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Author(s)	小境, 夕紀
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**Study on Cancer Inducible Mechanism via
Abnormal Nucleolar Formation by
Overexpression of Protein Phosphatase PPM1D**

(脱リン酸化酵素 PPM1D 過剰発現による
核小体形成異常を介した細胞癌化メカニズムに関する研究)

Laboratory of Biological Chemistry,
Graduate School of Chemical Sciences and Engineering,
Hokkaido University

Yuuki Kozakai

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Abbreviations:

ALCL	anaplastic large cell lymphoma
ALK	anaplastic lymphoma kinase
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ARF	alternate reading frame
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related protein
BLAST	basic local alignment search tool
CAD	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
CBP	cAMP response element binding protein
CDC25	cell division cycle 25
CDK1	cyclin-dependent kinase 1
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DFC	dense fibrillar component
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ER	estrogen receptor
FBS	fetal bovine serum
FC	fibrillar center

GC	granular component
H2AX	H2A histone family, member X
HA	hemagglutinin
HER2	human epidermal growth factor receptor 2
MAPK	Mitogen-activated Protein Kinase
MCF7	michigan cancer foundation-7
MDM2	murine double minute 2
MDS	myelodysplastic syndromes
MLF1	myeloid leukemia factor 1
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.
NES	nuclear export signal
NLS	nuclear localization signal
NPM	nucleophosmin
NuLS	nucleolar localization signal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PKR	protein kinase R
PLK1	polo-like kinase 1
PPM1D	protein phosphatase 1D
PP2A	protein phosphatase 2A
RAR α	retinoic acid receptor alpha
rDNA	ribosomal DNA
RNA	ribonucleic acid

rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
Tris	tris(hydroxymethyl)aminomethane

1. General Introduction

1.1. Protein phosphatase PPM1D

PPM1D (protein phosphatase magnesium-dependent 1, delta, also known as PP2C δ and Wip1) is a p53-inducible Ser/Thr phosphatase and a member of the type 2C phosphatase family. The *PPM1D* gene maps to 17q23.2 (**Figure 1-1**). PPM1D is composed of 605 amino acids and subdivided into two major domains, namely the catalytic and regulatory domains. In the catalytic domain, there are two unique loop regions (**Figure 1-1, 1-2, 1-3**). We have previously identified an alternative splice variant of PPM1D, PPM1D430 (*I*), which shares the catalytic domain (1–420 aa) and possesses 10 unique residues in the C-terminal domain (**Figure 1-1, 1-2**)

PPM1D was originally identified by Dr. Appella's group as a gene induced by tumor suppressor p53 after exposure to ionizing radiation (2). PPM1D dephosphorylates several proteins involved in DNA damage responses/repair. Furthermore, PPM1D is reported to dephosphorylate two distinct motifs: pTXpY and pS/pTQ (3). Acidic amino acids surrounding pS and pT are common features in PPM1D targets that include p53 (1, 2, 4, 5), ATM (6), ATR (7), Chk1 (8), Chk2 (9), p38 (10), and γ H2AX (11, 12) (**Figure 1-4**). In particular, Ser15 of p53 is a well-known substrate of PPM1D. Tumor suppressor p53 is a central factor that induces a number of proteins involved in cell cycle regulation and apoptosis by the DNA damage response (5). Ser15 in p53 is the site of phosphorylation by several kinases such as ATM and ATR (5). Phosphorylation of Ser15 recruits p300/CBP that acetylates lysine residues in in the C-terminus of p53 (5).

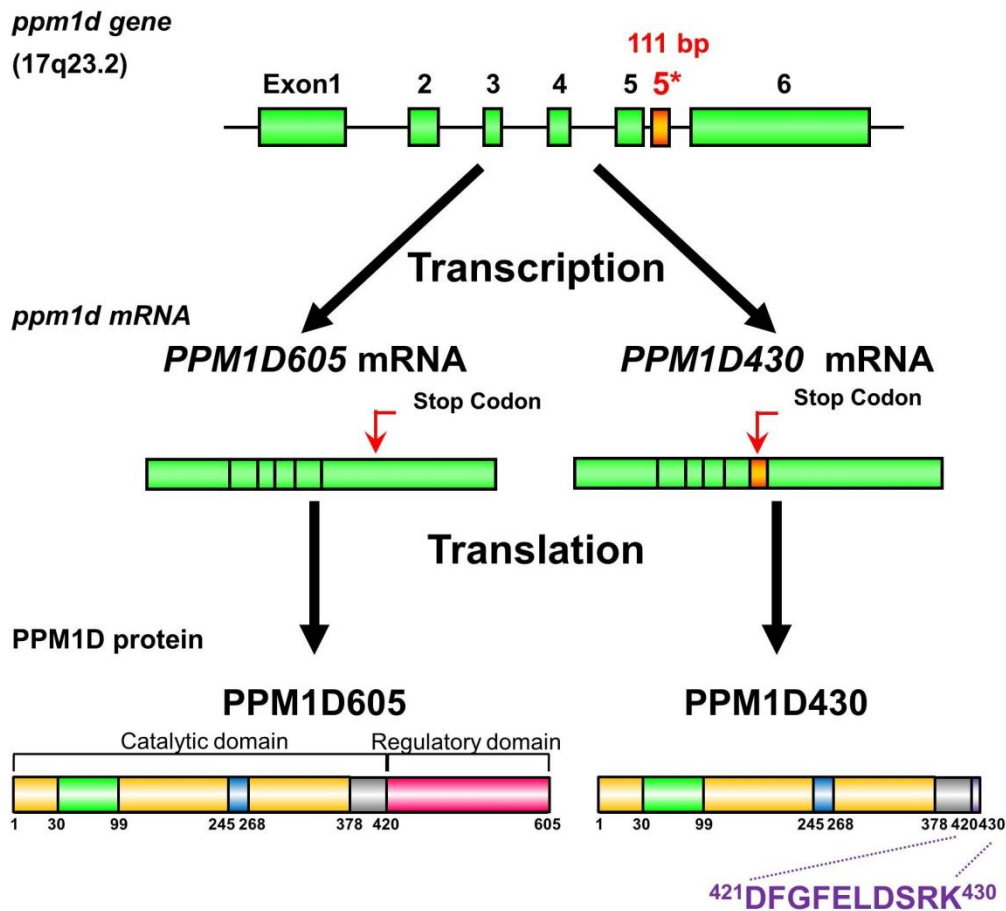


Figure 1-1. Schematic structure and amino acid sequence of PPM1D. PPM1D consists of two domains: catalytic domain (1-420) and regulatory domain (421-605). In the catalytic domain, there are two unique loop regions. The proline rich loop (P-loop) is represented in green and the basic rich loop (B-loop) is indicated in blue. Both loop regions are located within the catalytic domain of PPM1D.

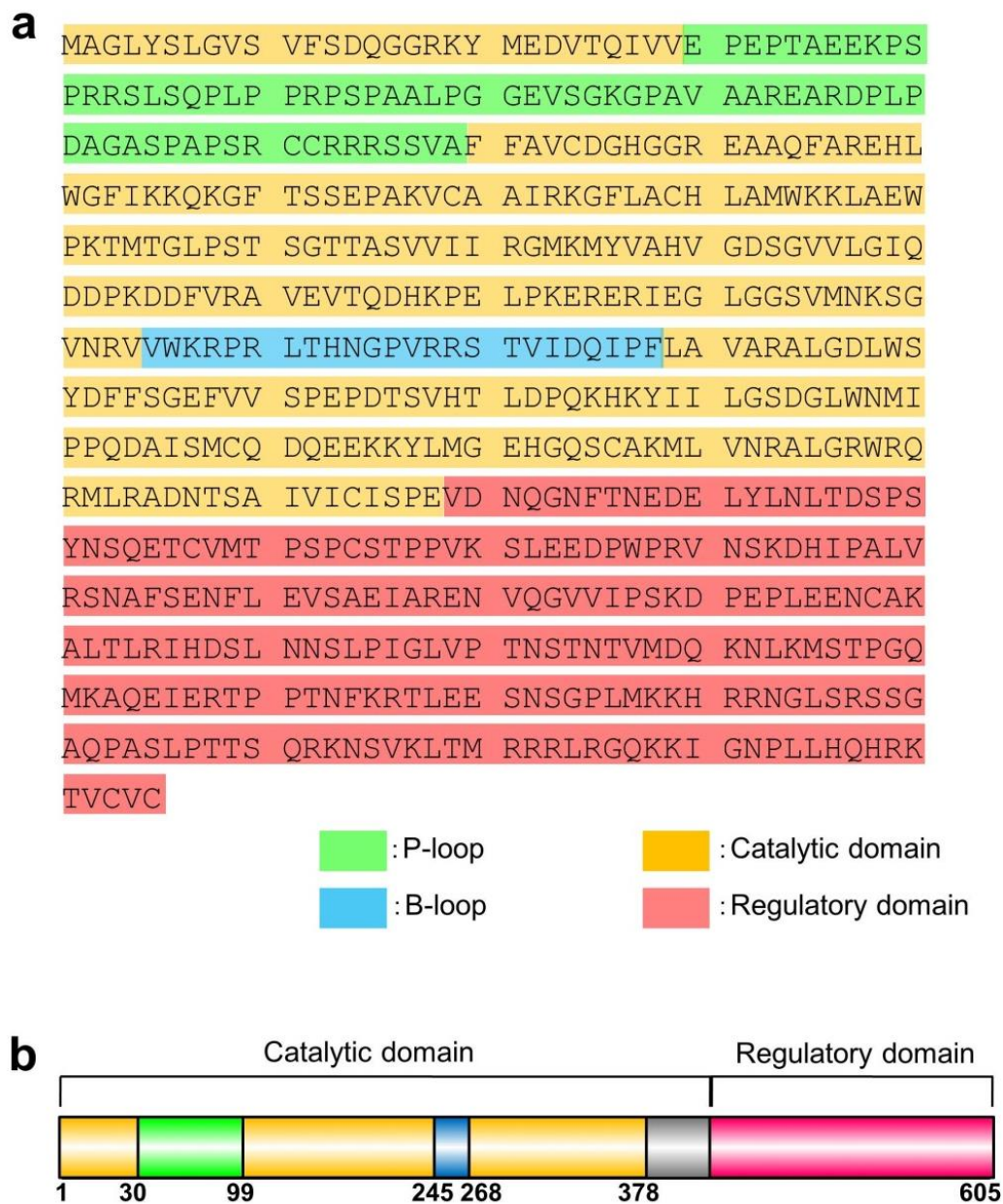


Figure 1-2. Amino acid sequence (a) and schematic structure (b) of PPM1D. PPM1D consists of two domains: catalytic domain (1-420) and regulatory domain (421-605). In the catalytic domain, there are two unique loop regions. The proline rich loop (P-loop) is represented in green and the basic rich loop (B-loop) is indicated in blue. Both loop regions are located within the catalytic domain of PPM1D.

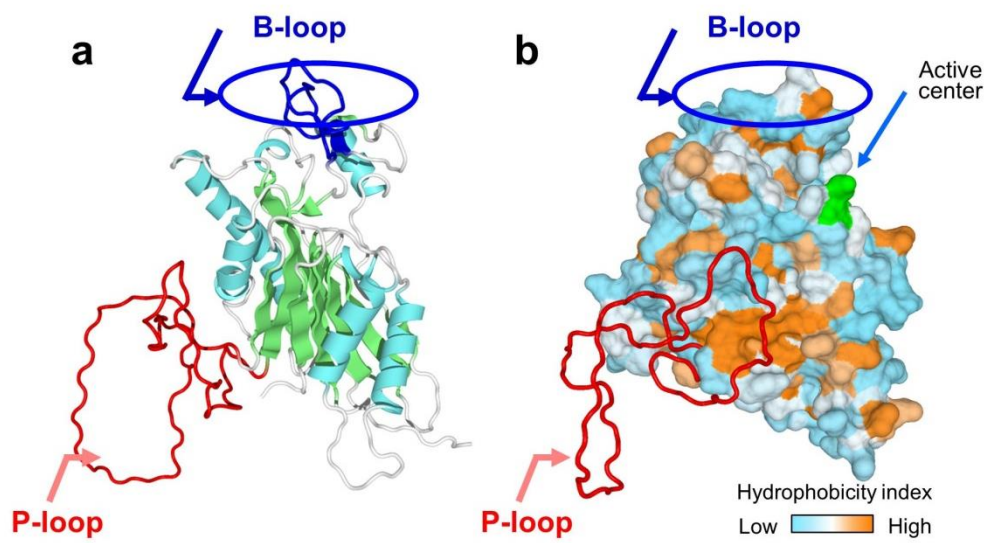


Figure 1-3. Structure model of PPM1D. Homology modeling of PPM1D was shown based on the structure of PPM1A, PPM1B and PPM1K. B-loop is located near by the active center, and P-loop is located at an opposite side of the active center.

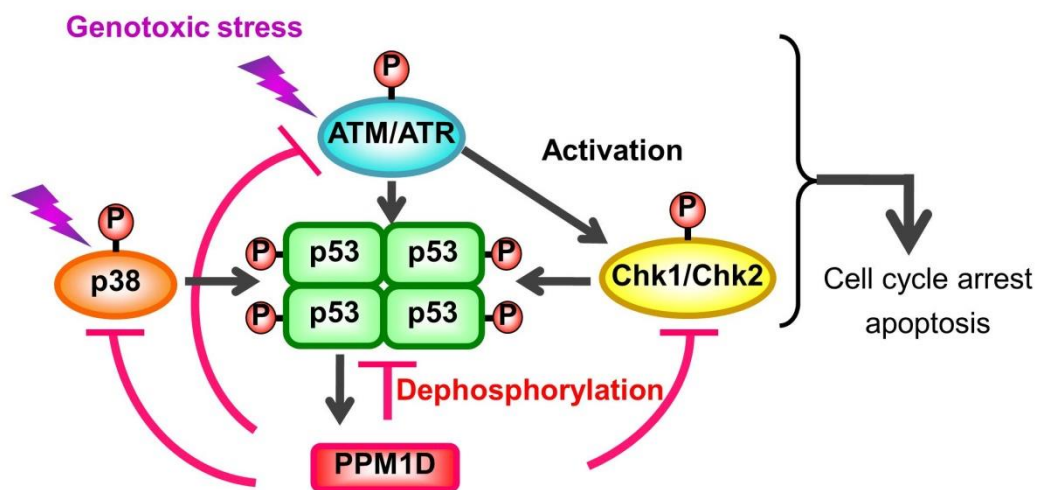


Figure 1-4. One of the main functions of Protein phosphatase PPM1D in normal cells. PPM1D is induced by a tumor suppressor p53 in response to genotoxic stress. PPM1D dephosphorylates and inactivates p53, Chk1, Chk2, ATM, ATR and p38.

Phosphorylation at the N-terminal region and acetylation at the C-terminal region is important to prevent the interaction with E3 ubiquitin ligase MDM2 (5). Therefore, dephosphorylation of p53 at Ser15 is important for homeostatic regulation after the DNA damage response (5).

1.4. *PPM1D* gene amplification, and *PPM1D* mRNA and protein overexpression in tumors

Gene amplification, and mRNA and protein overexpression of *PPM1D* have been reported in human tumors (**Table 1**) (3, 15-24). In particular, amplification of the *PPM1D* gene is often reported in breast and ovarian cancers. Breast cancers are one of the most common tumors in women. According to the American Cancer Society, general treatments for breast cancer patients include surgery, radiation therapy, chemotherapy, hormone therapy, targeted therapy, and bone-directed therapy. HER2 is frequently overexpressed at the surface of breast cancer cells and is a good target for targeted therapy. A cohort study of patients with invasive breast cancer showed that *PPM1D* is overexpressed in *PPM1D*-amplified cancers (25). It also showed gene amplification of *PPM1D* in tumors displaying luminal (ER-positive) and HER2-positive phenotypes (25).

