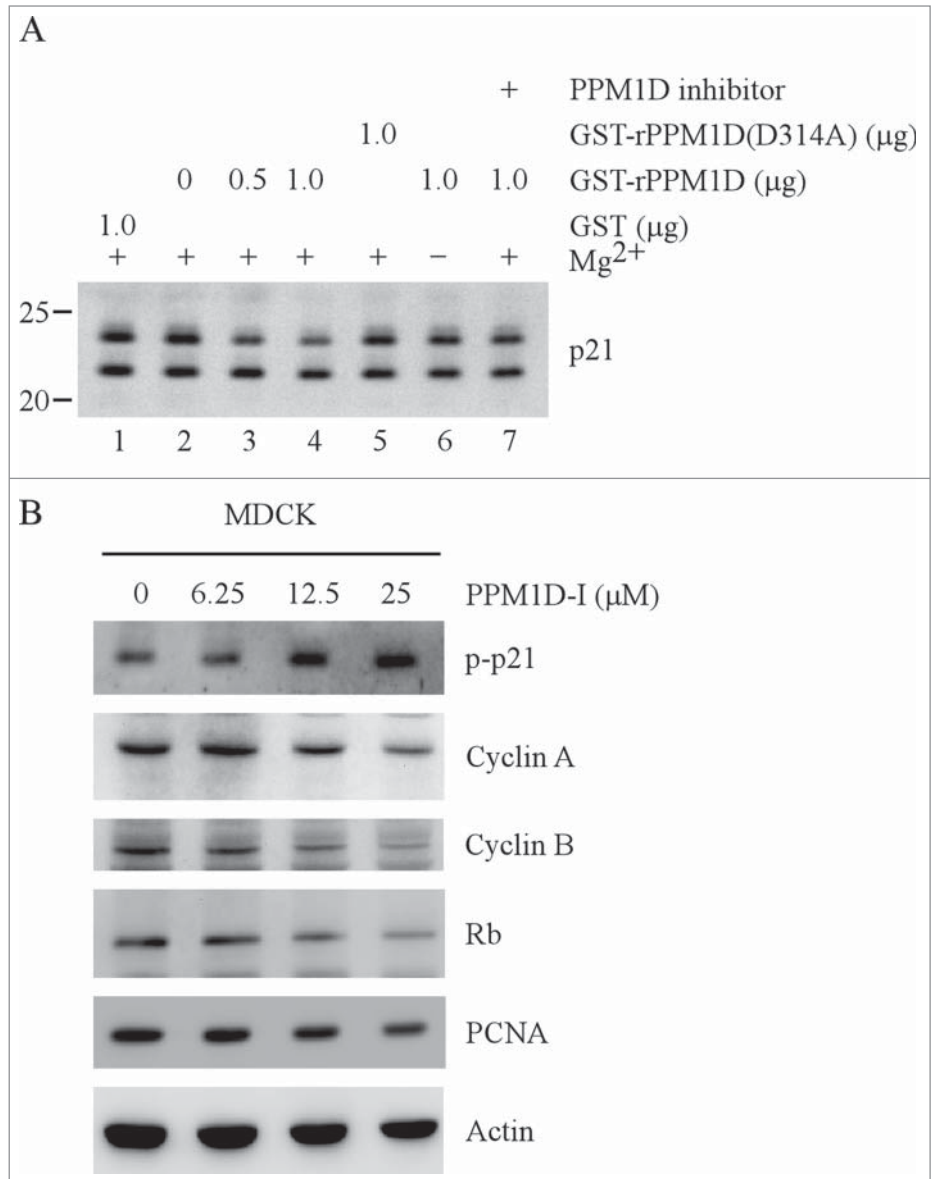

Figure 4. PPM1D dephosphorylates dog p21 at serine 123. (**A**) Cf2Th cells were induced to express dog p21 for 24 h, followed by immunoprecipatation with p21 antibody. The immunocomplexes were then incubated with recombinant GST or GST-tagged rPPM1D or rPPM1D (D314A) in the presence or absence of Mg^{2+} or PPM1D inhibitor for 4 h at 30°C, followed by Western blot analysis. (**B**) MDCK cells were treated with various amounts of PPM1D inhibitor for 24 h and the level of p-p21, cyclin A, cyclin B, Rb, and PCNA was determined by Western blot analysis.

We showed previously that in dog p21, serine 123 is followed by a proline, both of which form a consensus site for proline-directed phosphorylation.[19] While in human p21, serine 123 is followed by a glycine and thus, the proline-directed phosphorylation site is impaired. However, since serine 123 is present in human p21, it is possible that serine 123 can be targeted by a phosphatase or kinase other than proline-directed protein kinase. Interestingly, our preliminary data indicate that PPM1D inhibits human p21 expression independent of p53 expression. These data suggest that PPM1D may be a phosphatase of human p21.

However, it remains possible that PPM1D regulates human p21 expression via other mediators. For example, PP2Cγ, another member of PP2C family, was found to inhibit p21 expression via AKT.[24] Of particular interest, as both PPM1D and p21 are found to be deregulated in human cancers, thus, it is important to determine whether PPM1D-mediated p21 expression plays a role tumorigenesis. These questions need to be addressed in further studies.

