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TP53RK regulates DNA replication via DDK dependent pathway in colorectal cancer

대장암에서 DDK를 매개로 하는 TP53RK의 DNA 복제 조절 기전

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TP53RK regulates DNA replication via DDK dependent pathway in colorectal cancer

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Approved by thesis committee:

Professor _____ Chairperson

Professor _____ Vice Chairperson

Professor _____

Abstract

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In recent decades, colorectal cancer(CRC) is known as one of the cancerous type that represents highest incidence and mortality. Up to the present day, although studies on the genetic/epigenetic factors inducing colorectal cancer have been variously conducted, a more in-depth approach is needed to understand the pathogenesis of disease and effectively apply anticancer therapy. [1] In this context, next generation sequencing(NGS) and targeted therapy using biomarkers have become a core technology for popularization of precision medicine or personalized medicine that reflects environmental/biological information of individual patient.

In this study, CRISPR library screening was carried out based on a tyrosine kinase(TK) panel containing about 700 genes to identify novel colorectal cancer specific biomarker. As a result of knock out(KO) analysis for a total of six colon cancer cell lines, TP53RK(TP53 regulating kinase, PRPK) was showed topmost cell growth inhibition efficiency when its expression is reduced. TP53RK has been reported to be overexpressed in various cancer including multiple myeloma and skin cancer, however, the function of gene related to tumorigenesis is not clear. [2,3] Therefore, this study aimed to investigate the mechanism of cell death induced by TP53RK knock out through proteome analysis on a basis of mass spectrometry(MS) Indeed, when TP53RK was declined, we confirmed that phosphorylation status of CDC7 (Cell division cycle 7) kinase and MCM2/4, which is involved in initiation of DNA replication, was remarkably changed in addition to reduction of cell

viability in colon cancer cell line. [4,5] This consequence suggests that TP53RK regulates DNA replication via CDC7 and DBF4 (Dumbbell former 4 protein), a regulatory subunit of CDC7, dependent manner.

Key words: TP53RK/PRPK; CDC7; DBF4; DDK; MCM complex; DNA replication; CRISPR library screening; Colorectal cancer;

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Background and Purpose

1. Initiation of eukaryotic DNA replication

Eukaryotic DNA replication mechanism is highly conserved and strictly coupled to cell cycle regulation. [6.7] The initiation of replication occurs through discrete two stage. [8] First, hetero-hexameric minichromosome maintenance(MCM) complexes load on to origin recognition complex(ORC) bound template DNA to form pre-replicative complex(pre-RC) during G1 phase. [7] Second, assembly of replisome including CDC45-MCM-GINS(CMG) helicase proceeds. In that point, CDC7 and DBF4, as known as DBF4 dependent kinase(DDK), are activated at G1/S transition and phosphorylate the MCM2-7 complex. This process promotes recruitment of Sld3 and CDC45 to ORC. It is essential step for the generation of active replication fork structure. [9]

CDC7 is a conserved serine/threonine kinase, originally discovered in budding yeast. [10] A number of previous studies showed that CDC7 and DBF4 are required not only for initiation of genome replication but also for stable maintenance of replication fork. [9] Indeed, DDK is overexpressed in some human cancer cell lines and various primary tumors.

2. Purpose

Based on the background described above,

1) To identify the correlation between TP53RK and CDC7 in colorectal cancer

2) To investigate the possibility of TP53RK as a druggable target on the basis of its association with CDC7 in colorectal cancer

Introduction

Colorectal cancer is among the most prevalent malignancies, and it is also known to incur majority of cancer-related death in the world. [1,11,12] It is caused by mutations on proto-oncogenes, tumor suppressor genes, and genes related to DNA repair mechanism, but naturally, the personal features such as underlying condition or lifestyle also contribute to occurrence of disease. [13] Surgery and chemotherapy are considered the first choices, however such options have not been effective approaches in prognosis of colorectal cancer especially for patient with metastatic lesions. In recent years, targeted therapy new optional route that has efficiently prolonged overall survival for colorectal cancer patient. Worldwide guidelines are currently updating the recommended targeted drug, therefore, the needs for novel biomarkers are steadily emerging. [11]

TP53RK, the human homologue of yeast Bud32, is an subunit of EKC/KEOPS complex and serine/threonine kinase that phosphorylates p53 Ser15. This gene was first cloned from an interleukin-2-activated cytotoxic T cell subtraction library. [14.15] It is also known as PRPK, p53 related protein kinase, as the name suggests, early research on TP53RK focused on its relevance to p53. But the authors who first reported TP53RK later concluded

that the function of TP53RK is not limited to phosphorylation of p53. In fact, phosphorylation status of p53 is regulated by various kinases such as ATM and ATR, and p53 remains phosphorylated on Ser15 even after inhibition of TP53RK. [16] Although it is reported that the catalytic activity of TP53RK is related to phosphorylation Ser250 through AKT/PKB both *in vitro* and *in vivo*, downstream or binding molecules of TP53RK are hardly identified. [17]

Recent studies in the last decade have revealed, in several cancer, overexpression or phosphorylation of TP53RK is correlated with patient prognosis and metastasis. [16,18] However, the mechanism by which how TP53RK induces tumorigenesis is poorly understood. Here, we verified the p53 independent oncogenic properties of TP53RK in colon cancer cell lines by performing CRISPR library screening and proteomic analysis.