Gene amplification of *PPM1D* is also observed at high rates in neuroblastoma and medulloblastoma (92% and $\geq 37\%$, respectively) (**Table 1**) (3, 19-21). Neuroblastoma arises in early nerve cells. Medulloblastoma is a brain tumor located in the cerebellum. They are the most representative solid cancers in childhood and infancy. Their exact

Table 1. Gene amplification and RNA overexpression of *PPM1D*. In many types of human tumors, gene amplification and RNA overexpression of *PPM1D* are reported. Table1 was reported in *Cancer metastasis reviews* (3) and modified by our laboratory.

Tumors	Gene Amplification	RNA Overexpression	Reference
Breast Cancer	37/326 (11 %)		Bulavin <i>et al.</i> 2002.
	26/164 (16 %)		Li <i>et al.</i> 2002.
	13/117 (11 %)		Rauta <i>et al.</i> 2006.
		7/20 (35 %)	Yu <i>et al.</i> 2007.
Neuroblastoma	23/25 (92 %)	9/32 (28 %)	Saito-Ohara <i>et al.</i> 2003.
Medulloblastoma	24/47 (51 %)	148/168 (88 %)	Mendrzyk <i>et al.</i> 2005.
	6/16 (37 %)	3/11 (27 %)	Ehrbrecht <i>et al.</i> 2006.
Gastric carcinoma		35/39 (90 %)	Fuku <i>et al.</i> 2007.
Pancreatic adenocarcinoma	8/13 (62 %)		Loukopoulos <i>et al.</i> 2007.
Ovarian clear cell adenocarcinoma	8/20 (40 %)		Hirasawa <i>et al.</i> 2003.

Cancer Metastasis Rev., **27**, 123-35, 2008

Modified by our lab.

cause is unknown and effective molecular targeting drugs have not been developed for these tumors.

1.3. *PPM1D* mutations in tumors

Recently, *PPM1D* mutations were identified in breast, ovarian, and colorectal cancers as well as brainstem glioma (26-28). Most *PPM1D* mutations are found in the C-terminus, including nonsense mutations such as E472X, L484X, S516X, E525X, and E540X, and a frame shift mutation that disrupts Asn488 (28) (**Figure 1-5**). It has been reported that these mutations lead to C-terminal truncation of *PPM1D* and increases in both its protein stability and phosphatase activity (27). HCT-116, a human colon carcinoma cell line, harbors both C-terminus-truncated *PPM1D* and wild-type *PPM1D*605 alleles. Repair of mutated *PPM1D* in HCT-116 cells by homologous recombination results in inhibition of cell growth and colony formation (28). It has been reported that high doses of doxorubicin do not completely suppress HCT-116 cell growth (29). Therefore, C-terminal truncated *PPM1D* is also a good target molecule for anti-cancer treatments.

1.4. *PPM1D* inhibitors

In our laboratory, we have developed a small molecule inhibitor of *PPM1D*, SPI-001 (**Figure 1-6**). Several groups have reported *PPM1D*-specific inhibitors (**Table 1-2**). (30). SPI-001 is a specific inhibitor of *PPM1D* and strongly inhibits the phosphatase activity of *PPM1D* in a non-competitive manner. In addition, SPI-001 suppresses the

PPM1D605

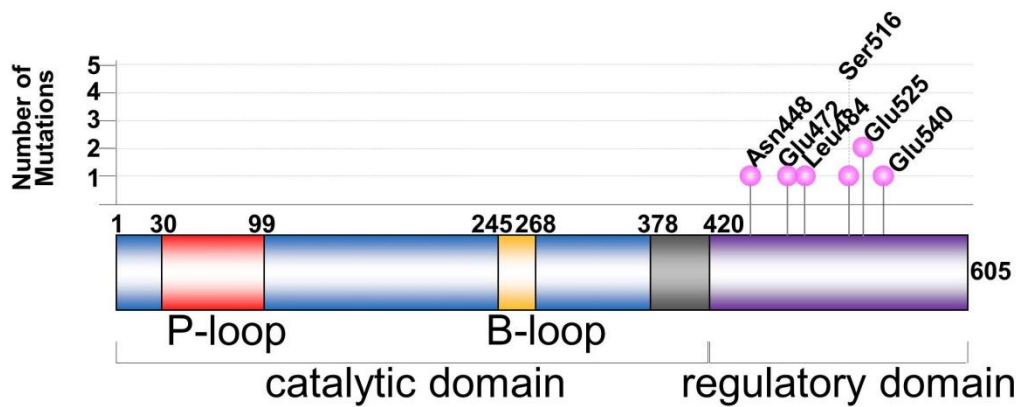


Figure 1-5. Reported mutations of PPM1D. Most of PPM1D mutations are found in C-terminal of PPM1D: nonsense mutations such as E472X, L484X, S516X, E525X E540X, and a frame shift mutation that disrupted Asn488 in glioma.

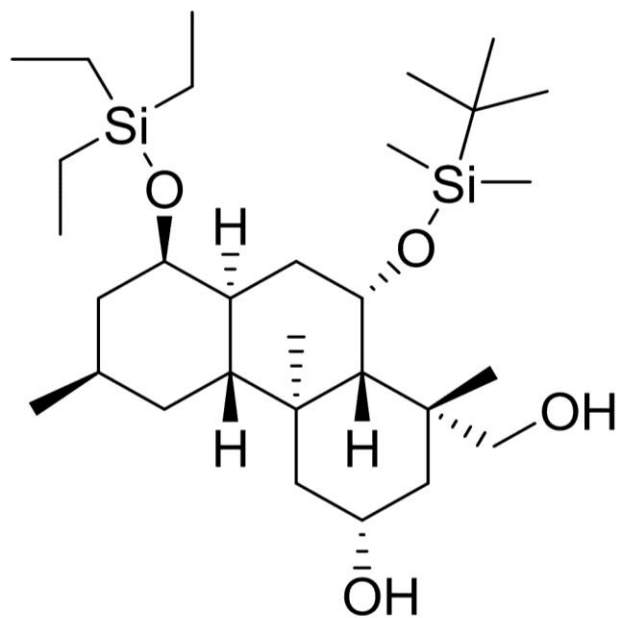
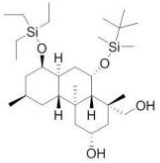
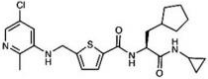
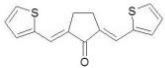
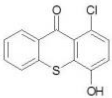
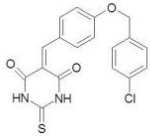
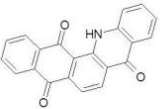
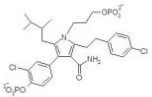


Figure 1-6. Structure of SPI-001.

Table 2. PPM1D inhibitors. Structure and inhibitory activity of PPM1D inhibitors were summarized.

Inhibitor	Structure	IC ₅₀	References
SPI-001		0.48 (μM)	<i>Bioorg. Med. Chem. Lett.</i> , 22 , 729-732 (2012)
not determined	c(MS(P)IY(P)VA)	3.7 (μM)	<i>Biochemistry</i> , 45 , 13193-13202 (2006)
not determined	c(FS(P)IY(P)DD)	0.1 (μM)	<i>Biochemistry</i> , 50 , 4537-4549 (2011)
GSK2830371		6 (nM)	<i>Nat. Chem. Biol.</i> , 10 , 181-187 (2014)
CCT007093		8.4 (μM)	<i>Oncogene</i> , 27 , 1036-1044 (2008)
CCT071835		1.5 (μM)	<i>Oncogene</i> , 27 , 1036-1044 (2008)
CCT021600		4.7 (μM)	<i>Oncogene</i> , 27 , 1036-1044 (2008)
CCT010971		6.1 (μM)	<i>Oncogene</i> , 27 , 1036-1044 (2008)
not determined		9.8 (μM)	<i>Chem. Med. Chem.</i> 3 , 230-232 (2008)

growth of the human breast cancer cell line MCF7 through activation of the p53 pathway. SPI-001 is one of the most potent small molecule inhibitors among the reported PPM1D inhibitors.

1.5. The nucleolus

The nucleolus, which is located in the nucleus, is the site of ribosome synthesis (**Figure 1-7**). It is divided into three subregions termed the fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC) (36). At the ends of chromosomes 13, 14, 15, 21, and 22, there are regions of genes encoding ribosomal DNA (rDNA). At the border between the FC and DFC, rDNA is transcribed to 47S ribosomal RNA (rRNA). It is then processed to form 28S, 18S, and 5.8S rRNAs and post-transcriptionally modified in the DFC. Finally, these rRNAs are assembled in the GC with ribosomal proteins before transport to the cytoplasm. Although the nucleolus has been considered to be involved in ribosomal synthesis, several lines of evidence suggest that it is also related to other cellular activities such as stress responses and cell cycle progression (36-38).

1.6. Abnormal nucleoli in tumors

Cancer cells have specific features in terms of cell morphology, such as abnormal nucleolar formation (**Figure 1-8**) (31, 32). In breast cancers, an increase in the nucleolar number has been observed in high-grade tumors (33, 34). Increases in the number or size of nucleoli are important parameters in cancer prognostics (35). However, the

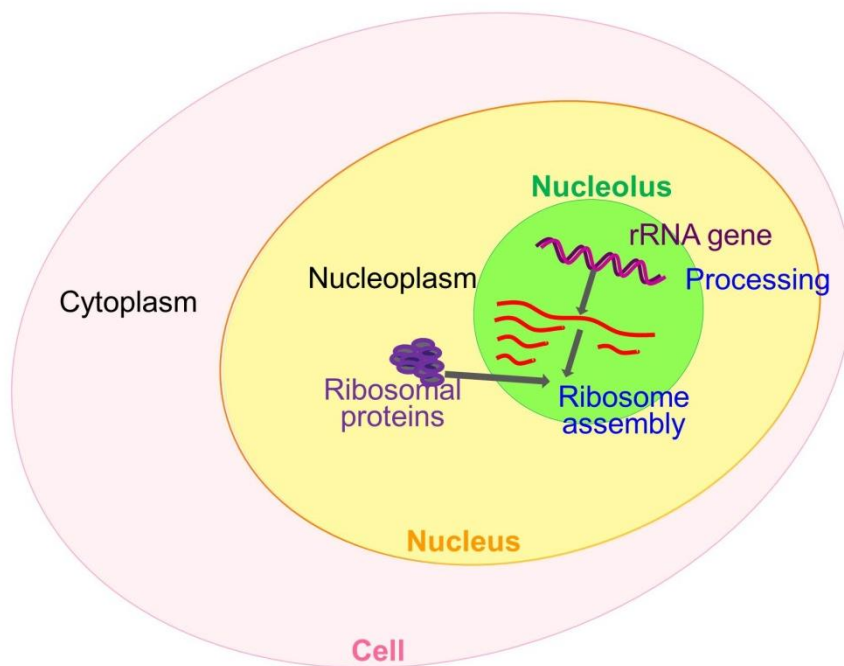


Figure 1-7. The nucleolus. In the nucleolus, rDNA is transcribed to 47S ribosomal RNA. It is processed to form 28S, 18S and 5.8S ribosomal RNAs (rRNA) and post-transcriptionally modified. Finally, they are assembled in GC before transporting to the cytoplasm.

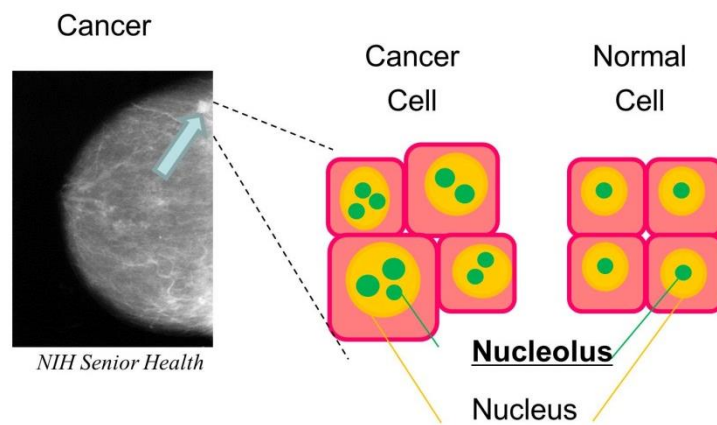


Figure 1-8. Morphological characteristics of cancers. Cancer cells is characterized by: having irregular size or shape of cells, a large nucleus and abnormal nucleoli. An increase in the number or size of nucleoli is often observed in tumor cells.

underlying molecular mechanisms of their structural integrity and abundance are still unclear (35).

1.7. Nucleolar protein nucleophosmin

Proteomic analysis of the nucleolus has revealed the presence of over 700 proteins, including ribosomal proteins and nucleolar proteins such as nucleophosmin (NPM) (39). NPM, also called B23, numatrin, and NO38, is an abundant phosphoprotein. It is localized at the GC of the nucleolus and shuttles between the nucleus and cytoplasm (40). NPM is composed of 294 amino acids and consists of four main domains: oligomerization, molecular chaperone, histone-binding, and DNA/RNA-binding domains. It also contains a nuclear export signal (NES), bipartite nuclear localization signal (NLS), and nucleolar localization signal (NuLS) (**Figure 1-9**) (40). NPM is overexpressed in solid tumors, whereas the *NPM1* locus is deleted or translocated in hematopoietic tumors (40). Two different functions of NPM have been reported in tumorigenesis. In tumors, overexpressed NPM can function as a proto-oncogene by suppression of apoptosis through inhibiting phosphorylation of tumor suppressor p53 in response to stress stimuli (40, 41). Overexpression of NPM is also reported to induce cell growth and proliferation through its own activity of ribosome biogenesis (40). In contrast, other reports show that NPM directly binds to p53 and induces its stability and activity (40, 42). NPM also interacts with and stabilizes tumor suppressor ARF (40, 43). Several kinases such as CDK1 (44) and PLK1 phosphorylate NPM at multiple sites

A

MEDSMDMDMS PLRPQNYLFG CELKADKDYH FKVDNDENEH
 QLSLRTVSLG AGAKDELHIV EAEAMNYEGS PIKVTLATLK
 MSVQPTVSLG GFEITPPVVL RLKCGSGPVH ISGQHLVAVE
 EDAESEDEEEE EDVKLLSISG KRSAPGGGSK VPQKKVKLAA
 DEDDDDDDEE DDEDDDDDDD FDDEEAEEKA PVKKSIRDTP
 AKNAQKSNQN GKDSKPSSTP RSKGQESFKK QEKTPKTPKG
 PSSVEDIKAK MQASIEKGG S LPKVEAKFIN YVKNCFRMTD
 QEAIQDLWQW RKSL

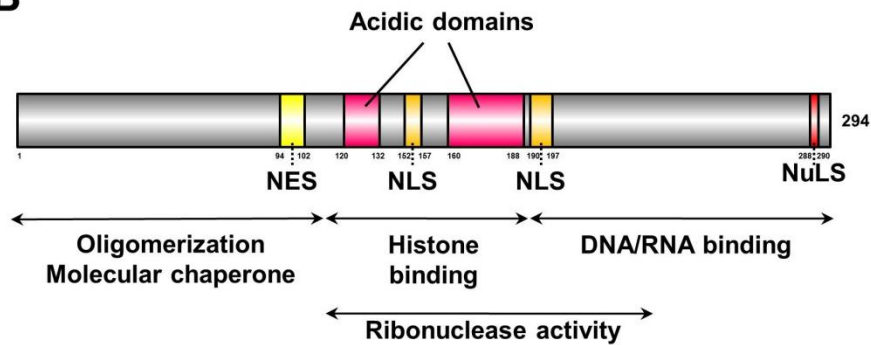
B

Figure 1-9. Schematic structure and amino acid sequence of NPM. (A) Primary sequence of NPM. Red letters represent reported phosphor-Ser/Thr. (B) NPM consists of three main domains: oligomerization domain as well as molecular chaperone domain, histone binding domain and DNA/RNA binding domain. It also contains nuclear export signal (NES), bipartite nuclear localization signal (NLS), nucleolar localization signal (NuLS).