Materials and Methods

Reagents

Anti-p21 (sx118), anti-PPM1D (H300), anti-Cyclin A (H432), anti-Cyclin B (H433), anti-Rb (C-15), and anti-PCNA (F-2) was purchased from Santa Cruz Biotechnology. Anti-actin and PPM1D inhibitor CCT007093 were purchased from Sigma. Scrambled siRNA (GCA GUG UCU CCA CGU ACU A) and siRNA against dog PPM1D (GCA UAG ACG AAA UGG UUU A) were purchased from Dharmacon RNA Technologies. The phospho-S123 dog p21 antibody (α-p-dog p21) was generated by OpenBiosystems by using a phosphopeptide span serine123; CTLLPH(p)SPERPEA.

Cell culture and transfection

MDCK (ATCC CCL-34) and Cf2Th (ATCC CRL-1430) cell lines were purchased from ATCC. Canine melanoma-36 cell line was generated from primary tumors of dogs with melanoma as previously described.[19] Cf2Th cells that can inducibly express dog p21 under the control of tetracycline promoter were previously generated.[19] To induce dog p21 expression, tetracycline (250–500 ng/ml) was added to the culture medium. All cells were maintained in DMEM (Dulbecco-Vogt modified Eagle's MEM) supplemented with 10% fetal bovine serum and 1% nonessential amino acid. For transient transfection, ExpressFect (Denville Scientific, Inc.) was used according to the manufacturer's instructions.

Plasmids and mutagenesis

Vector expressing dog p21 was generated as previously described.[19] To generate dog p21(S137A) mutant, a 2-step PCR strategy was used. The first-step PCR was performed to separately amplify 2 DNA fragments by using dog p21 expression vector as a template. Fragment 1 was amplified with P1, 5′ ATC CAA GCT TCC GCC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT TCG GAG CCG TCC AGG GA 3′, and P2, 5′ CGC CGT TTT CGG CCC TGA GCG GTG CCA GGC ACA CC 3′. Fragment 2 was amplified with P3, 5′ CCC CGG GTG TGC CTG GCA CCG CTC AGG GCC GAA AA 3′, and P4, 5′ ATC CGA ATT CAG ATT AGG GCT TCC TCT TGG AG 3′. The second-step PCR was performed using a mixture of fragment 1 and 2 as a template with P1 and P4 primers. This PCR product was cloned into pGEMT vector for sequence validation and then subcloned to pcDNA3 vector via HindIII and EcoRI sites to generate pcDNA3-dog p21(S137A). To generate dog PPM1D expression vector, the same 2-step PCR strategy was used except the cDNAs from MDCK cells was used as a template. To amplify Fragment 1, the primers used were P1, 5′ TAA AGC TTA TGG CGG GGC TGT ACT CG 3′, and P2, 5′ TTT CTC CTC TTG GTC CTG GCA C 3′. To amplify Fragment 2, the primers used were P3, 5′ CAC CGC AAG ATG CCA TCT CAA T 3′, and P4, 5′ CGC TCT AGA TCA GAC ACA AAC AGT 3′. To generate a vector expressing GST-tagged the catalytic domain (residue 1–420) of human PPM1D (rPPM1D), PCR products were amplified by using cDNAs from MCF7 cells as a template with P1, 5′ AAA GGA TCC AAA TGG CGG GGC TGT ACT CGC 3′, and P2, 5′ AAA CTC GAG TTA CTT GAC TGG TGG TGT AG AAC 3′. These PCR products were inserted into pGEX vector through BamH1 and XhoI sites and confirmed by sequencing. To generate GST-tagged human rPPM1D (D314A), the same strategy was used except that PCR products were amplified with 4 primers (P1, P2, P3, and P4). P1 and P2 are the same as the ones for human PPM1D whereas P3 is 5′ TTG GGG AGT GCT GGA CTT TGG AAT ATG 3′, and P4 is, 5′ AAG TCC AGC ACT CCC CAA TAT AAT ATA C 3′.

Western blot analysis

This assay was performed as previously described.[25] Briefly, cells lysates were resolved in 8–12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed with indicated antibodies. The proteins were visualized by SuperSignal West Femto Chemiluminescent Substrate (Pierce) using the ChemiDoc-It imaging system (UVP). The relative level of protein expression was quantified by densitometry using VisonworksLS software (UVP).

RNA isolation and RT-PCR analysis

The assay was performed as previously described.[2] Briefly, the primers for dog PPM1D are forward primer, 5′ CCA GAA CTT CCC AAG GAA AG 3′, and reverse primer, 5′ GTC TGG TTC AGG TGA CAC CAC 3′. The primers for dog actin were forward primer, 5′ CCT GAA TCC CAA AGC CAA CCG 3′, and reverse primer, 5′ TTG ATG TCA CGC ACG ATC TCC C 3′.

Recombinant protein purification and *In vitro* phosphatase assay

Recombinant proteins were expressed in bacteria BL21 and purified by glutathione sepharose beads. For in vitro phosphatase assay, extracts were collected from Cf2Th cells induced to express dog p21 for 24 h, followed by immunoprecipitation with antibody against p21. The immunocomplexes were suspended in 50 μl phosphatase buffer (50 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 0.05% 2-mercaptoethanol), and then incubated with recombinant GST, GST-rPPM1D or GST-rPPM1D (D314A) for 4 h at 30°C. Samples were subjected to Western blot analysis with antibody against p21. To inhibit the phosphatase activity of PPM1D, the phosphatase buffer was used without $MgCl_2$ or PPM1D inhibitor CCT007093 was added at 50 μM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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