Through the phospho-proteome analysis after TP53RK knock out, it is confirmed that the phosphorylation of CDC7 substrates was significantly decreased due to TP53RK depletion. CDC7 is a serine/threonine kinase and its main substrate in mammalian cell is the MCM complex. [19] For activation, CDC7 requires binding with DBF4 for cell cycle. The expression of DBF4, which is low during G1 and G2/M phases, is maintained high level in S phase. On the other hands, expression of CDC7 is almost constant throughout cell cycle. [20] It means that DBF4 plays a role as the regulator of CDC7 activity. These two molecules, as known as DDK, are

involved in initiation of DNA replication by phosphorylating MCM complex at the G1/S transition. Since published data indicated that the phosphorylation of CDC7 abrogates DNA replication, we hypothesized TP53RK is concerned with cell proliferation by regulating phosphorylation of CDC7. [21] Indeed, out data shows the reduction of TP53RK caused increase of CDC7 phosphorylation and decrease of MCM2/4 phosphorylation, but not total CDC7 expression, and induced apoptosis in colon cancer cell lines. This result demonstrates that TP53RK regulates DNA replication via DDK dependent pathway, at least through the phosphorylation of CDC7, in colon cancer cell lines.

Materials and Methods

Antibodys

Rabbit monoclonal antibody recognizing CDC7(ab229187), DBF4(ab124707), phospho-MCM2 S40(ab133243), phospho-MCM4 S54(ab74014), cleaved caspase3(ab214430) were purchased from abcam. Rabbit monoclonal antibody recognizing Histone H2AX(9714S) was purchased from Cell Signaling Technology. Rabbit polyclonal antibody recognizing MCM2(ab4461), MCM4(ab4459), Histone H3(ab1791), HA(ab9110) were purchased from abcam. Rabbit polyclonal antibody recognizing phospho-CDC7 T376(PK558) was purchased from KINEXUS. Rabbit polyclonal antibody recognizing Caspase3(9662S) was purchased from Cell Signaling Technology. Rabbit polyclonal antibody recognizing HA-probe(sc-805) was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody recognizing TP53RK(sc-514703), p53(sc-126) were purchased from Santa Cruz Biotechnology. Goat polyclonal antibody recognizing Actin(sc-1616) was purchased from Santa Cruz Biotechnology. Mouse IgG(ab37355) was obtained from abcam. Alexa Flour 488 goat anti-mouse antibody(A11001), Alexa Flour 594 goat anti-rabbit(A11012), 4',6-Diamidino-2-Phenylindole(DAPI; D3571) were obtained from Thermo Fisher Scientific.

DNA Constructs and mutagenesis

TP53RK-HA was cloned into pBabe-puro(addgene; #1764) vector. pBabe-puro-TP53RK-HA 213G>C, 214C>T, 729C>G, 730C>T in CDS sequence were constructed using PfuUltra High-Fidelity DNA polymerase(Agilent; #600380).

Cell culture

SW480, HT29, CaCO2, H508, HCT116, CRL1459 were obtained from the Korea Cell Line Bank or American Tissue Culture Collection. All cell lines were not cultured for longer than two months. Cells were grown in RPMI-1640 or DMEM with 10% fetal bovine serum(FBS; Welgene) and gentamicin(10㎍/mL) at 37°C in a humidified 5% CO_2 atmosphere.

Colony formation assay (CFA)

Colony formation assays were conducted in 6-well plates. A total of 2×10^5 cells suspended in 3mL of medium were seeded per well and incubated for 3 days. After 2 washes with phosphate-buffered saline(PBS), the cells were stained with Coomassie Brilliant Blue(Sigma Aldrich) for 6 hours and photographed using GelCountTM automatic plate scanner(Oxford Optronix GelCount, Abingdon, UK).

Viral transfection for engineering cell lines

The LentiCRISPRv2 vector system was obtained from addgene(#52961) to deliver Cas9, a sgRNA, and a selective marker(Puromycin) into target cells. Plasmid expressing TP53RK was purchased from addgene(#23774) too. Transfection was performed at a final concentration of 6μg/ml using Lipofectamine 2000(Invitrogen). Lentiviruses were produced by transducing 293FT cells with lentiCRISPRv2 plasmids(TP53RK, CDC7) as previously described. [22] The viruses were harvested on day 3, and cells were incubated with viruses in the presence of 6μg/ml polybrene(Sigma Aldrich). After a day incubation, the transduced cells were cultured with 1μg/ml puromycin(Sigma Aldrich) for another 5−7 days before harvest. The overexpression experiment using retro−viral system was also carried out with same process. Expression change of target genes was validated by western blot. Two distinct sgRNAs were designed for each genes. The oligo sequences used for sgRNA are noted in Table 1. The primer sequences used in TP53RK mutagenesis cloning are noted in Table 2.

Table 1. Oligo sequences for sgRNA used in CRISPR KO

Gene	Sequence (5' to 3')
TP53RK-3	(F) CACCGCTACCGGCACCCGGCGCTGG
	(R) AAACCCAGCGCCGGGTGCCGGTAGC
TP53RK-5	(F) CACCGTTAGATGAAGTGCGCCTGAG
	(R) AAACCTCAGGCGCACTTCATCTAAC
CDC7-1	(F) CACCGAGTTATTGCTATGCCATATC
	(R) AAACGATATGGCATAGCAATAACTC
CDC7-4	(F) CACCGTTGAAACGCATTCATCAGTT
	(R) AAACAACTGATGAATGCGTTTCAAC

Table 2. Primer sequences used in mutagenesis cloning

Gene	Sequence (5' to 3')
for TP53RK-3	(F) CGGCACCCGGCCTTGGAGGCGCGG
	(R) CCGCGCCTCCAAGGCCGGGTGCCG
for TP53RK-5	(F) CTAAAAAAATTAGATGAAGTGCG GTTGAGAGGAAGAAAG AGGTCCATG
	(R) CATGGACCTCTTTCTTCCTCTCAA CCGCACTTCATCTAATTTTTTTAG

Western blot analysis

Cultured cells were washed with PBS and lysed with lysis buffer (50mM Tris-HCl, pH 7.5 1% NP40, 0.1% sodium deoxycholate, 150mM NaCl, 50mM NaF, 1mM sodium pyrophosphate, 1mM EDTA and protease/phosphatase inhibitors) for 30 minutes at 4°C. Lysates were clearly by centrifugation at 13,000 rpm for 20 minutes. Protein concentrations were quantified with a Bicinchoninic Acid Protein Assay Reagent(Pierce, Rockford, IL), according to manufacturer's instructions. Samples containing equal quantities of total proteins were resolved in 12% SDS-PAGE gel, transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer containing 1% skim milk and 1% bovine serum albumin(BSA) or 4% skim milk for an hour at room temperature and probed overnight at 4°C with primary antibodies. This was followed by incubation with HRP-conjugated secondary antibody for an hour at room temperature.