(**Figure 1-9**) (45). However, the sequential relationship among these phosphorylation sites is not fully understood.

1.8. Aims of this study

An increase in the nucleolar number is one of the important parameters for cytology. However, underlying basis for the molecular mechanism is still unknown. Proto-oncogene *PPM1D* is often amplified and mutated in many tumors, suggests that PPM1D is a promising target for anti-cancer treatments. In this study, I am going to reveal the cancer inducible molecular mechanism via abnormal nucleolar formation in PPM1D overexpressed tumors and develop a novel anti-cancer treatment in PPM1D mutated tumors.

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2. Development of anti-cancer treatments against truncated PPM1D

2.1. Abstract

PPM1D is a p53-inducible protein phosphatase. Gene amplification and mRNA overexpression of PPM1D are reported in various types of tumors. We have previously reported that a PPM1D inhibitor suppresses the growth of PPM1D-overexpressing cancer cells. Recent reports have shown PPM1D mutations in several types of tumors including breast, ovarian, colorectal, and brainstem tumors. It has been also reported that C-terminal truncation of PPM1D resulting from gene mutation leads to increases in protein stability and phosphatase activity. Therefore, truncated PPM1D may be a good target for anti-cancer treatments. HCT-116 human colorectal carcinoma cells overexpress C-terminus-truncated PPM1D and express wild-type PPM1D. In this study, I found that co-treatment with the PPM1D inhibitor SPI-001 and doxorubicin is more effective to decrease the viability of HCT-116 cells than doxorubicin alone. Furthermore, co-treatment with SPI-001 and doxorubicin increased the phosphorylation level of p53 at Ser15, one of the targets of PPM1D. My results suggest that combinatorial treatment with the PPM1D inhibitor and doxorubicin may be a powerful anti-cancer treatment for C-terminal truncated PPM1D-overexpressing cancer cells.

2.2 Introduction

PPM1D is a promising target for anti-cancer drugs. Gene amplification and mRNA overexpression of PPM1D are reported in many human tumors (1-4). PPM1D-overexpressing mouse embryonic fibroblasts (MEFs) show enhanced oncogenic activity of H-rasV12 for transformation (2). PPM1D-deficient mice show defects in cell cycle regulation (5). The function of PPM1D as a proto-oncogene is further supported by several findings showing that PPM1D dephosphorylates and inactivates tumor suppressor p53 and p53-related proteins such as ATM (6), ATR (7), Chk1 (8), Chk2 (9), and p38 (10). In particular, Ser15 in p53 is a well-known target of PPM1D (11-14). Tumor suppressor p53 plays an important role in the DNA damage response and induces a number of proteins involved in cell cycle regulation and apoptosis (14).

Recently, PPM1D mutations were found in several types of tumors including breast, ovarian, and colorectal tumors, as well as brainstem glioma (15-17). PPM1D is composed of 605 amino acids and subdivided into two domains: the N-terminal catalytic domain and C-terminal regulatory domain. Most PPM1D mutations are found in the C-terminus of PPM1D, including nonsense mutations such as E472X, L484X, S516X, E525X, and E540X, and a frame shift mutation that disrupts Asn488 (17). HCT-116, a human colorectal carcinoma cell line, possesses PPM1D605 and PPM1D L450X mutations (**Figure 2-1**) (16). Doxorubicin is a representative drug used for cancer treatment. It has been reported that a high concentration of doxorubicin does not suppress the growth of HCT-116 cells completely.

In our laboratory, we have developed a small molecule inhibitor of PPM1D, SPI-001 (**Figure 1-4**) (18). This small molecule inhibitor shows strong inhibitory activity against PPM1D but not PPM1A or PP2A. SPI-001 has a tricyclo[8.4.0.0^{2,7}]tetradecane (perhydrophenanthrene) core as a backbone with two hydrophobic silyl groups and hydroxyl groups. We found that the two silyl groups were responsible for its inhibitory activity. It also suppressed the growth of MCF7 cells, a human breast cancer cell line that overexpresses PPM1D605 (**Figure 2-2**), through elevation of p53 phosphorylation and induction of apoptosis (18).

In this study, I revealed the synergistic effect of SPI-001 and doxorubicin on growth inhibition of HCT-116 cells. I also found that this inhibitory effect on the growth of HCT-116 cells was mediated by activation of p53. These findings indicate that combinatorial treatment with SPI-001 and doxorubicin may be a potential and useful anti-cancer treatment for PPM1D-mutated cancer cells.

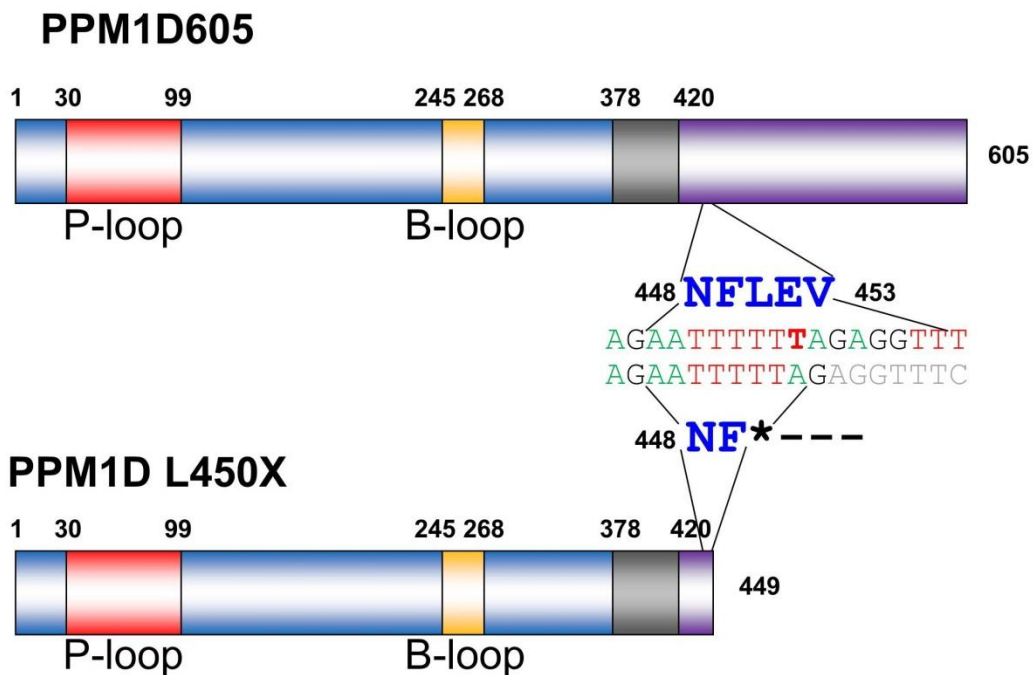


Figure 2-1. Schematic diagram of protein structures of PPM1D605 and PPM1D L450X. PPM1D605 is composed of 605 amino acids and PPM1D L450X is composed of 449 amino acids. Heterozygous mutations 1349delT within the *PPM1D* gene result in PPM1D L450X. The blue box represents the catalytic domain. In the catalytic domain, the proline rich loop (P-loop) and the basic rich loop (B-loop) are located. P-loop is represented in red and B-loop is indicated in yellow.

2.3. Experimental procedures

2.3.1. Cell culture

A549 lung carcinoma cells and HCT-116 human colorectal carcinoma cells were obtained from ATCC (Rockville, MD). Cell lines were cultured in Dulbecco's modified Eagles medium or McCoy's Medium supplemented with 10% v/v fetal bovine serum with 100 units/ml of penicillin and 100 µg/ml of streptomycin in a humidified atmosphere of 5% CO₂. When required, cells were treated with a range of concentration of 0.5-2 µM of doxorubicin and 40 µM of SPI-001 for 24 h or 48 h.

2.3.2. Antibodies

Rabbit polyclonal anti-PPM1D was prepared as previously described (13). Other antibodies used include: Mouse monoclonal anti-p53 (DO-1) and anti-p21 (F-5) from Santa Cruz Biotechnology; Mouse monoclonal Phospho-p53 (Ser15) (16G8) from Cell Signaling Technology, Mouse monoclonal Anti-Actin, clone C4 from Millipore; Secondary antibodies for Western blotting include: Anti-mouse IgG HRP-linked antibody from GE healthcare; Anti-rabbit IgG HRP-linked antibody from New England Bio Labs.

2.3.3. Western blotting analysis

1x10⁵ cells were plated onto 35 mm dishes with 2 ml of medium and incubated for 24 h or 48 h before drug treatment. In figure 3, cells were treated with SPI-001 and

doxorubicin for 48 h and then stained with 8.1 μM of Hoechst 33342. In figure 5, cells were treated with SPI-001 and doxorubicin for 24 h or 48 h and then total cell extracts were prepared by collecting attached and floating cells in 1x sample buffer (50 mM Tris-HCl, pH6.8, 10% Glycerol, 2% SDS, 6% 2-mercaptoethanol). In figure S1, attached cells were collected and sampled with 1x sample buffer. Equivalent amount of total cellular protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Proteins were detected by enhanced chemiluminescence with the following antibodies.

2.3.4. Cell viability assay (MTS assay)

1×10^4 cells were plated onto 96-well flat bottomed plates with 100 μl of medium and incubated for 48 h after drug treatment. Cell viability was measured by commercially available kit (CellTiter 96[®] AQueous One Solution Cell Proliferation Assay, Promega) in accordance with manufacturer's instructions.

2.4. Results

2.4.1. Effect of SPI-001 on the viability of HCT-116 cells overexpressing C-terminal truncated PPM1D

I compared the expression levels of PPM1D among MCF7, HCT-116, and A549 cells (**Figure 2-2**). Compared with the other cell lines, the human breast cancer cell line MCF7 overexpressed PPM1D605. HCT-116 cells overexpressed C-terminal truncated PPM1D and PPM1D L450X together with a normal level of PPM1D605. Doxorubicin is an anti-cancer drug used for treatment of many kinds of cancer (19). By binding to DNA-associated enzymes, it intercalates the base pairs of DNA (19). To investigate the inhibitory effect of doxorubicin on the viability of A549 and HCT-116 cells, I conducted MTS assays. In HCT-116 cells, even high doses of doxorubicin did not lead to significant cell death, although it suppressed A549 cell growth in a dose-dependent manner (**Figure 2-3**). I also examined the effect of SPI-001 on the viability of HCT-116 cells in MTS assays. As a result, 40- μ M SPI-001 alone showed little effect on the viability of HCT-116 cells (**Table 2-1**). This result may be because MCF7 and HCT-116 cells express different protein levels of PPM1D (**Figure 2-2**).

2.4.2. Effects of co-treatment with SPI-001 and doxorubicin on cell viability of HCT-116

Compared with doxorubicin alone, I found that co-treatment with doxorubicin and SPI-001 suppressed cell growth (**Figure 2-4**). To investigate the details of the inhibitory

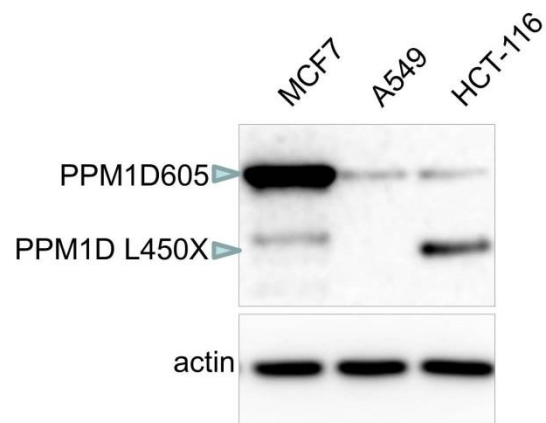


Figure 2-2. Comparison of protein level of PPM1D among MCF7, A549 and HCT-116 cells. Attached cells were collected and lysed with 1x sample buffer. Both PPM1D and actin were detected by western blotting. The detection of actin was used as loading control.

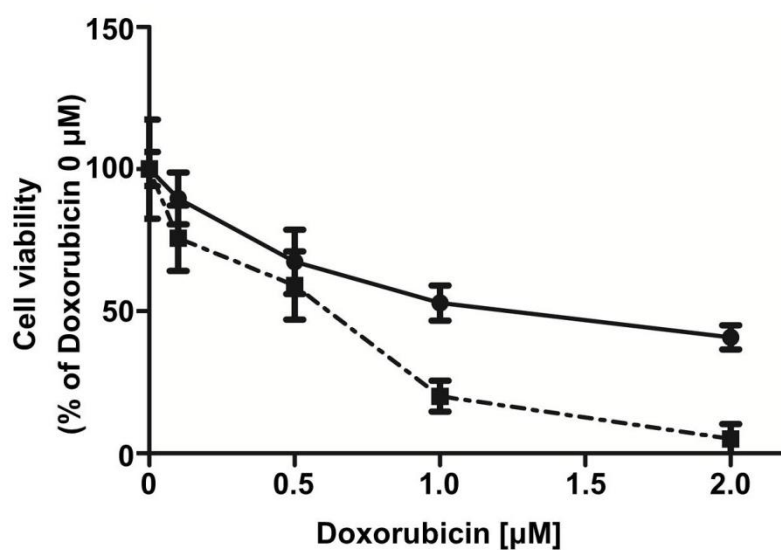


Figure 2-3. Cell viability of HCT-116 and A549 cells under the treatment with a range of concentration of doxorubicin. Both HCT-116 and A549 cells were incubated with a range of doxorubicin for 48h. MTS assay was conducted after treatment in HCT-166 (bold line) or A549 cells (dot line). Data represent the mean \pm S.D. of eight or six points, respectively, from independent experiments.

Table 2-1. Cell viability of co-treatment with 2- μ M doxorubicin and 40- μ M SPI-001 in HCT-116

	Cell viability (% of no treatment)	
	No treatment	Doxorubicin
No treatment	100.0 \pm 16.3	40.7 \pm 3.7
SPI-001	94.2 \pm 11.9	26.3 \pm 3.4

HCT-116 cells were incubated under the treatment of 2- μ M doxorubicin and 40- μ M SPI-001. MTS assay was performed 48 h after treatment with doxorubicin alone or in combination with SPI-001. Data represent the mean \pm S.D. of eight points from independent experiments.

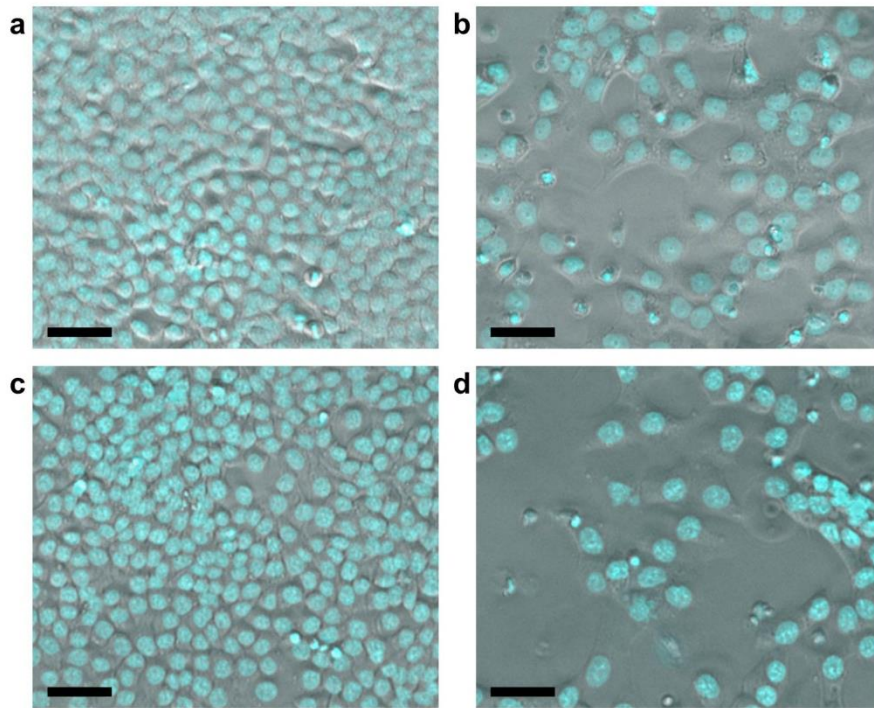


Figure 2-4. Phase contrast images of HCT-116 cells under the treatment with doxorubicin and SPI-001. HCT-116 cells were incubated with vehicle (a), 2- μ M of doxorubicin alone (b), with 40- μ M SPI-001 (c), or with co-treatment of doxorubicin and SPI-001 (d) and then nuclei were stained with 8.1- μ M of Hoechst 33342. Scale bar represents 50 μ m.

effect of co-treatment with doxorubicin and SPI-001, I treated HCT-116 with various concentrations of doxorubicin and 40- μ M SPI-001 for 48 h and then performed MTS assays (**Figure 2-5 and Table 2-1**). Co-treatment with 2- μ M doxorubicin and 40- μ M SPI-001 suppressed cell viability by up to 26%, even though 41% of cells treated with doxorubicin alone remained viable (**Figure 2-5 and Table 2-1**).