Immunoprecipitation analysis

Nuclear extract was obtained by incubating trypsinized cells first in Buffer A(10mM Tris-HCl, 10mM NaCl, 3mM $MgCl_2$ and protease/phosphatase inhibitors) for 15 minutes, followed by addition of 10% NP40. The pellets were collected after centrifugation at 2000 rpm for 5 minutes, and resuspended in Buffer B(10mM Tris-HCl, 10mM KCl, 1mM DTT, 1mM $MgCl_2$ and protease/phosphatase inhibitors). The pellets were collected and lysed with Buffer C(50mM Tris-HCl, 150mM NaCl, 1mM sodium pyrophosphate, 1% NP40, 0.1% sodium deoxycholate and protease/phosphatase inhibitors). Total nuclear extract was pro-cleared with Dynabead (Invitrogen) for 4 hours, and rotated overnight with Dynabead and 5~10μg antibodies or mouse IgG.

Immunofluorescence analysis

Cells were cultured in Poly-L-Lysin(Sigma Aldrich) coated plate to 50% confluency. Cells wore washed with PBS, incubated in 3.7% formalin solution(Sigma Aldrich) for 30 minutes at room temperature, permeabilized in 0.5% TX-100 solution for 5 minutes, and washed with PBS, then blocked in 2% BSA buffer for an hour. Antibodies were diluted in 2% BSA buffer and incubated overnight. Cells were washed with PBS and incubated with secondary antibody for an hour. Slides were mounted with Faramount Aqueous Mounting Medium(Dako).

Flow cytometric analysis

For detection of Annexin V and PI double positive population, the cells were harvested via trypsinization and stained using FITC Annexin V Apoptosis Detection Kit(BD pharmingen; 556547). Stained cells were subjected to flow cytometric analysis using a BD FACSCanto Ⅱ 6 colors(BD Bioscience).

Proteomic analysis using mass spectrometry

The harvested and digested protein samples were TMT labeled using TMTsixplex Isobaric Label Reagent Set(Thermo Fisher Scientific; 90062). After fractionation, 5% of samples were used for global-proteome analysis and 95% of samples were used for phospho-proteome analysis.

Statistical analysis

All experiments were conducted in triplicate with at least two biological replicates. $P<0.05$ was considered statistically significant. *$P<0.05$, **$P<0.01$, ***$P<0.001$,

Result

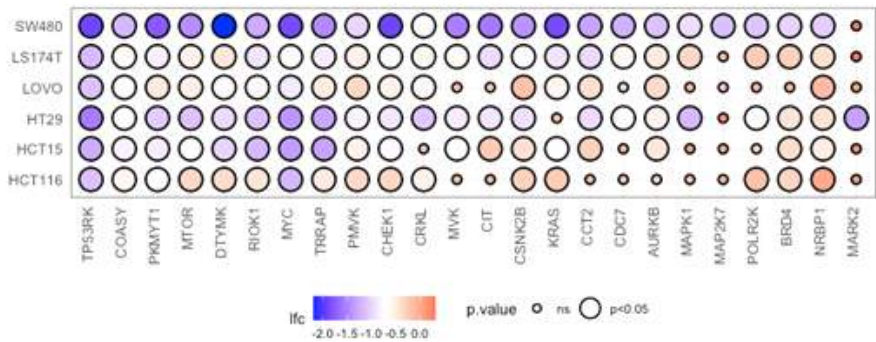
Overexpression and gene amplification of TP53RK in colorectal cancer patients

To select a biomarker candidate specific for colorectal cancer, we performed CRISPR library screening using MAGeCK algorithm, 700 gene-set TK panel and 6 sgRNAs per gene on a total of 6 colon cancer cell lines. This algorithm is suitable for analyzing the effect of each gene expression on cell viability cause it contains process for normalization through the replicate, validation of p-value and $\log_2$ fold change(FC), and verification of actual sgRNAs binding efficiency. [23] As a result, TP53RK was ranked as a gene showing the highest cell death efficiency in all 6 colon cancer cell lines when its expression was downregulated. (Figure 1A)

According to previous study, TP53RK is frequently amplified at chromosome 20q13.12 along with HNF4A, a nuclear transcriptional factor, in colorectal cancer patient. [24] Therefore, we examined the expression and amplification pattern of TP53RK through colorectal cancer patient data. Indeed, tumor presented high level of TP53RK expression compared to normal in our patients data(Center for Precision Medicine in Seoul National University Hospital; CoPM). (Figure 1B) COACY and MTOR showed similar expression in tumor

and normal. PKMYT1 had very low basal expression of the gene. Moreover, in 143 of 225 patients, overexpression and CNVs of TP53RK were identified together. (Figure 1C) These results suggest that there is certain correlation between expression pattern of TP53RK and its abnormal amplification in colorectal cancer patients.