2.4.3. Effect of co-treatment with SPI-001 and doxorubicin on the p53 pathway

Tumor suppressor p53 is a well-known substrate of PPM1D. p53 induces cell cycle regulator p21 or apoptotic proteins in response to DNA stress. Phosphorylation of p53 at Ser15 is dephosphorylated by PPM1D *in vitro* and *in vivo* (12-14). To investigate the effect of co-treatment on the p53 pathway, I treated HCT-116 cells with 40- μ M SPI-001 and 2- μ M doxorubicin for 24 or 48 h and then analyzed the phosphorylation level of p53 at Ser15. Doxorubicin alone increased the phosphorylation levels of p53 and p21 compared with those in untreated cells. However, compared with doxorubicin alone, co-treatment with SPI-001 and doxorubicin enhanced phosphorylation of p53 at Ser15 and p21 (**Figure 2-6**). PPM1D is also a downstream target of p53. Therefore, an increase in PPM1D protein was observed in cells treated with doxorubicin and SPI-001.

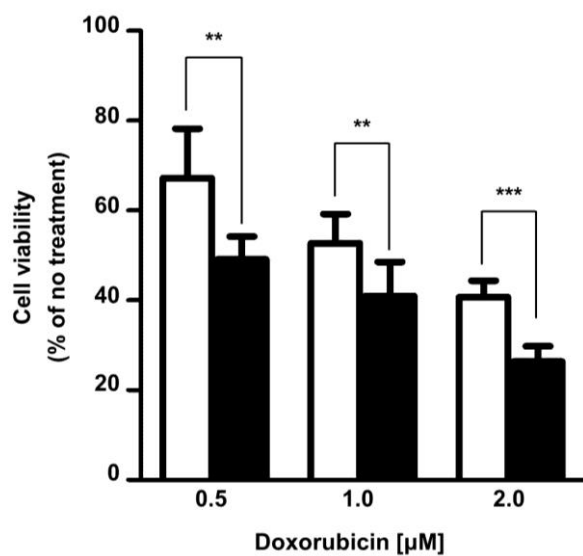


Figure 2-5. Effect of co-treatment with doxorubicin and SPI-001 on cell viability of HCT-116. HCT-116 cells were incubated with a range of doxorubicin and 40- μM of SPI-001 for 48 h. MTS assay was performed after treatment with doxorubicin alone (open column) or in combination with SPI-001 (closed column). Data represent the mean \pm S.D. of eight points from independent experiments. Statistical significance was determined by student's T-test and established at *** $p < 0.001$ or ** $p < 0.01$.

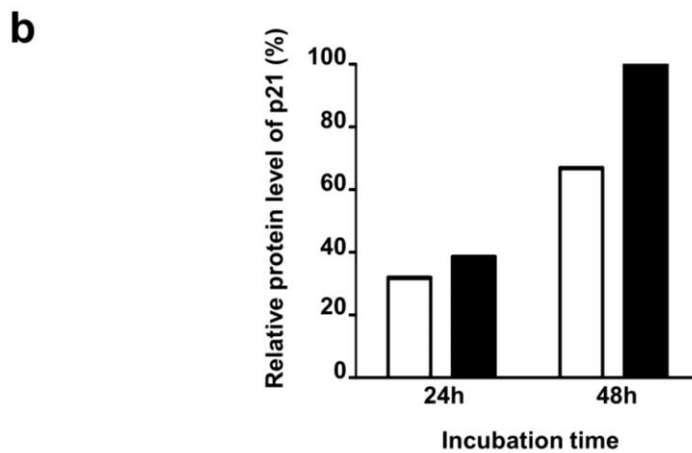
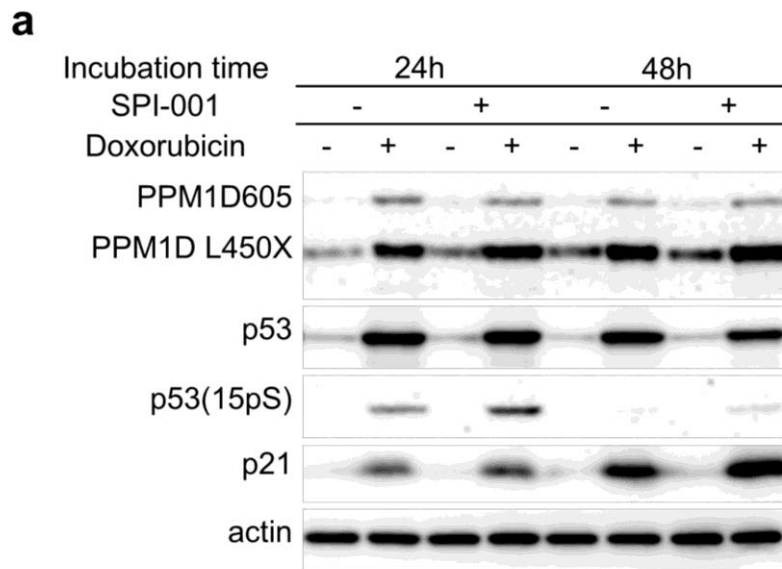


Figure 2-6. Effect of co-treatment with doxorubicin and SPI-001 on p53 activity in HCT-116 cells. HCT-116 cells were incubated with 2- μ M doxorubicin and 40- μ M SPI-001 for 24 h or 48 h. Attached and floating cells were collected and lysed with 1x sample buffer. (a) Western blotting for phosphorylated p53 at Ser15, total p53 protein, p21, PPM1D and actin. The detection of actin was used as loading control. (b) Relative expression level of p21 was quantified from western blotting (a). Each column indicates relative p21 expression in the treatment with doxorubicin alone (open column) and in combination with SPI-001 (closed column).

2.5 Discussion

In summary, I found that the PPM1D inhibitor SPI-001 supported the effects of doxorubicin in HCT-116 cells that overexpress C-terminal truncated PPM1D. Furthermore, compared with doxorubicin alone, I demonstrated that co-treatment with SPI-001 and doxorubicin increased phosphorylation of p53 at Ser15 and downstream targets of p53 in HCT-116 cells.

Recent reports show that the stability and phosphatase activity of C-terminal truncated PPM1D are higher than those of PPM1D605. In brainstem glioma patients, C-terminal truncated *PPM1D* mutations have been identified at high rates, such as nonsense mutations E472X, L484X, S516X, E525X, and E540X, and a frame shift mutation that disrupts Asn488 resulting in a *de novo* stop codon. It is important to develop anti-cancer treatments against cancer cells that express C-terminal truncated PPM1D. Our results suggest that co-treatment with the PPM1D inhibitor and doxorubicin is an effective treatment strategy for these cancers.

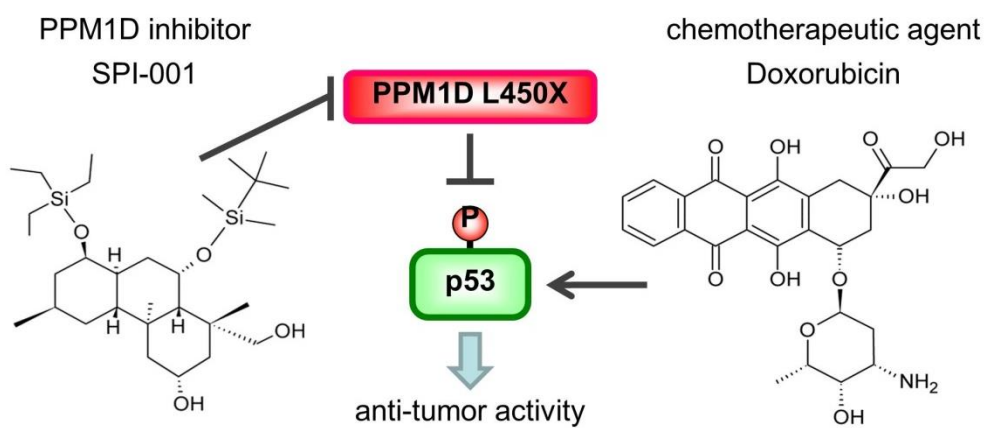


Figure 2-7. Co-treatment with PPM1D inhibitor and SPI-001 in C-terminal truncated PPM1D overexpressed cancer cells.

2.6. References

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3. An increase in the nucleolar number by PPM1D overexpression

3.1. Abstract

Morphological changes of the nucleolus have been seen in most cancers. Increases of the nucleolar number and size are important markers for cytology and tumor development. However, the underlying mechanisms of their structure and abundance are still unclear. Protein phosphatase PPM1D is upregulated in various types of carcinoma including breast cancers. The *PPM1D* gene maps to 17q23.2 and is within a region of frequent rearrangement or amplification in tumors. PPM1D overexpression is thought to be involved in cancer progression and tumorigenesis through hyper-dephosphorylation, and delineation of the related signaling pathways in cancer is needed to develop treatments targeting PPM1D. NPM is a ubiquitously expressed phosphoprotein involved in nucleolar formation and ribosomal synthesis.

I revealed that a PPM1D-specific inhibitor and PPM1D knockdown decreased the number of nucleoli under UV stimulation. I also found that PPM1D knockdown induced a decrease in the nucleolar number. In H1299 clones that express different level of HA-PPM1D, PPM1D overexpression led to an increase in the nucleolar number. Furthermore, PPM1D overexpression increased the size of nucleoli not only the number of them. These results suggested that PPM1D is involved in the nucleolar formation.

3.2. Introduction

Cancer cells exhibit specific morphology: a large nucleus, prominent nucleoli, and scarce cytoplasm. In particular, an increase in the nucleolar number has been used in cytology as a prognostic marker for many kinds of human tumors (1). In breast tumors, high-grade tumors show an increase in nucleoli (2, 3). During investigations of the effect of the PPM1D inhibitor SPI-001 on cell viability, I found that SPI-001 decreased the nucleolar number in MCF7 cells, a human breast cancer cell line. MCF7 cells overexpress protein phosphatase PPM1D and harbor a large number of nucleoli.

PPM1D (also known as PP2C δ and Wip1) is a protein phosphatase that regulates cellular homeostasis by dephosphorylation of tumor suppressor p53 (4-7) and p53-related proteins. Reported substrates of PPM1D include ATM (8), ATR (9), Chk1 (10), Chk2 (11), p38 (12), and γ H2AX (13, 14). It is also known to suppress p16^{INK4a} (15) and ARF (16). Conversely, the *PPM1D* gene is amplified in many types of human tumors including breast and ovarian cancers (17-20). Therefore, PPM1D is considered to be a proto-oncogene. Transfection of MEFs with *PPM1D* complements the oncogene activity of H-rasV12 (18). *PPM1D*-amplified breast tumors often show luminal or HER2 phenotypes, suggesting a link between them (20). Amplification of PPM1D is also observed in p53 mutant breast cancers (20). Because PPM1D has various substrates and is overexpressed in most carcinomas, it has been suggested that PPM1D is involved in various cellular activities. All evidence suggests that there is an alternative pathway in cancer cells independent of the p53 status.

I showed that the PPM1D inhibitor SPI-001 decreased the nucleolar number in the human breast cancer cell line MCF7. In this study, I found that PPM1D overexpression increased the nucleolar number in both p53-wt and p53-null cell lines. These results suggest that PPM1D overexpression increases the nucleolar number and leads to tumorigenesis.

3.3. Experimental procedures

3.3.1. Cell lines and materials

MCF7 human breast carcinoma cells, H1299 human non-small-cell lung cancer carcinoma cells were obtained from ATCC (Rockville, MD). No.9 and No.12 were derived from H1299 in our laboratory. Rabbit polyclonal anti-PPM1D was prepared as previously described (6). Other antibodies used include: either a rabbit or goat polyclonal anti-HA (HA-probe (Y-11)) from Santa Cruz Biotechnology; Mouse monoclonal anti-actin (Ab-1) from Calbiochem; Mouse monoclonal anti-NPM (Mouse anti-Nucleophosmin (FC-61991)) from Invitrogen. Secondary antibodies for Immunocytochemistry include: Alexa Fluor488 goat anti-mouse IgG and Alexa Fluor568 goat anti-rabbit IgG from Invitrogen.

3.3.2. Cell manipulation

Cell lines were cultured in Dulbecco's modified Eagles medium supplemented with 10% v/v foetal bovine serum with 100 units/ml of penicillin and 100 µg/ml of streptomycin in a humidified atmosphere of 5% CO₂. When required, cells were treated 24 h prior to fixation with up to 10 µM of the PPM1D inhibitor SPI-001 developed in our laboratory(21) and/or UV irradiation at 15 J/m². For PPM1D knockdown in MCF7, 48 h after the transfection, cells were subject to immunocytochemistry and analysis of the nucleolar number (see immunofluorescence and quantification section). Target specific siRNA duplexes were purchased from Invitrogen and the sequence information

for PPM1D was as followed: 5'-GAAGUGGACAAUCAGGGAAACUUUA-3'. SiRNA duplexes were subject to a BLAST search against the human genome sequence to ensure that the specificity towards the targets by Invitrogen BLOCK-iTTM RNAi Designer. Transient transfection of siRNAs was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For each experiment, specific silencing was confirmed by Western Blotting and immunofluorescence.

3.3.3. Western Blotting

All cells were rinsed with ice-cold PBS and lysed in high salt lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 25 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 1 mM p-Amidinophenyl Methansulfonyl Fluoride) or sampled with 1x sample buffer (50 mM Tris-HCl, pH6.8, 10% Glycerol, 2% SDS, 6% 2-mercaptoethanol). Equivalent amount of total cellular protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Proteins were detected by enhanced chemiluminescence with the above antibodies.

3.3.4. Flow cytometry

Cells were fixed with 70% ethanol, harvested and resuspended in PI/RNase staining buffer (BD Pharmingen, San Diego, CA). DNA cell cycle analysis was done by flow cytometry (FACSCalibur, FACStation ver 1.1) and Flow Jo 7.5 software (Tree Star Inc., Ashland, OR).

3.3.5. Immunofluorescence studies and quantification

In order to visualize nucleoli content, cells were cultured on glass coverslips and incubated for 48 h or 72 h. Cells were fixed with 3.5% formaldehyde for 15 min, washed in PBS twice, permeabilized with 0.2% Triton X-100/PBS for 15 min, and blocked with 10% FBS/PBS for 1h. After incubation overnight with primary antibodies, Rabbit polyclonal anti-PPM1D and Mouse monoclonal anti-NPM (Mouse anti-Nucleophosmin (FC-61991)), cells were stained with secondary antibodies, Alexa Fluor488 goat anti-mouse IgG and Alexa Fluor568 goat anti-rabbit IgG, along with 4',6-diamidino-2-phenylindole. Cells were then observed by fluorescence microscope (BZ-9000, KEYENCE). For the quantitative analysis of the nucleolar number or size, stained-NPM was used as an indicator of the nucleoli. Data were analyzed by hand with counter or tracing paper and by using the statistical student's t test. Significance was established at $P < 0.001$.

3.4. Results

3.4.1. A decrease in the nucleolar number by PPM1D inhibitor SPI-001

MCF7 cells overexpress PPM1D and harbor a large number of nucleoli. NPM is localized in granular components as reported previously. However, PPM1D is localized in the dense fibrillar components or the fibrillar center of the nucleolus. I found that the PPM1D inhibitor SPI-001 decreased the nucleolar number in MCF7 cells when the nucleoli were visualized by NPM staining (**Figure 3-1, Table 3-1**). SPI-001, which was developed in our laboratory, suppressed cell growth and the phosphatase activity of PPM1D *in vitro* and *in vivo* (21). These results suggest that PPM1D is involved in nucleolar formation.

3.4.2. An increase in the nucleolar number in PPM1D overexpressed cells

To analyze the effect of PPM1D on the nucleolar number, I counted nucleolar numbers in MCF7 cells treated with PPM1D siRNA. I confirmed that PPM1D was successfully knocked down (**Figure 3-2A, 3-2B**). Depletion of PPM1D induced a significant decrease in the nucleolar number. Quantitative analysis showed that PPM1D siRNA-treated cells contained an average of 3.7 nucleoli per cell, whereas control siRNA-treated cells had 4.4 nucleoli per cell (**Figure 3-2A, Table 3-2**). MCF7 cells harbor wild-type p53. To analyze whether this phenomenon was observed in p53-null cells, I next established stable H1299 cell lines expressing various levels of HA-PPM1D. H1299 clone No. 9 and 12 overexpressed PPM1D by 20- and 2.2-fold,

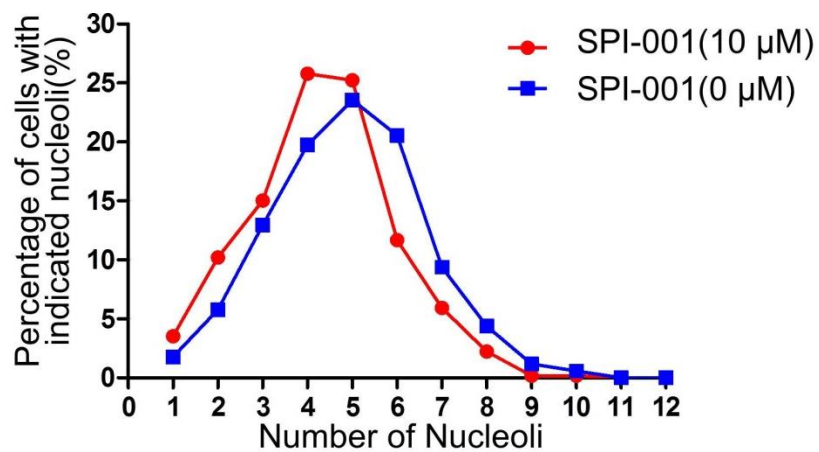


Figure 3-1. Effect of PPM1D inhibitor SPI-001 on nucleolar number in MCF7. MCF7 cells were fixed with formalin subsequent to treatment with 10- μ M of the PPM1D inhibitor SPI-001 and UV irradiation at 15 J/m² for 24 h. Fixed cells were stained with rabbit polyclonal anti-PPM1D, mouse monoclonal anti-NPM, secondary antibodies and DAPI. Data were analyzed by counteracting the nucleolar number based on the signal of NPM.

Table 3-1. Effect of PPM1D inhibitor SPI-001 on nucleolar number in MCF7

Data were analyzed by counteracting the nucleolar number based on the signal of NPM. Statistical significance was determined by Student's t-test and established at *** $p < 0.001$. Total cell numbers were 539, 501 in MCF7 cells treated without or with SPI-001, respectively.

SPI-001 (μM)	Number of Nucleoli (Mean \pm SE)	n
10	4.3 \pm 0.07***	539
0	4.9 \pm 0.08	501

$p^{***} < 0.001$

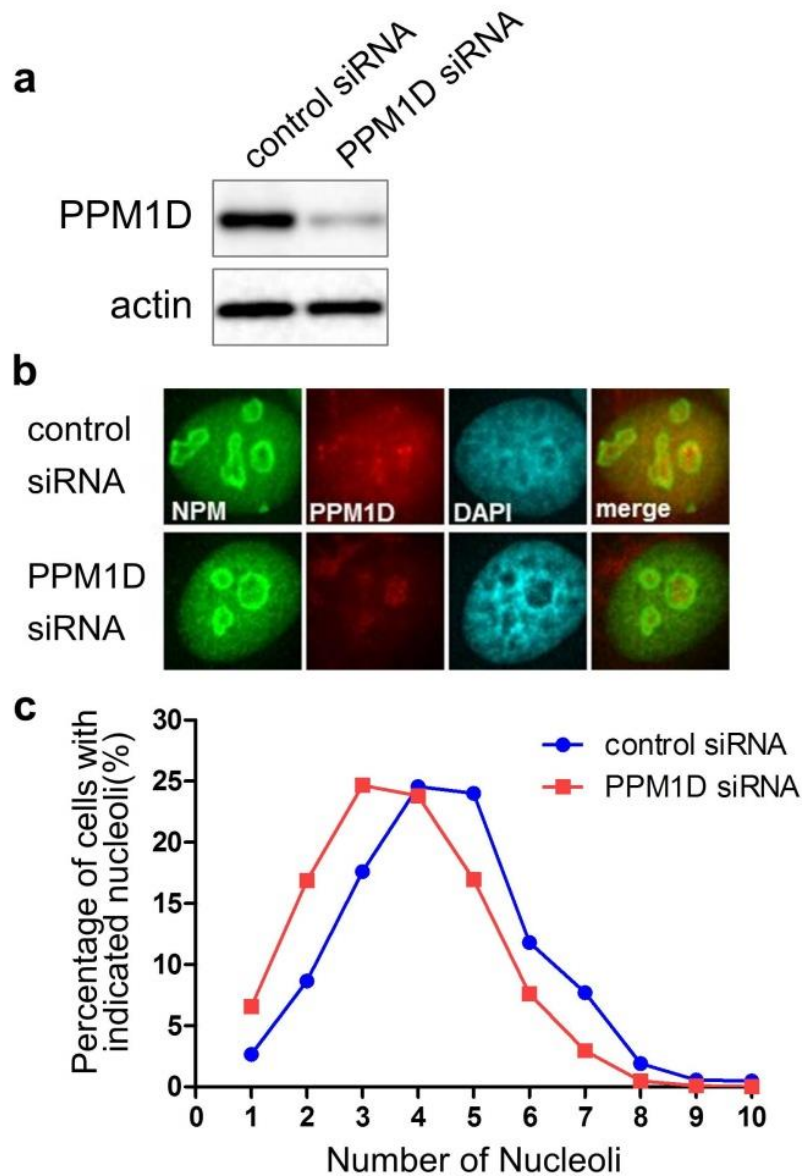


Figure 3-2A. Effect of PPM1D knockdown on nucleolar number. MCF7 cells were solubilized with 1x sample buffer subsequent to the treatment with PPM1D siRNA or control siRNA for 48h (a). (a) Actin and PPM1D proteins were analyzed by Western blotting with mouse monoclonal anti-actin and rabbit polyclonal anti-PPM1D. MCF7 cells were fixed with formalin subsequent to the treatment with PPM1D siRNA or control siRNA for 48h (b). (b) Fixed cells were stained with rabbit polyclonal anti-PPM1D, mouse monoclonal anti-NPM, secondary antibodies and DAPI. (c) Data were analyzed by counting the nucleolar number based on the signal of NPM. Figure shows the percentage of cells with indicated nucleoli in PPM1D siRNA- or control siRNA-treated MCF7 cells.

Table 3-2. Effect of PPM1D knockdown on nucleolar number in MCF7

Data were analyzed by counteracting the nucleolar number based on the signal of NPM. Statistical significance was determined by Student's t-test and established at *** p<0.001. Total cell numbers were 1050, 1050 in control siRNA treated cells or in PPM1D siRNA treated cells, respectively.

siRNA	Number of Nucleoli (Mean \pm SE)	n
PPM1D siRNA	3.7 \pm 0.05***	1050
Control siRNA	4.4 \pm 0.05	1050

p***<0.001

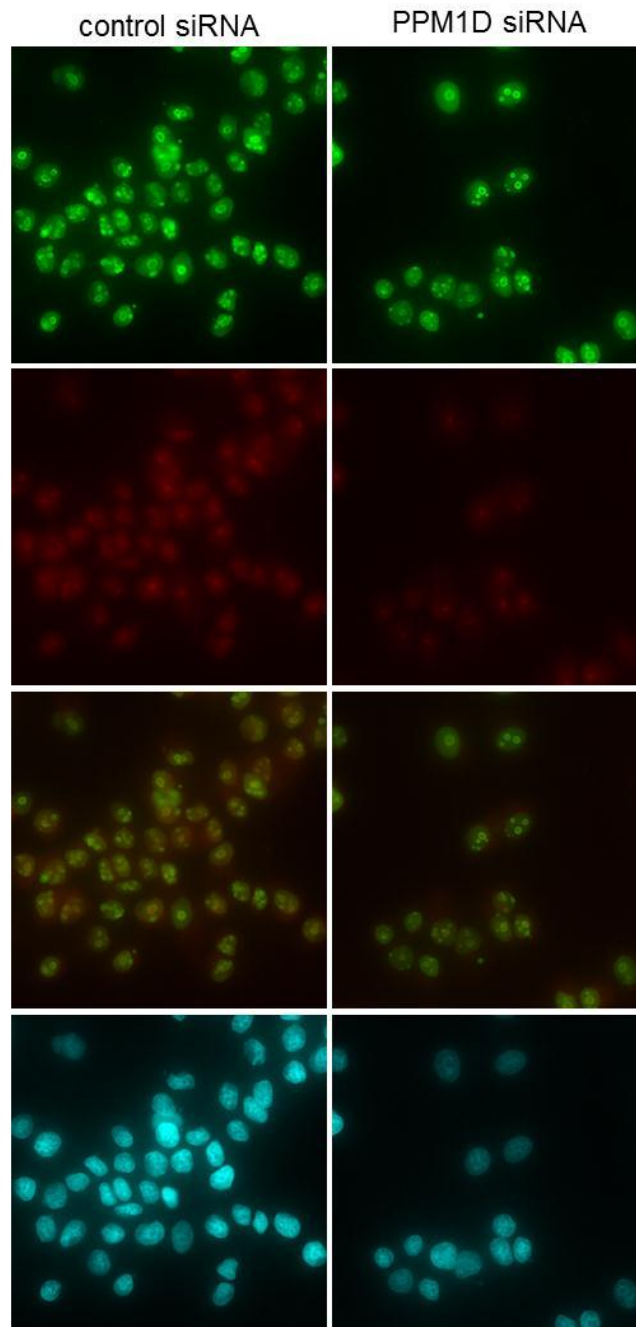


Figure 3-2B. Effect of PPM1D knockdown on nucleolar number. MCF7 cells were fixed with formalin subsequent to the treatment with PPM1D siRNA or control siRNA for 48h. Fixed cells were stained with rabbit polyclonal anti-PPM1D, mouse monoclonal anti-NPM, secondary antibodies and DAPI.

respectively. The p53-null human lung carcinoma cell line H1299 expressed a low level of endogenous PPM1D and contained an average of 3.8 nucleoli per cell. In H1299 clones, increases in the nucleolar number corresponded with PPM1D levels (**Figure 3-3A, 3-3B, Table 3-3**). Taken together, these results indicate that PPM1D overexpression induces an increase in the nucleolar number of wild-type p53 and p53-null cancer cells.

3.4.3. An increase in the nucleolar size in PPM1D overexpressed cells

In addition to the nucleolar number, the nucleolar size is a useful parameter in cytology. I found that PPM1D overexpression induced an increase in the size of nucleoli in H1299 cells. H1299 cells have an average of 50 μm^2 of nucleoli per cell. However, the size of nucleoli in H1299 clone No. 9, which overexpressed HA-PPM1D, was 61 μm^2 (**Table 3-4**). Therefore, PPM1D overexpression induced a 1.2-fold increase in the size of nucleoli. These results suggest that increases in the nucleolar number and size were induced by PPM1D overexpression.

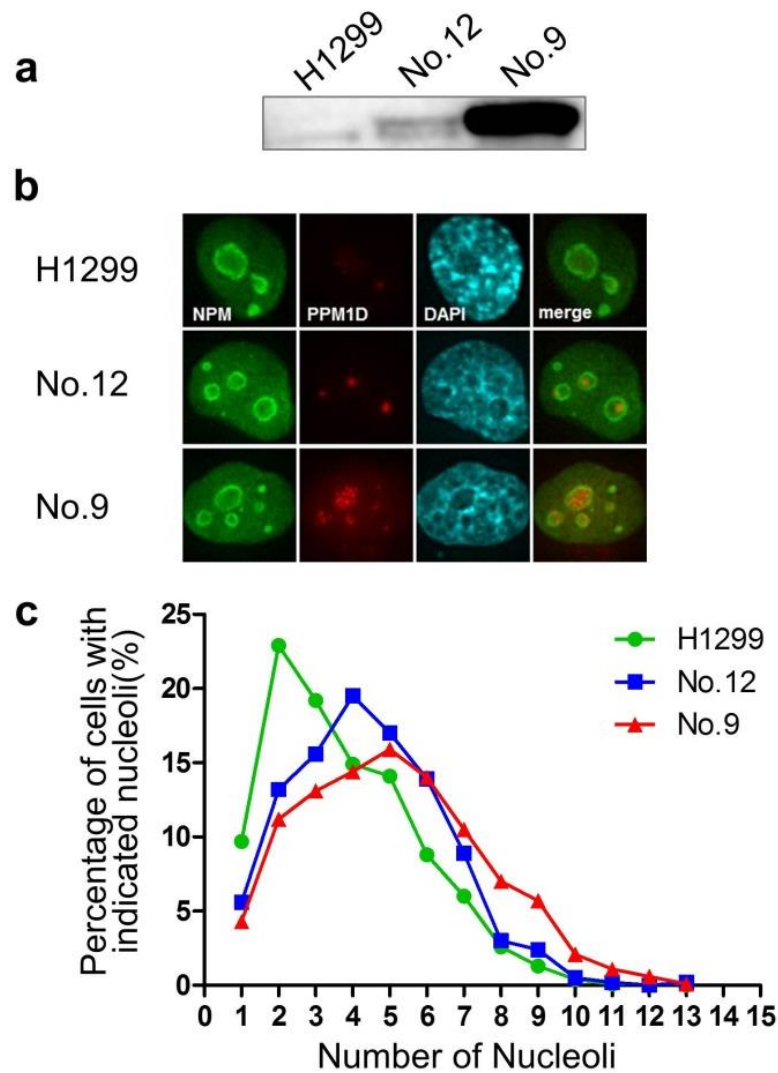


Figure 3-3A. Effect of PPM1D overexpression on nucleolar number in H1299. I derived two kinds of H1299 stable clone No.9 and No.12 that express different level of HA-PPM1D605. H1299 clone cells were collected and solubilized with 1x sample buffer (a) or fixed with formaldehyde for Immunocytochemistry (b). (a) Actin and PPM1D proteins were analyzed by Western blotting with rabbit polyclonal anti-PPM1D. (b) Fixed cells were fixed and stained with rabbit polyclonal anti-PPM1D and mouse monoclonal anti-NPM. (c) Data were analyzed by counting the nucleolar number based on the signal of NPM. Figure shows the percentage of cells with indicated nucleoli in H1299 clones.