A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TP53RK
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CHEK1
CRKL
MVK
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CCT2
CDC7
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MAP2K7
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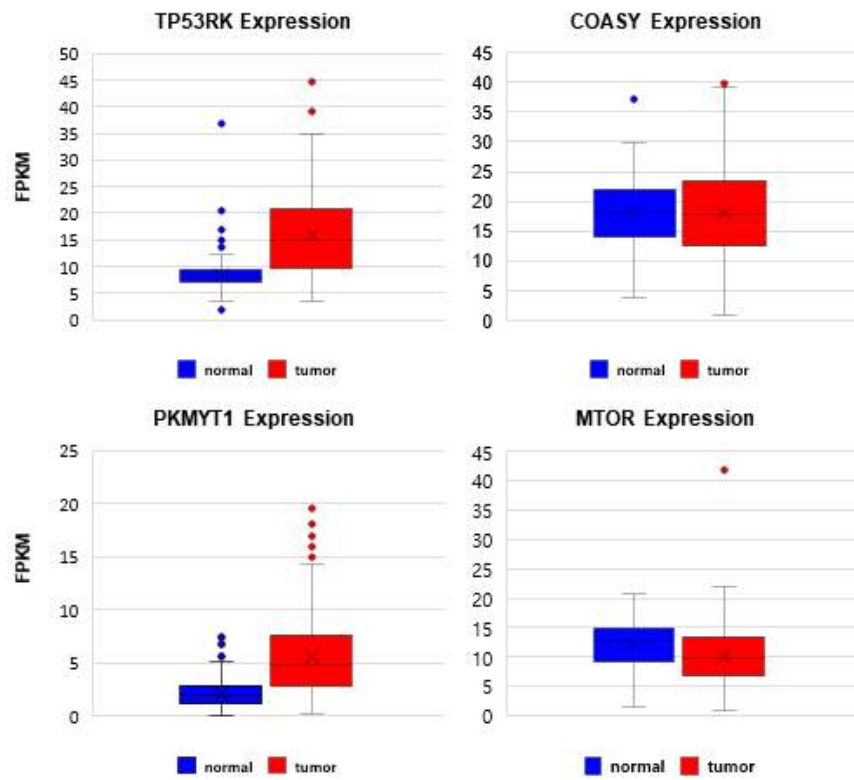

TP53RK Expression
COASY Expression
PKMYT1 Expression
MTOR Expression
FPKM
normal
tumor

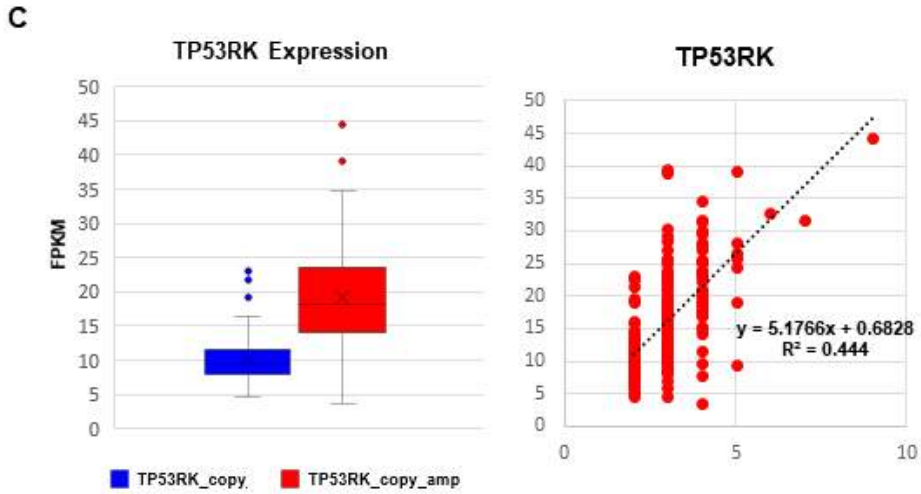


Figure 1. Overexpression and gene amplification of TP53RK in colorectal cancer patient

(A) CRISPR library screening result analyzed by MAGeCK algorithm. Heatmap depicts log2 FC of sgRNA abundance(averaging each sgRNA). The statistical significance of each sgRNA was measured using p-value. FDR<0.05 and *P<0.033, **P<0.002, ***P<0.001 were considered statistically significant.

(B) Expression patterns of TP53RK, COASY, PKMYT1 and MTOR in tumor compared to normal group. Patients data was provided from the CoPM in Seoul National University Hospital.

(C) Correlation between TP53RK expression and its CNVs. X axis is copy number and Y axis is mRNA expression. (right)

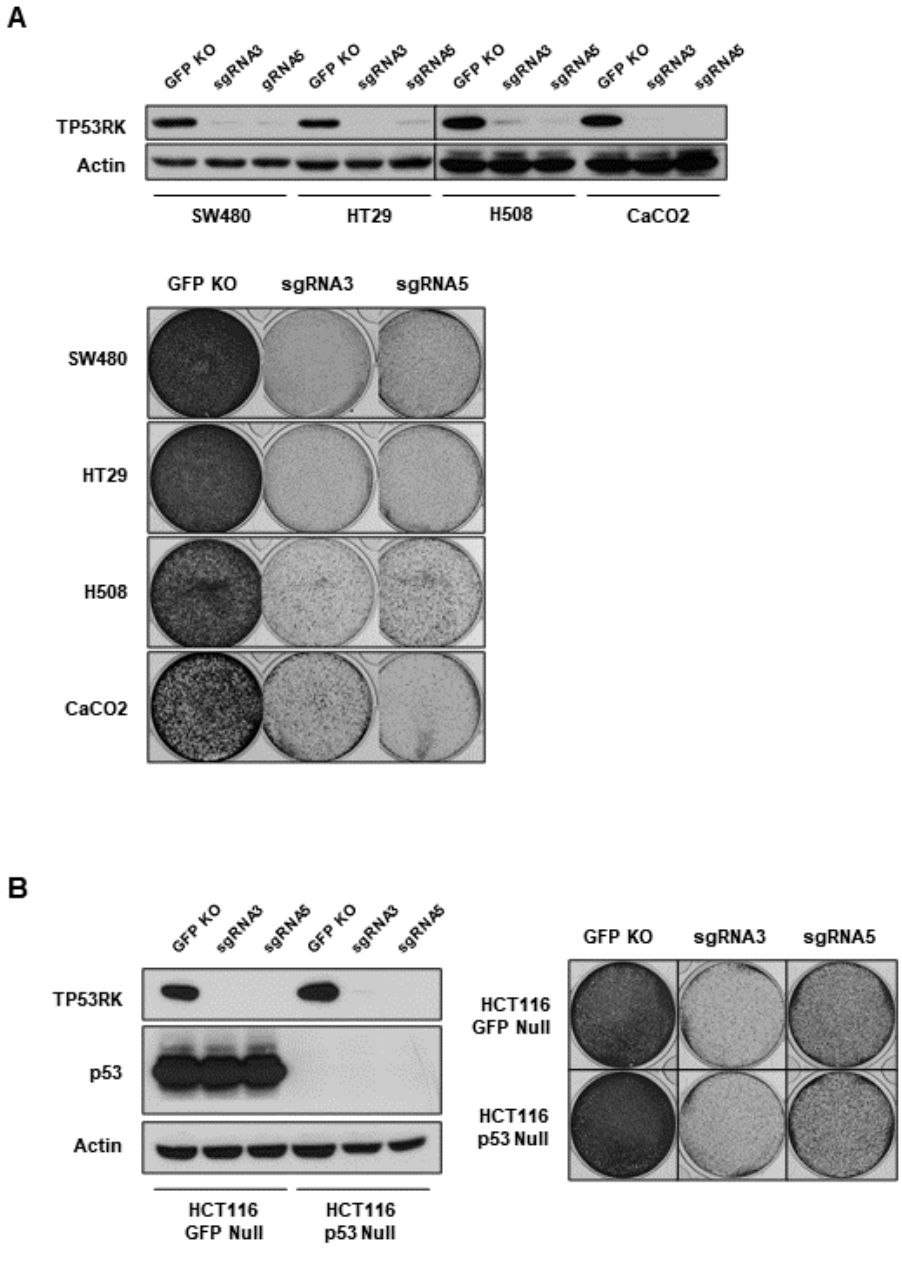
Depletion of TP53RK induces apoptosis with or without p53 in colon cancer cell lines but not for normal colon fibroblast

To validate the result of CRISPR library screening, we conducted TP53RK knock out using 2 sgRNAs on a total of 4 colon cancer cell lines. Consistent with the results of screening, depletion of TP53RK effectively reduced cell viability in all cell lines. (Figure 2A) All of these cell lines have a mutation in p53. Furthermore, by engineering p53 null HCT116 cell line, we confirmed whether cell death due to decrease of TP53RK expression is related to p53. As expected, the effect of TP53RK knock out was not significantly up or downregulated in p53 null condition. (Figure 2B) When the expression of TP53RK was inhibited, although cells maintained viability, *r*H2AX signal was observed in TP53RK knock out group. (Figure 2C) Annexin V staining also revealed that most of cells entered apoptosis about 2 weeks after TP53RK inhibition. (Figure 2D) Altogether, these data indicated that depletion of TP53RK induces apoptosis due to DNA damage accumulation in colon cancer cell line.

In addition, to determine whether this viability alteration is definitely affected by the presence or absence of TP53RK, we constructed TP53RK-HA overexpression vectors including single nucleotide alteration for sgRNAs evasion. Consequently, when sgRNA and overexpression vector were infected at one time, cell

proliferation was remarkably restored compared to knock out condition. (Figure 2E) It means that decrease of the cell viability is directly related to TP53RK function, not any other bypass factor.

Next, we performed a TP53RK knock out with the same scheme in normal colon fibroblast cell line CRL1459 for checking tumor specificity. In this case, even after depletion of TP53RK, cell viability was almost the same as that of the control. (Figure 2F) Therefore, we expect that a decrease of TP53RK may present tumor specific anticancer effect, at least *in vitro* condition.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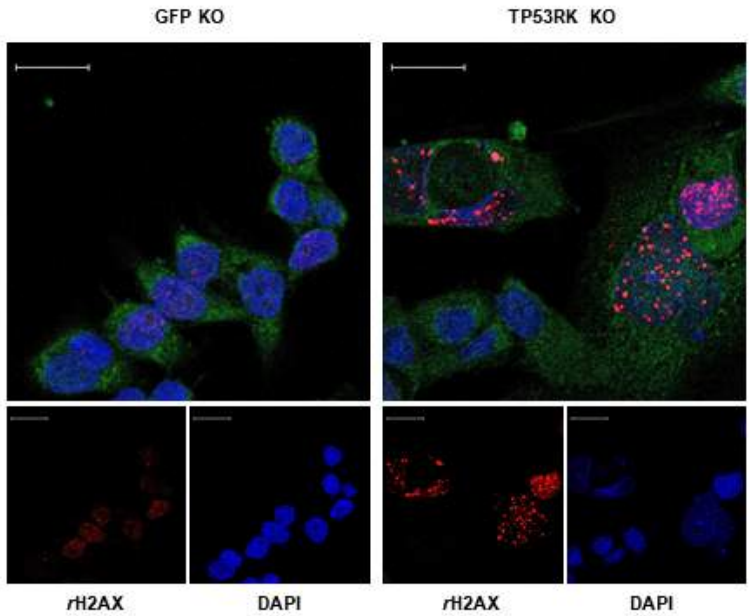
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DAPI