Table 3-2. Effect of PPM1D knockdown on nucleolar number in MCF7

Data were analyzed by counteracting the nucleolar number based on the signal of NPM. Statistical significance was determined by Student's t-test and established at *** p<0.001. Total cell numbers were 838, 801, 717 in H1299, No.12, No.9, respectively.

Cells	PPM1D levels	Number of Nucleoli (Mean \pm SE)	n
H1299	1	3.8 \pm 0.07	838
No.12	2.2	4.4 \pm 0.07***	801
No.9	20	5.1 \pm 0.09***	717

p***<0.001

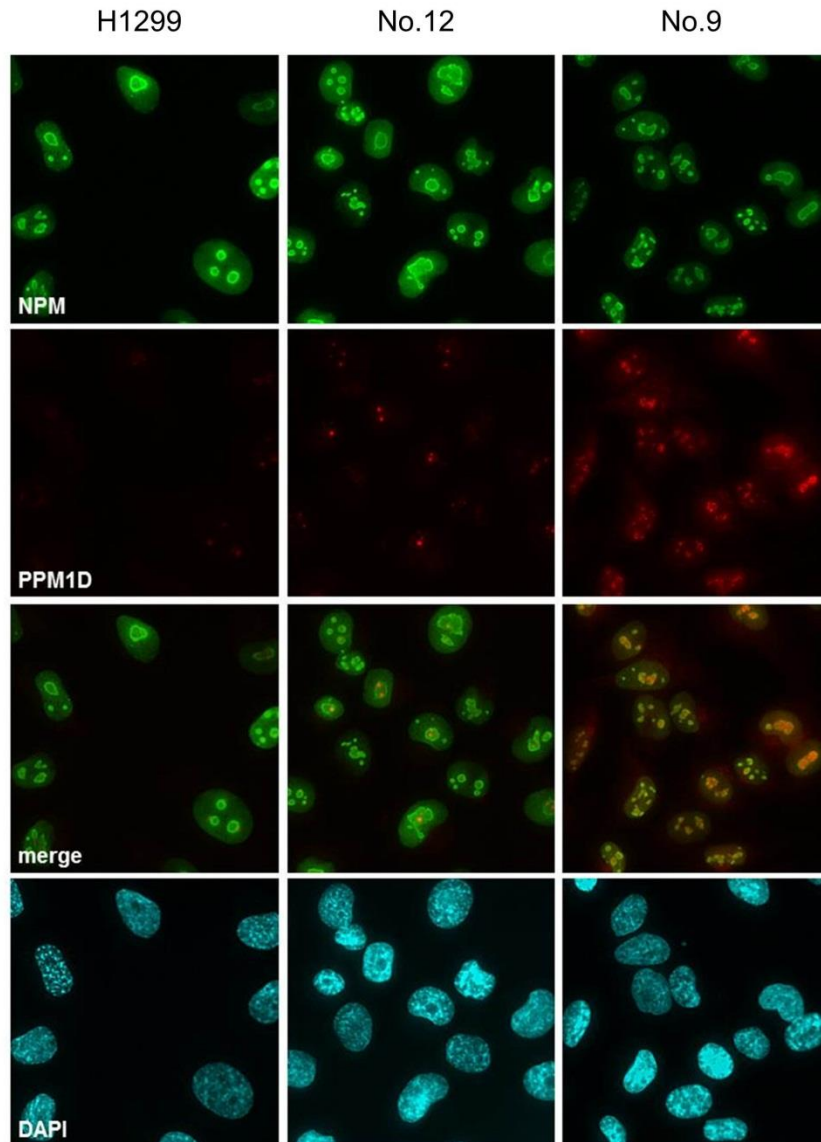


Figure 3-3B. Effect of PPM1D overexpression on nucleolar number in H1299. H1299 clone cells were fixed with formaldehyde for Immunocytochemistry. Fixed cells were fixed and stained with rabbit polyclonal anti-PPM1D and mouse monoclonal anti-NPM.

Table 3-4 Effect of PPM1D overexpression on nucleolar size in H1299

The size of nucleoli was estimated from the weight of tracing paper subsequent to copy the image of nucleoli on the paper. Significance was established at *P<0.1 from three independent experiments.

Cells	PPM1D levels	Area of Nucleolus in a Nucleus (μm^2) (Mean \pm SE)	n
H1299	1	50 \pm 5.6	205,205,215
No.9	20	61 \pm 0.8	119,205,205

*P<0.1

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4. Cancer inducible mechanism by PPM1D overexpression

4.1. Abstract

Morphological changes of the nucleolus have been seen in most cancers. Increases of the nucleolar number and size are important markers for cytology and tumor development. However, the underlying mechanisms of their structure and abundance are still unclear. Protein phosphatase PPM1D is upregulated in various types of carcinoma including breast cancers. NPM is a ubiquitously expressed phosphoprotein involved in nucleolar formation and ribosomal synthesis. I found that PPM1D overexpression led to an increase in the nucleolar number.

I demonstrated the molecular mechanism of the effect of PPM1D overexpression on phosphorylation of NPM. I also revealed that specific phosphorylation sites of NPM are important to increase the nucleolar number, and found a novel sequential cascade of these phosphorylation sites by CDC25-CDK1-PLK1. These results may improve our understanding of the molecular mechanisms that govern nucleoli formation, and this novel signaling pathway indicates that PPM1D is a promising target for anti-cancer drugs.

4.2. Introduction

The nucleolus is the site of ribosomal synthesis. It is also reported that the nucleolus is involved in other cellular activities including M phase regulation, cell proliferation, and apoptosis (1). It is important to determine the mechanisms of the structural integrity and abundance of nucleoli (2). In the nucleolus, there is rDNA, rRNA, ribosomal proteins, and nucleolar proteins such as NPM. NPM, which is also known as B23, is a ubiquitously expressed phosphoprotein (3). Dysregulation of NPM has been reported in many types of human tumors (4). The general functions of NPM are considered to be ribosomal synthesis and nucleolar formation based on several findings: shuttling between the nucleolus and cytoplasm (5, 6), binding to DNA (7), and transportation of ribosomal proteins (3, 8). NPM is considered to function as a shuttling protein and molecular chaperone. Knockdown of NPM leads to distortion of nucleolar formation (4). Post-translational modification of NPM is often reported. In particular, NPM is frequently phosphorylated by several kinases such as PLK1 (9) and CDK1 (10). However, information on sequential phosphorylation of NPM has not been revealed yet. It has been reported that NPM regulates the activities of several proteins including p53 (11, 12), CAD (13), and PKR (14). NPM is also reported to form fusion proteins with ALK, RAR α , and MLF1 in acute promyelocytic leukemia (APL), anaplastic large cell lymphoma (ALCL), myelodysplastic syndrome (MDS), and acute myeloid leukemia (AML) (3).

In this study, I found that PPM1D overexpression increased the nucleolar number

through regulating phosphorylation of NPM. I also revealed that phosphorylation of NPM at Thr199 and Ser4 was induced by activation of the CDK1-PLK1 cascade in PPM1D-overexpressing cells. Alanine mutant experiments showed that phosphorylation of NPM at Thr199 and Ser4 was important to increase the nucleolar number. These results suggest that PPM1D overexpression increases the nucleolar number and leads to tumorigenesis. Therefore, PPM1D may be a promising target for anti-cancer treatments.

4.3. Experimental procedures

4.3.1. Cell lines and materials

MCF7 human breast carcinoma cells, H1299 human non-small-cell lung cancer carcinoma cells were obtained from ATCC (Rockville, MD). No.9 and No.12 were derived from H1299 in our laboratory. The plasmids containing HA-NPM or empty vector were used for transient expression of NPM. NPM variants were generated by the following oligonucleotides are used for mutations; 5'-CATGTCCATGGCATCTTCCA T-3' for HA-NPM(S4A), 5'-TTGGCTGGAGCATCTCGTAT-3' for HA-NPM(T199A), 5'-CATGTCCATATCATCTTCCAT-3' for HA-NPM(S4D), and 5'-TTGGCTGGTTCATCTCGTAT-3' for HA-NPM(T199E). All variants of NPM were cloned into pCMV2 vector (Gene Therapy Systems, Inc., San Diego, CA, USA) for expression of HA-tagged protein in mammalian cells. Rabbit polyclonal anti-PPM1D was prepared as previously described (15). Other antibodies used include: Mouse monoclonal anti-p53 (DO-1) and either a rabbit or goat polyclonal anti-HA (HA-probe (Y-11)) from Santa Cruz Biotechnology; Mouse monoclonal anti-actin (Ab-1) from Calbiochem; Mouse monoclonal anti-NPM (Mouse anti-Nucleophosmin (FC-61991)) from Invitrogen; Rabbit monoclonal anti-pNPM(S4) (Phospho-NPM (Ser4) (D19C1) XP[®] Rabbit mAb), rabbit polyclonal anti-pNPM(T199) (Phospho-NPM (Thr199) Antibody), rabbit monoclonal anti-pCDC25C(S216) (Phospho-cdc25C (Ser216)(63F9) Rabbit mAb) and rabbit monoclonal anti-CDC25C (cdc25C (5H9) Rabbit mAb) from Cell Signalling Technology; Rat monoclonal anti-HA (Anti-HA affinity clone3F10) from Roche

Applied Science. Secondary antibodies for Western blotting include: Anti-mouse IgM HRP-linked antibody from KPL; Anti-mouse IgG HRP-linked antibody from GE healthcare; Anti-rabbit IgG HRP-linked antibody from New England Bio Labs. And finally, secondary antibodies for Immunocytochemistry include: Alexa Fluor488 goat anti-mouse IgG and Alexa Fluor568 goat anti-rabbit IgG from Invitrogen. His-NPM were expressed in *E. coli* and purified. CDK1-cyclinB and PLK1 were obtained from New England BioLabs and Millipore respectively. His-PPM1D(1-420) were expressed in *E. coli* and purified. Phosphorylated peptide analogues as substrate are as follows: CDC25C(210–212)216P : Ac-W-GLYRSPS(P)MPENLN-NH₂, p53(373-386)378P : Ac-W-KGQSTS(P)RHKKLMFK-NH₂, p53(381-393)392P : Ac-W-KKLMFKTEGPD S(P)D-OH.

4.3.2. Cell manipulation

Cell lines were cultured in Dulbecco's modified Eagles medium supplemented with 10% v/v foetal bovine serum with 100 units/ml of penicillin and 100 µg/ml of streptomycin in a humidified atmosphere of 5% CO₂. Transfection of cells with HA-NPM or empty vector was done with Lipofectamine 2000 (Invitrogen) under conditions as described by the manufacturer following endogenous NPM knockdown. 24h after the transfection, cells were subject to immunocytochemistry and analysis of the nucleolar number and size (see immunofluorescence and quantification section). Target specific siRNA duplexes were purchased from Invitrogen and the sequence information for both PPM1D and endogenous NPM were as followed: 5'-GAAGUGGACAAUCAGGGAA

ACUUUA-3' and 5'-AUAUAUAGACCCUGAAGAUCUCGCG-3', respectively. siRNA duplexes were subject to a BLAST search against the human genome sequence to ensure that the specificity towards the targets by Invitrogen BLOCK-iTTM RNAi Designer. Transient transfection of siRNAs was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells were assayed after 48h of transfection. For each experiment, specific silencing was confirmed by immunoprecipitation and immunofluorescence. To synchronize cells at M phase, Cells transfected with siRNA for 48h were treated with 4 µg/ml nocodazole for 16h. Cells were then harvested by mitotic shake off and analysed by flow cytometry and Western blotting. For the experiments with CDC25 inhibitor, cells were treated with 3 µM CDC25 Phosphatase Inhibitor II (NSC663284, Santa Cruz Biotechnology) for 6h and subsequent to treatment with 4 µg/ml nocodazole for 16h. Cells were then harvested by mitotic shake off and analysed by Western blotting.

4.3.3. Western Blotting

All cells were rinsed with ice-cold PBS and lysed in high salt lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 1 mM p-Amidinophenyl Methansulfonyl Fluoride) or sampled with 1x sample buffer (50 mM Tris-HCl, pH6.8, 10% Glycerol, 2% SDS, 6% 2-mercaptoethanol). Equivalent amount of total cellular protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Proteins were detected

by enhanced chemiluminescence with the above antibodies.

4.3.4. Immunoprecipitation

Cell lysates were immunoprecipitated with anti-HA (3F10) antibody using protein G agarose beads and followed by Western Blotting with the above antibodies.

4.3.5. Immunofluorescence studies and quantification

In order to visualize nucleoli content, cells were cultured on glass coverslips and incubated for 48h or 72h. Cells were fixed with 3.5% formaldehyde for 15min, washed in PBS, permeabilized with 0.2% Triton X-100/PBS for 15min, and blocked with 10% FBS/PBS for 1h. After incubation with primary antibodies, Rabbit polyclonal anti-HA and Mouse monoclonal anti-NPM (Mouse anti-Nucleophosmin (FC-61991)), cells were stained with secondary antibodies, Alexa Fluor488 goat anti-mouse IgG and Alexa Fluor568 goat anti-rabbit IgG, along with 4',6-diamidino-2-phenylindole. Cells were then observed by fluorescence microscope (BZ-9000, KEYENCE). For the quantitative analysis of the nucleolar number or size, stained-HA-NPM was used as an indicator of the nucleoli. Data were analyzed by hand with counter or tracing paper and by using the statistical student's t test. Significance was established at $P < 0.001$.

4.3.6. *In vitro* kinase assay

2 μ g of His-NPM were incubated with 10 units of CDK1-cyclinB for 60min at 30°C, prior to incubation with 100 ng of PLK1 for 60min at 30°C. 2x sample buffer (100 mM

Tris-HCl, pH6.8, 20% Glycerol, 4% SDS, 12% 2-mercaptoethanol) was added as a reaction stop. Samples were separated by SDS-PAGE and were subjected to western blotting with the above antibodies.

4.3.7. *In vitro* phosphatase assay

Phosphatase activity was assayed by measuring the released free phosphate by BIOMOL GREEN Reagent following protocol provided by BIOMOL. The amount of phosphatase released was calculated using a phosphate standard curve. All assays were carried out in Tris buffer (50 mM Tris-HCl pH 7.4, 30 mM MgCl₂, 0.1 mM EGTA, 0.02% 2-mercaptoethanol) by incubation with phosphopeptides and His-PPM1D420 (4, 10, 20 nM) for 5min at 30°C.

4.4. Results

4.4.1. Regulation of NPM phosphorylation by PPM1D overexpression

To determine the molecular mechanisms of an increase in the nucleolar number by PPM1D overexpression, post-translational modifications of NPM were detected in PPM1D siRNA-treated cells. Nocodazole is a new synthetic microtubule inhibitor that leads to prometaphase arrest. Control and PPM1D siRNA-treated MCF7 cells were collected by mitotic shake off after nocodazole treatment. Phosphorylated NPM at Thr199 and Ser4 was increased at M phase. However, in the presence of PPM1D siRNA, phosphorylated NPM at Thr199 was decreased and the phosphorylation level of Ser4 was decreased significantly (**Figure 4-1**). Cell cycle analysis confirmed that 58 and 36% of control siRNA- and PPM1D siRNA-treated cells were synchronized at G2/M phase, respectively (**Table 4-1**).

4.4.2. Effect of NPM phosphorylation on nucleolar number

The results suggested that phosphorylation might play an important role in the increase of the nucleolar number. Next, I examined the effect of NPM phosphorylation on the nucleolar number in MCF7 cells using substitution mutants. I prepared alanine substitution mutants for each phosphorylation site and phosphomimetic mutants. After knockdown of endogenous NPM, HA-tagged NPM mutants were expressed in MCF7 cells and then immunocytochemistry was performed with anti-NPM and -HA antibodies. I confirmed that endogenous NPM was partially knocked down, and all mutants

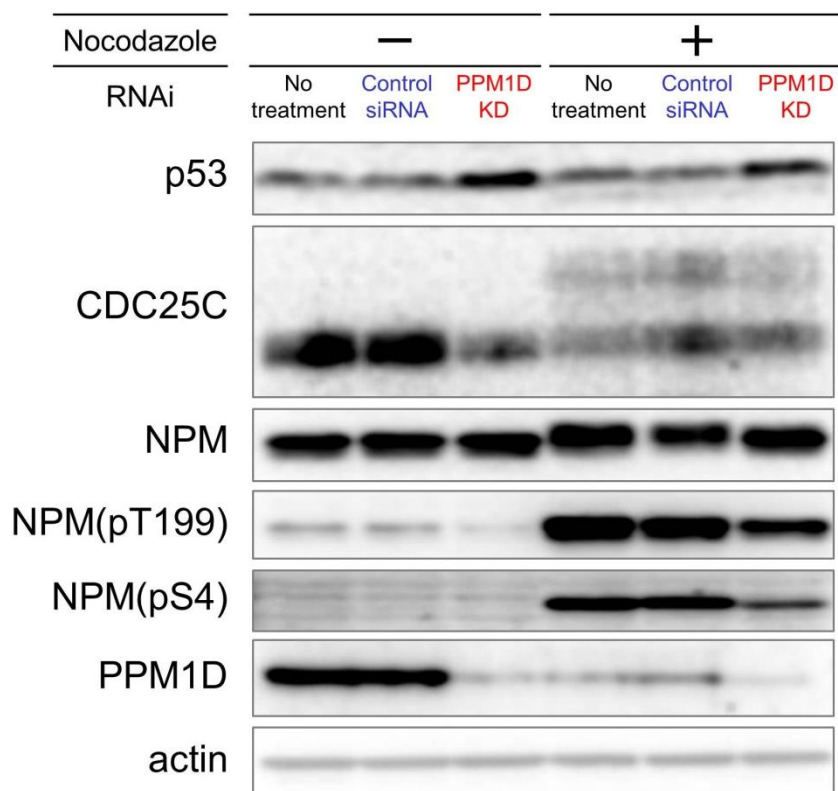


Figure 4-1. Effect of PPM1D knockdown on phosphorylation of NPM in MCF7 cells. MCF7 cells were treated with siRNA for 48h subsequent to treatment 4 μ g/ml nocodazole for 16h. Cells were collected by mitotic shake off and solubilized with 1x sample buffer. p53, CDC25C, NPM, phosphorylated-NPM at Thr199 and Ser4, PPM1D and actin were analyzed by Western blotting with mouse monoclonal anti-p53, rabbit monoclonal anti-CDC25C, mouse monoclonal anti-NPM, rabbit polyclonal anti NPM(pT199), rabbit monoclonal anti-NPM(pS4), rabbit polyclonal anti-PPM1D, mouse monoclonal anti-actin and secondary antibodies.

Table 4-1 Effect of PPM1D knockdown on cell cycle in MCF7. MCF7 cells were treated with siRNA for 48h subsequent to treatment 4 µg/ml nocodazole for 16h. Cells were collected by mitotic shake off, fixed with ethanol. Fixed cells were resuspended in PI/RNase staining buffer and analyzed by Flow cytometry. Analysis of cell cycle analysis was performed by flow cytometry and Flow Jo 7.5 software.

Nocodazole	-		+	
PPM1D knockdown	-	+	-	+
G2/M	20.1	19.5	57.7	36.1
S	20.0	15.7	17.5	22.6
G1	55.0	61.5	15.8	35.6
subG1	4.9	3.2	9.0	5.8

localized in the nucleoli (**Figure 4-2A**). HA-NPM(WT) cells showed an average of 4.5 nucleoli per cell (**Figure 4-2B, Table 4-2**). Cells that were transfected with both alanine mutants showed a decrease in the nucleolar number to 4.0 (**Figure 4-2B, Table 4-2**). The nucleolar number in phosphomimetic mutant-expressing cells was an average of 4.5. These results suggest that phosphorylation of NPM at Ser4 and Thr199 is important to increase the nucleolar number.

4.4.3. Sequential phosphorylation of NPM by CDK1-PLK1

It has been reported that G2/M checkpoint kinase CDK1 (10) phosphorylates NPM at Thr199, and PLK1 (9) phosphorylates NPM at Ser4. To reveal a link between CDK1 and PLK1, His-tagged NPM was expressed in *E.coli* and purified with a His-tag affinity column (**Figure 4-3A**). Using commercially available active CDK1 and PLK1, I found that NPM at Thr199 was phosphorylated by CDK1 prior to phosphorylation of NPM at Ser4 by PLK1 *in vitro* (**Figure 4-3A**). Both sites were successfully phosphorylated by CDK1 and PLK1 as revealed by western blotting with phospho-specific NPM antibodies. Surprisingly, NPM phosphorylated at Ser4 by PLK1 was increased by NPM phosphorylated at Thr199 by CDK1. I also expressed HA-NPM(WT) or HA-NPM(T199A) in MCF7 cells after knockdown of endogenous NPM, followed by immunoprecipitation (IP) with the anti-HA antibody. Similar to the *in vitro* kinase assay results, IP showed sequential phosphorylation of NPM by CDK1-PLK1 in MCF7 cells (**Figure 4-3B**). These results suggested that CDK1 phosphorylates NPM at Thr199 and then PLK1 phosphorylates NPM at Ser4.

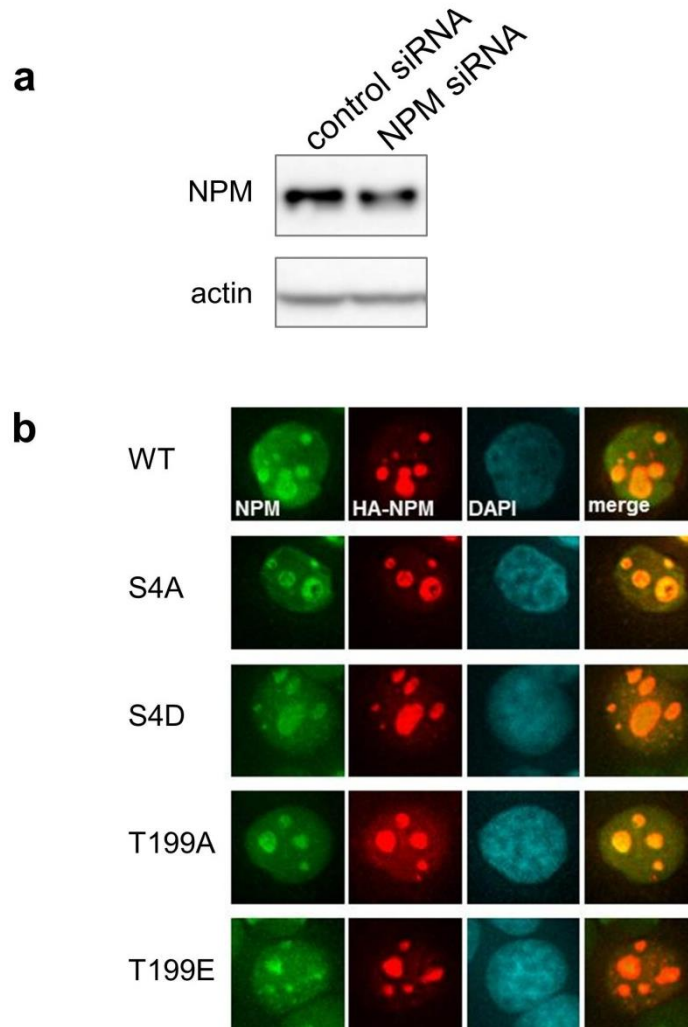


Figure 4-2A. Effect of phosphorylated-NPM on nucleolar number. MCF7 cells were transfected with HA-NPM mutant for 24h subsequent to the treatment with knockdown of endogenous NPM for 24h and changed the medium. (a) NPM and actin were analyzed by Western Blotting for samples from MCF7 treated with NPM siRNA for 24h. (b) MCF7 cells were fixed subsequent to the treatment with NPM siRNA, changed medium, transfected with HA-NPM mutants. Fixed cells were stained with rabbit polyclonal anti-HA, mouse monoclonal anti-NPM, secondary antibodies, and DAPI.

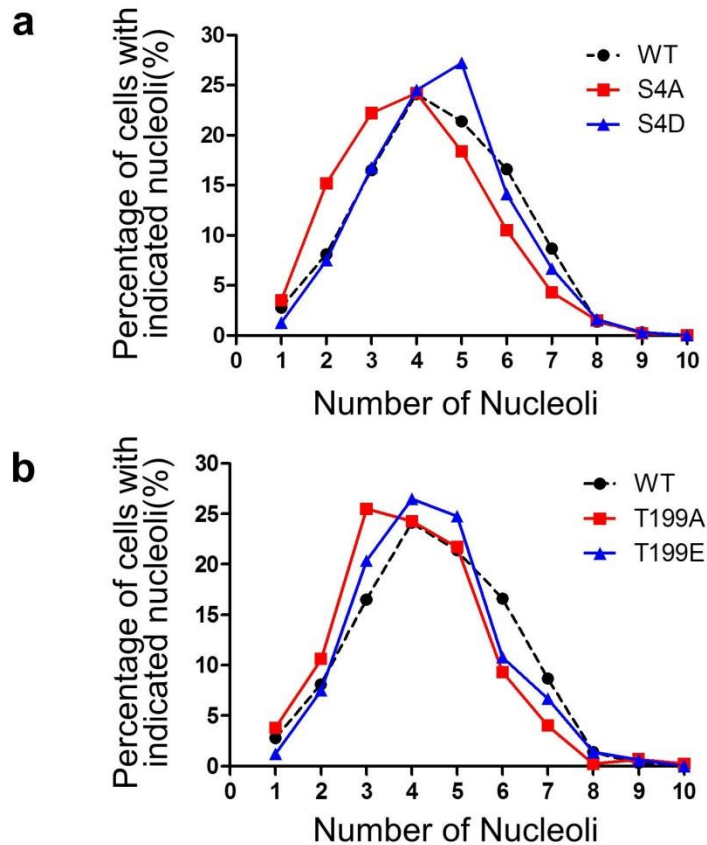


Figure 4-2B. Effect of phosphorylated-NPM on nucleolar number. MCF7 cells were transfected with HA-NPM mutant for 24h subsequent to the treatment with knockdown of endogenous NPM for 24h and changed the medium. (a) Data were analyzed by counting the nucleolar number based on the signal of HA. Figure shows the percentage of cells with indicated nucleoli in MCF7 cells transfected with HA-NPM(WT), HA-NPM(S4A) or HA-NPM(S4D). (b) Data were analyzed by counting the nucleolar number based on the signal of HA. Figure shows the percentage of cells with indicated nucleoli in MCF7 cells transfected with HA-NPM(WT), HA-NPM(T199A) or HA-NPM(T199E).

Table 4-2 Effect of phosphorylated-NPM on nucleolar number

Summary of number of nucleoli in MCF7 cells transfected with HA-NPM(WT), HA-NPM(S4A) or HA-NPM(S4D), HA-NPM(T199A) or HA-NPM(T199E). Statistical significance was determined by Student's t-test and established at ^{***}p<0.001. Total cell numbers were 704, 599, 375, 605, 491 in HA-NPM(WT, S4A, S4D, T199A, T199E) transfected MCF7 cells, respectively.

Cells	Number of Nucleoli (Mean ± SE)	n
WT	4.5 ± 0.06	704
S4A	4.0 ± 0.06	599
S4D	4.5 ± 0.08	375
T199A	4.0 ± 0.06	605
T199E	4.5 ± 0.07	491

p^{***}<0.001

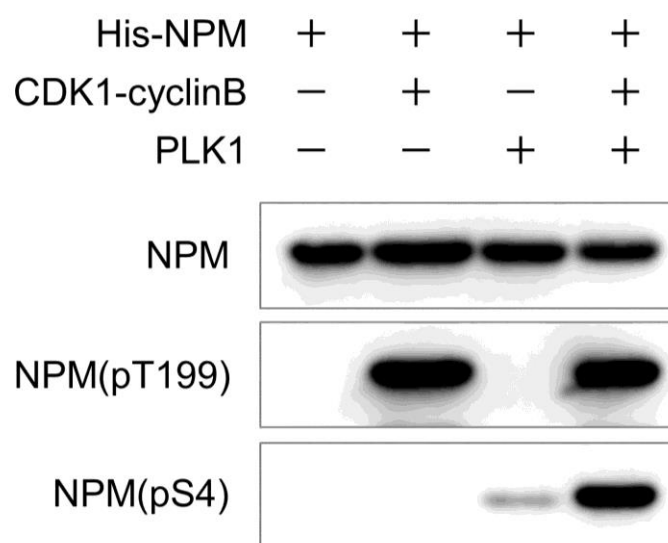


Figure 4-3A. Sequential phosphorylation of NPM by CDK1-PLK1. His-NPM was prepared by *E-coli* expression. 10 units of CDK1-cyclinB was added with 2 μ g of His-NPM and incubated for 60 min at 30°C subsequently 100 ng of PLK1 was added and incubated for 60 min at 30°C. 2x sample buffer was added as a reaction stop. Samples were separated by SDS-PAGE and were subjected to western blotting with anti-NPM, anti-NPM(pT199) and anti-NPM(pS4) antibodies.

4.4.4. Inhibition of CDC25C activity by PPM1D overexpression

CDC25 is a well-known phosphatase and activator of CDK1. To examine the effect of PPM1D on CDC25 protein levels, I treated MCF7 cells with a CDC25 inhibitor and then analyzed the phosphorylation levels of NPM at Ser4 and Thr199. As a result, the CDC25 inhibitor decreased the phosphorylation levels of NPM at Thr199 and Ser4 (**Figure 4-1**). Furthermore, in MCF7 cells, PPM1D knockdown induced a significant decrease in protein expression of CDC25C, a representative CDC25 phosphatase. I also found that PPM1D knockdown increased the p53 protein level as reported previously. It has been suggested that CDC25C is up-regulated by PPM1D overexpression through suppression of p53. In p53-null H1299 cells, PPM1D overexpression did not change CDC25C protein levels. However, in H1299 clones, phosphorylation of CDC25C at Ser216 was decreased in PPM1D-overexpressing cells (**Figure 4-4**). It has been reported that phosphorylated CDC25C at Ser216 decreases the activity of CDC25C itself. Therefore, I expressed and purified recombinant His-PPM1D (1-420) and synthesized phosphopeptides corresponding to residues surrounding Ser216 of human CDC25C and the C-terminus of p53 as a negative control. *In vitro* phosphatase activity showed that phosphorylated CDC25C at Ser216 was dephosphorylated by PPM1D (**Figure 4-5**). These data suggest that the activity of CDC25 is up-regulated by PPM1D in both p53-dependent and -independent manners. Activated CDC25 leads to activation of CDK1. CDK1 phosphorylates NPM at Thr199, leading to sequential phosphorylation of NPM at Ser4 by PLK1. This molecular mechanism might also explain the increase in the nucleolar number of PPM1D-overexpressed cells independently the p53 status.

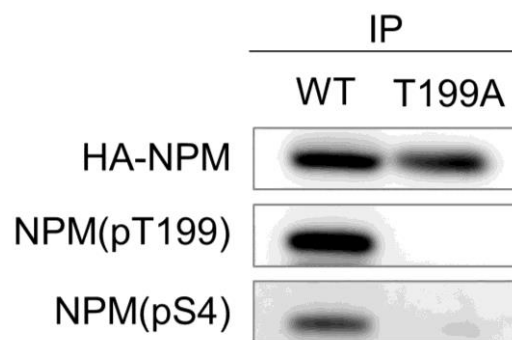


Figure 4-3B. Sequential phosphorylation of NPM by CDK1-PLK1. MCF7 cells were immunoprecipitated with mouse monoclonal anti-HA subsequent to transfected with either HA-NPM(WT) or HA-NPM(T199A) for 40 h and treated with 4 μ g/ml nocodazole for 16 h. IP samples were analyzed by Western blotting with polyclonal anti-HA, anti-NPM(pT199) and anti-NPM(pS4) antibodies.