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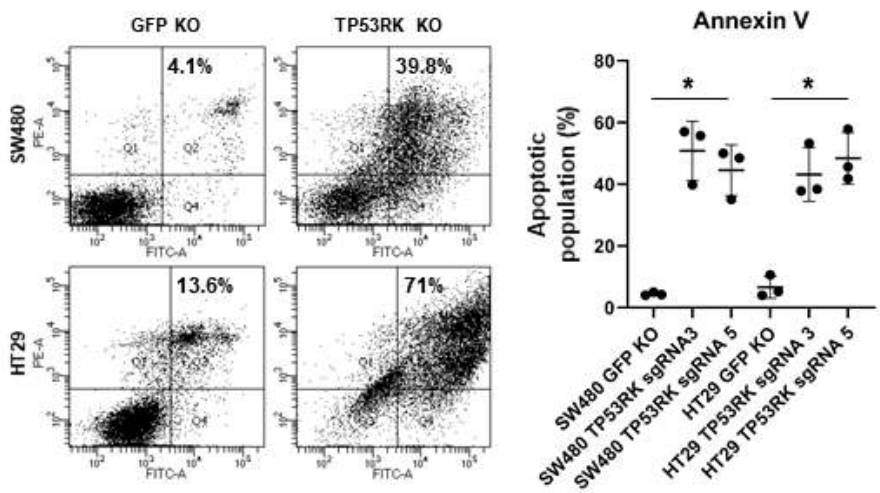
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SW480
HT29
PE-A
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4.1%
39.8%
13.6%
71%
Annexin V
Apoptotic population (%)
*
*
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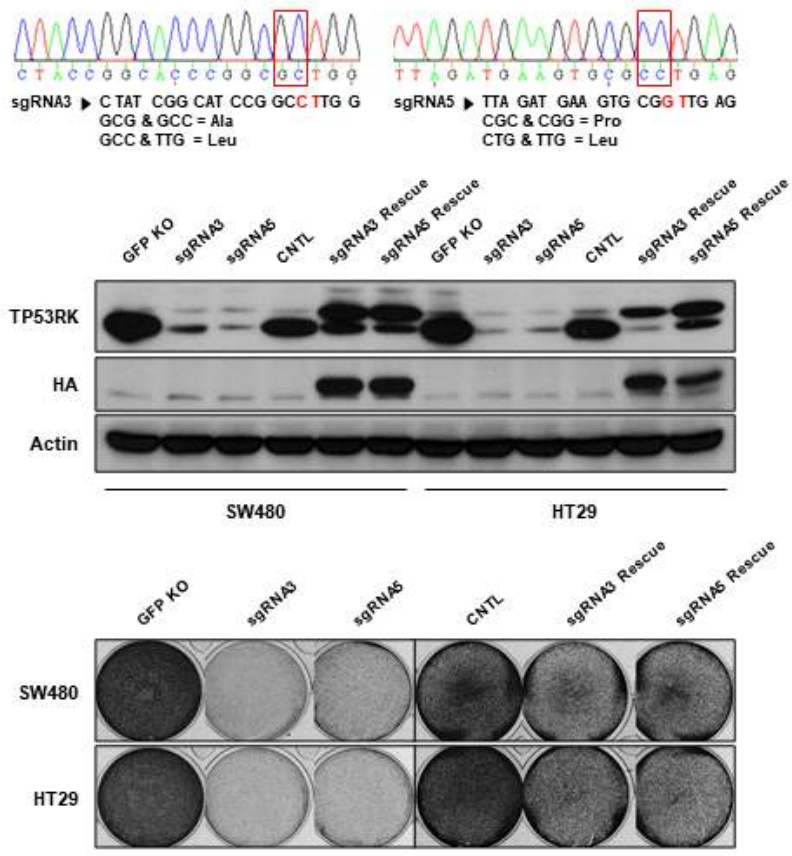
SW480 TP53RK sgRNA 5
HT29 GFP KO
HT29 TP53RK sgRNA 3
HT29 TP53RK sgRNA 5

E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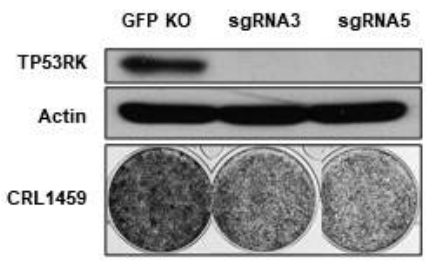
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sgRNA3
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TP53RK
Actin
CRL1459

Figure 2. Depletion of TP53RK induces apoptosis with or without p53 in colon cancer cell lines but not for normal colon fibroblast

(A) Western blot and CFA after TP53RK knock out in 6 colon cancer cell lines. Cells were harvested at least 4 days after sgRNA infection. sgRNA targeting GFP was used as a negative control. CFA staining was performed for 3 days from 8 days after sgRNA infection.

(B) Western blot and CFA after TP53RK knock out in p53 null HCT116. Experimental workflow is the same as Figure A.

(C) Confocal immunofluorescence images of basal HT29 stained for the different antibody as indicated. Pictures are representative of 2 different experiments reproduced conducting biological triplicate trial. Scale bars, 20 ㎛.

(D) Graph presenting apoptotic population after TP53RK knock out. Numerical value was obtained by FITC-Annexin V and propidium iodide co-staining with flow cytometric analysis. *$P<0.05$

(E) Western blot and CFA after infection of TP53RK-HA overexpression vector including single nucleotide alteration and sgRNA targeting TP53RK. Experimental workflow is the same as Figure A.

(F) Western blot and CFA after TP53RK knock out in normal colon fibroblast CRL1459. Cells were harvested 11 days after sgRNA infection. CFA staining was performed for 3 days from 15 days after sgRNA infection.

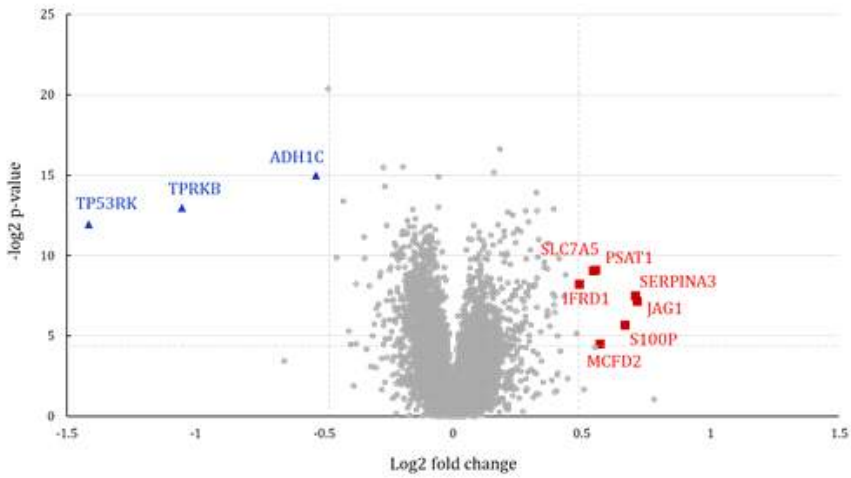
TP53RK regulates phosphorylation of CDC7 substrates