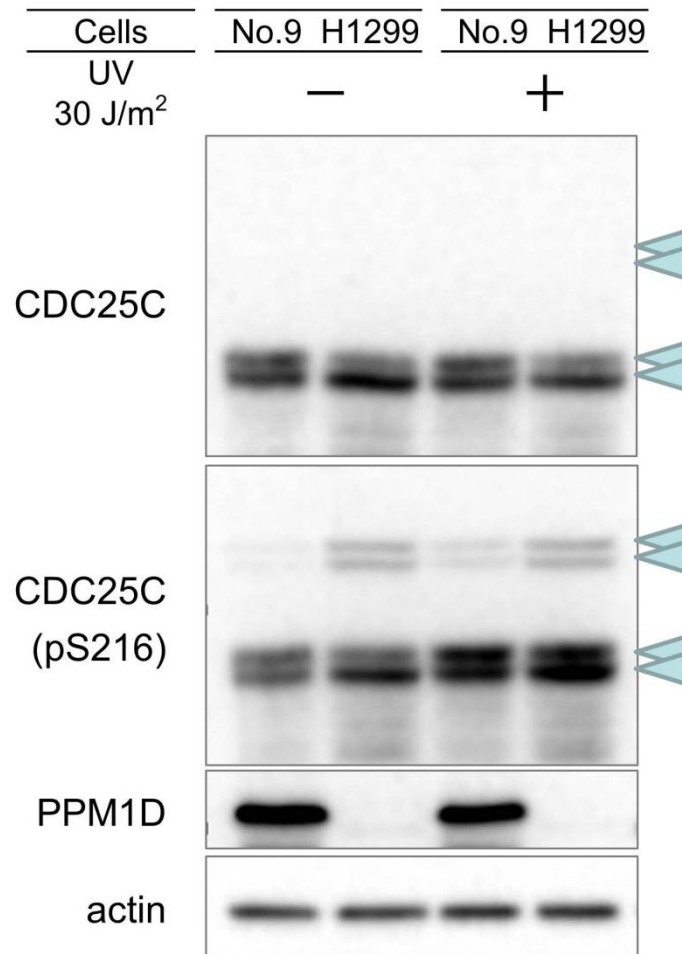


Figure 4-4. Effect of PPM1D overexpression on phosphorylation of CDC25C at Ser216 in H1299 clones. H1299 clones were lysed 2h after UV irradiation at 30 J/m² and analyzed by Western blotting with rabbit monoclonal anti-CDC25C, anti-CDC25C(pS216), anti-PPM1D and anti-actin antibodies.

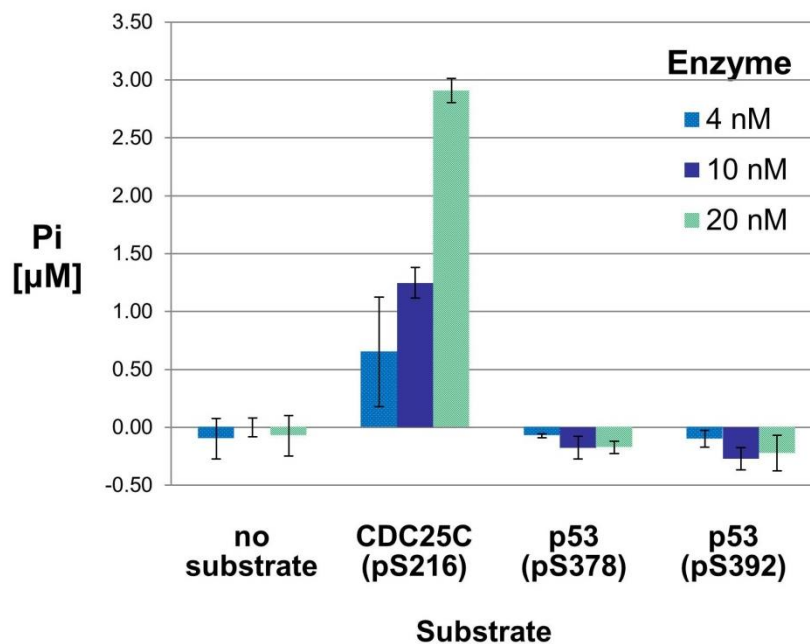


Figure 4-5. Phosphatase activity of PPM1D on phosphopeptides derived from CDC25C at Ser216. Activity was measured by the released free phosphate by BIOMOL GREEN Reagent following BIOMOL protocol. The amount of released phosphate was estimated using a phosphate standard curve. All assays were performed in Tris buffer (50 mM Tris-HCl pH 7.4, 30 mM MgCl₂, 0.1 mM EGTA, 0.02% 2-mercaptoethanol) subsequent to incubation with phosphopeptides (40 µM) and His-PPM1D420 (4, 10, 20 nM) for 5min at 30°

4.5 Discussion

Cancer cells often exhibit specific morphological characteristics such as an increase in the nucleolar number and size. Therefore, these characteristics are useful cytological and prognostic parameters (2). However, the molecular mechanism regulating nucleolar formation has not been fully understood.

Protein phosphatase PPM1D is a p53-inducible Ser/Thr phosphatase. Gene amplification and mRNA overexpression of *PPM1D* are reported in various types of human cancers (16-19). PPM1D dephosphorylates cell cycle regulators and apoptotic proteins such as ATM (20), ATR (21), Chk1 (22), Chk2 (23), p38 (24), and p53 (15, 25-27). Furthermore, *PPM1D* has been reported to complement *H-rasV12*, *MYC*, and *NEU1* in transformation of wild-type MEFs (17). This evidence confirms that PPM1D is a proto-oncogene. It is therefore necessary to examine the molecular mechanisms in PPM1D-overexpressing cancer cells and develop molecular agents against PPM1D.

In this study, I demonstrated that PPM1D overexpression increased the nucleolar number in both p53-dependent and -independent manners (**Figure 4-6**). I also showed that PPM1D overexpression increased the phosphorylation of NPM through the CDC25C-CDK1-PLK1 cascade. Additionally, I revealed that two phosphorylation sites of NPM are important to increase the nucleolar number. CDC25C is a well-known cell cycle regulator by dephosphorylating G2/M checkpoint kinase CDK1. Tumor suppressor p53 down-regulates CDC25C mRNA expression induced by DNA damage. It is possible that PPM1D overexpression leads to an increase in the CDC25C protein

level by inhibition of p53 expression. In p53-null cells, phosphorylation of CDC25C at Ser216 was decreased in PPM1D-overexpressed cells, even though the protein level of CDC25C did not change. I also showed that Ser216 in CDC25C may be a target of PPM1D by an *in vitro* phosphatase activity assay. It has been reported that phosphorylation of CDC25C at Ser216 inactivates CDC25C dephosphorylation activity. Therefore, PPM1D activates CDC25C in p53-dependent or -independent manners. Activated CDC25C dephosphorylates and activates CDK1. Activated CDK1 phosphorylates NPM at Thr199 as reported previously. Polo-like kinase PLK1 is expressed at a high level during mitosis. PLK1 binds to a phosphorylated substrate by CDK1 through its binding domain and then it phosphorylates the other site of the same substrate. In this signaling cascade, PLK1 binds to phosphorylated NPM at Thr199 and phosphorylates NPM at Ser4. Taken together, regardless of the p53 status, PPM1D would increase the phosphorylation level of NPM through the CDC25C-CDK1-PLK1 cascade. This study first revealed a link between PPM1D overexpression and the nucleolar number. Based on the signaling cascade model in PPM1D-overexpressing cells, a relationship is suggested between PPM1D overexpression and both CDK1 and PLK1. Both CDK1 and PLK1 are well-known target molecules for anti-cancer treatments.

Nucleolar assembly and disassembly are well-regulated processes. Nucleolar disassembly starts at the beginning of mitosis, and nucleolar assembly starts at telophase (28). In the process of nucleolar disassembly during prophase, the molecular

mechanism has been revealed gradually. Nucleolar disassembly is suggested to be linked to suppression of rDNA transcription. It has been also suggested that the inhibition of rDNA transcription is mediated by phosphorylation of factors of the rDNA machinery by CDK1-cyclin B (29). In the assembly of nucleoli at the exit of M phase, it is reported to occur independently of rDNA transcription (30). These findings demonstrate that nucleolar assembly and disassembly are processes regulated by phosphorylation levels and the kinds of substrates of CDKs. NPM is phosphorylated at Thr199 and Ser4 by CDK1 (10) and PLK1 (9) at M phase. My study showed that these phosphorylation sites are important for the nucleolar structure. These data suggest that phosphorylation of NPM at these two sites might be crucial for nucleolar disassembly, and dephosphorylation of NPM at these sites might be important for assembly of nucleoli. These results are also supported by the fact that phosphorylated NPM at Thr199 needs to be released from chromatin. Overexpression during interphase of cells expressing phosphomimetic NPM mutants, which included Thr199, increased the mobility of NPM. I showed that phosphorylated NPM at Thr199 enhanced the phosphorylation level of Ser4. These data suggest that phosphorylated NPM at Thr199 and Ser4 is involved in nucleolar assembly. Phosphorylated NPM due to PPM1D overexpression might prevent nucleolar assembly, resulting in the increase in the nucleolar number.

In growing cells with an increase in the number of nucleoli, ribosome synthesis is also active (31). Therefore, it is possible that the increase in the nucleolar number is a consequence of increased demand for ribosome biogenesis. However, another group has

reported that phosphorylation of NPM at Thr199 inhibits ribosome biogenesis (32). These findings suggest a link between increases in the nucleolar number and ribosome biogenesis. Thus, I also have to investigate the effect of PPM1D on ribosomal biogenesis.

I have reported a small molecule inhibitor of p53-inducible protein phosphatase PPM1D, namely SPI-001. SPI-001 suppresses the growth of MCF7 cells overexpressing PPM1D605 (33) and enhances the effect of doxorubicin in HCT-116 cells overexpressing PPM1D L450X (34). Furthermore, I revealed that an increase in nucleolar numbers were occurred by PPM1D overexpression, which contributes to its proto-oncogene activity. These findings are important to understanding the molecular mechanism of the nucleolar integrity in PPM1D-overexpressing cells. These findings also indicate that PPM1D is a promising target for cancer therapy.

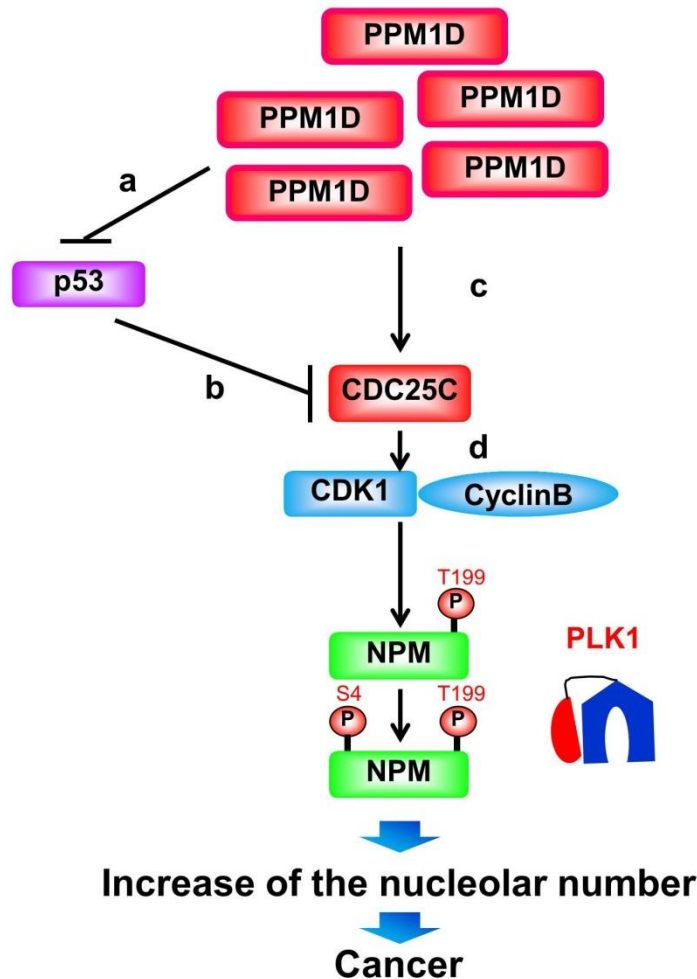


Figure 4-6. Cancer inducible mechanism in PPM1D-overexpressed cells. (a) PPM1D dephosphorylates and inactivates tumor suppressor p53. (b) p53 downregulates CDC25C induced by DNA damage. (c) Phosphorylated-CDC25C at Ser216 might be dephosphorylated by PPM1D. CDC25C is phosphorylated at Ser216 when CDC25C is inactive. In both p53 dependent and independent manner, PPM1D might upregulate CDC25C activity. (d) CDK1 is dephosphorylated and inactivated by CDC25C. PLK1 is known to bind to a phosphorylated target by CDK1 through its binding domain and then it phosphorylates the other site of same target protein. Activated CDK1 phosphorylates NPM at Thr199 and, PLK1 phosphorylates NPM at Ser4.

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5. Conclusions

Protein phosphatase PPM1D is a remarkable target protein for anti-cancer treatments. Gene amplification as well as mRNA and protein overexpression of PPM1D has been reported in many types of human tumors. Recent reports have also identified PPM1D mutations in several types of tumors. To develop molecular targeting agents against PPM1D and cancer induction mechanisms, this study revealed the effects of a PPM1D inhibitor in cell lines overexpressing C-terminal truncated PPM1D and on the nucleolar number. Furthermore, I demonstrated a cancer induction mechanism based on the nucleolar number which is a parameter in cytology.

In Chapter 2, I examined the effects of a small molecule inhibitor of PPM1D, SPI-001, on the human colorectal carcinoma cell line HCT-116 overexpressing C-terminal truncated PPM1D, which was developed in our laboratory. High concentrations of doxorubicin did not suppress the viability of HCT-116 cells compared with that of A549 cells expressing a normal level of PPM1D. I revealed that SPI-001 enhanced the anti-tumor effect of doxorubicin on the viability of HCT-116 cells through activation of the p53-p21 pathway. These observations suggest that combinatorial treatment with the PPM1D inhibitor and doxorubicin may be a novel anti-cancer treatment.

In Chapter 3, I found that SPI-001 suppressed the nucleolar number in the human breast cancer cell line MCF7. I therefore expanded my study to investigate the effect of PPM1D on the nucleolar structure and a link with cancer induction. I demonstrated that PPM1D overexpression induced an increase in the nucleolar number in p53-dependent or -independent manners.

In Chapter 4, I revealed the molecular mechanism in PPM1D-overexpressing cells. PPM1D overexpression increased the phosphorylation level of NPM at two target sites of CDK1 and PLK1. I also found that these two sites in NPM are important to control the nucleolar number as shown in experiment with NPM mutants. PPM1D also increased the protein level and activity of CDC25C, leading to activation of the CDC25-CDK1-PLK1 cascade in p53-dependent or -independent manners. These findings showed not only the involvement of PPM1D in nucleolar structure by regulating the phosphorylation of nucleolar proteins, but also that PPM1D up-regulates therapeutic target proteins such as CDC25, CDK1, and PLK1.

In summary, I have demonstrated the effects of a PPM1D inhibitor on the viability of cancer cell lines overexpressing C-terminal truncated PPM1D and the cancer induction mechanism in PPM1D-overexpressing cells. These results suggest that the PPM1D inhibitor SPI-001 is a useful tool for investigating cancer induction mechanisms and a potential anti-cancer drug.

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