To identify binding partner or downstream target of TP53RK, we conducted global and phospho-proteome analysis based on mass spectrometry. In the global-proteome analysis, as confirmed by volcano plot, TP53RK knock out did not affect a expression change of specific protein except for TPRKB(TP53RK binding protein), a member of EKC/KEOPS complex. [25](Figure 3A) It is known to bind to TP53RK, however, interaction mechanism with TP53RK and definite function is poorly characterized. Goswami et al. (2019) reported that loss of TPRKB leads to decrease of proliferation in TP53 mutant cancer cell lines, but our data presented a rather different tendency after TPRKB knock out. [26](data not shown)

As previous mentioned, cause TP53RK is a kinase, probability that it is directly involved in regulation of a protein expression is relatively low. Through the kinase-substrate enrichment analysis with phospho-proteome analysis, a technique for prediction which kinases regulate substrates related to target protein by identifying alteration of phosphosites, we found that when expression of TP53RK is suppressed, the phosphorylation of CDC7 substrates is significantly decreased. (Figure 3B) This result suggests that depletion of TP53RK triggers hypophosphorylation of CDC7 substrates, for example MCM complex, a main target of CDC7. (Figure 3C) Although CDC7/DBF4, also known as DDK, is a kinase

and important regulator essential for firing DNA replication, just a few substrates or binding partners of CDC7 have been identified up to now. [27] Therefore, we hypothesized that CDC7 and TP53RK would bind directly or at least co-localize.

A



B

	Node Name	Corrected P-value with Benjamini-Hochberg
Upregulated in TP53RK KO	RPS6KB1	0.0417
	PAK2	0.0417
	PAK1	0.0417
	PDK3	0.0417
	PDPK1	0.0417
	PDK1	0.0417
	EEF2K	0.0417
	OXSR1	0.0417
	PDK2	0.0417
	RPS6KB2	0.0597
	PAK3	0.101
	MTOR	0.146
	RPS6KA2	0.146
	MAPK9	0.152
	PRKCA	0.200
	TRPM7	0.211
	CDK2	0.211
	RPS6KA1	0.268
	SGK1	0.286
	PRKCD	0.362
Upregulated in GFP KO	CDC7	0.006997
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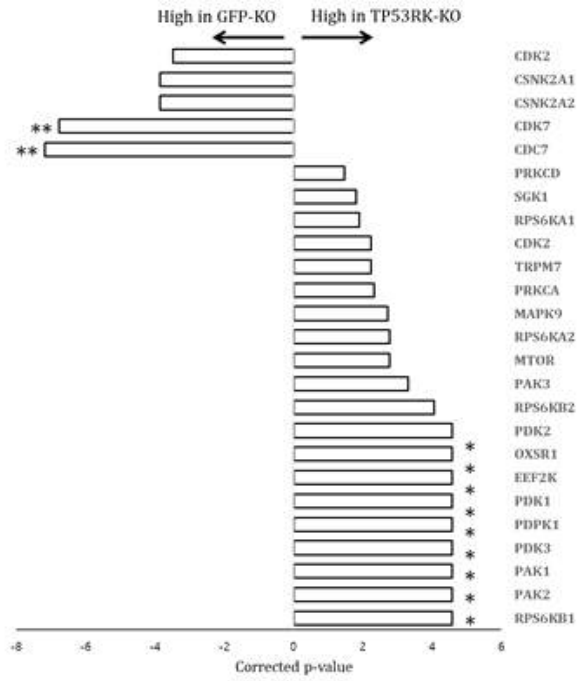
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High in TP53RK-KO
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CSNK2A1
CSNK2A2
CDK7
CDC7
PRKCD
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PRKCA
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RPS6KA2
MTOR
PAK3
RPS6KB2
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*
-8
-6
-4
-2
0
2
4
6
Corrected p-value

C

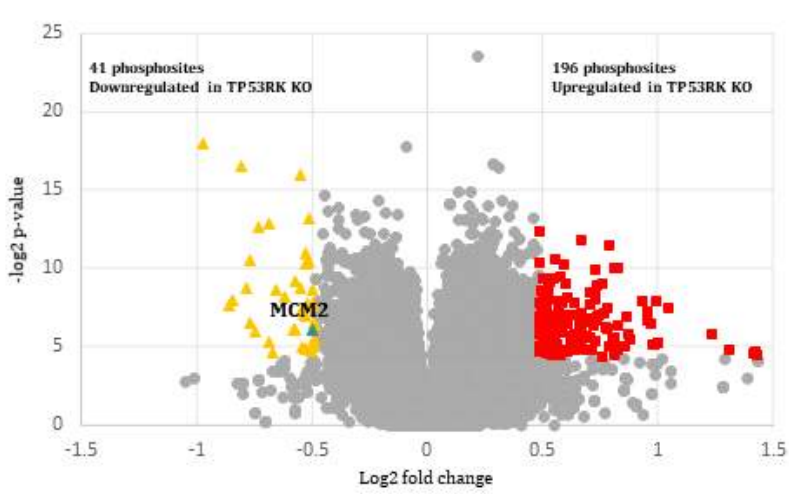

41 phosphosites
Downregulated in TP53RK KO
196 phosphosites
Upregulated in TP53RK KO
MCM2
-log2 p-value
Log2 fold change
25
20
15
10
5
0
-1.5
-1
-0.5
0
0.5
1
1.5

Figure 3. TP53RK regulates phosphorylation of CDC7 substrates

(A) Volcano plot showing the protein with altered expression of GFP knock out versus TP53RK knock out samples in global-proteome analysis. Cells were harvested day 7 after sgRNA infection. Each dot represents a protein.

(B) Result of kinase-substrate enrichment analysis on a basis of phospho-proteome analysis after TP53RK knock out in HT29. Cells were harvested day 7 after sgRNA infection. *P<0.05, **P<0.01 were considured statistically significant. (Input; Phosphosites of differentially expressed phosphopeptides, FC>1.4, p-value<0.05)

(C) Volcano plot showing the phosphosite with altered phosphorylation of GFP knock out versus TP53RK knock out samples. On the left, blue color triangle highlights MCM2 phosphosite. Each dot represents a phosphosite of protein.

TP53RK depletion causes phosphorylation of CDC7 leading to DNA replication disorder

To confirm whether TP53RK and CDC7 co-localize in same intracellular compartment, we carried out immunofluorescence staining. TP53RK was evenly located in nucleus and cytosol. On the other hand, as is known, main location of CDC7 was nucleus. (Figure 4A) This result suggests nucleic co-localization between TP53RK and CDC7. Accordingly, fractionation studies also verified positional distribution of two molecules and DBF4. (Figure 4B) Next, we immunoprecipitated CDC7 in TP53RK overexpressing SW480 and HT29. Indeed, result of immunoprecipitation assay demonstrated the binding of CDC7 with TP53RK, which is expected to occur inside nucleus. (Figure 4B)

CDC7 is involved in DNA replication by phosphorylating MCM complex through the binding to DBF4. Therefore, to confirm finding of the proteomic analysis, we examined expression change of MCM2 and MCM4, a substrates of CDC7, caused by TP53RK knock out. As expected, there was a striking reduction of MCM2 and MCM4 phosphorylation although the total expression of those proteins did not change notably. (Figure 4C) And most importantly, the level of CDC7 phosphorylation was increased not only on the immunoblotting but also on the immunofluorescence staining. (Figure 4D) In a previous studies, Knockleby et al. (2016) reported that phosphorylation status of CDC7 takes a regulatory switch role for

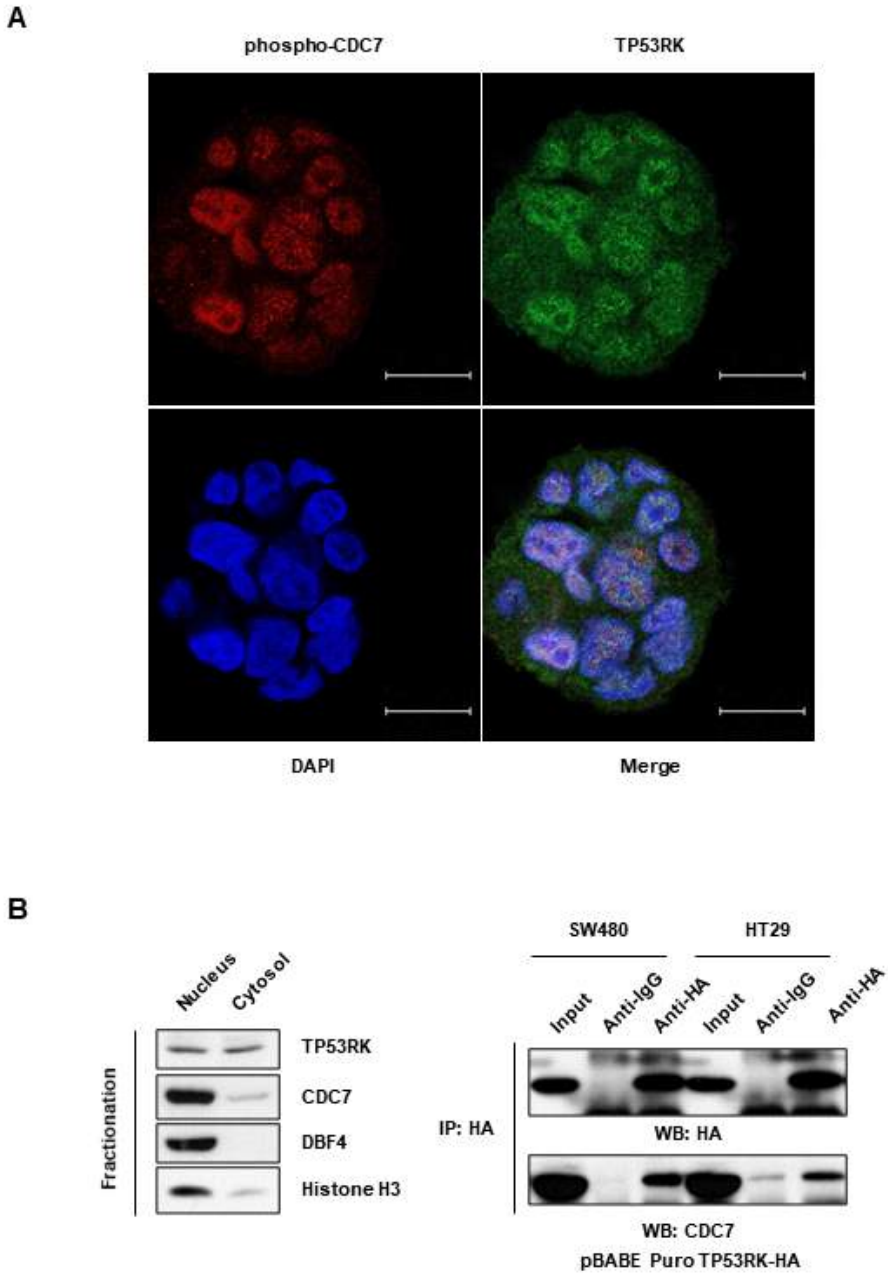
DNA replication in mitosis, especially prometaphase. [21] Upon phosphorylation, CDC7 is eliminated from origins, and it causes interference of replication initiation. Our results also indicate that phosphorylation of CDC7 caused by TP53RK knock out relates to replication disorder. Moreover, DNA damage signal was remarkably enhanced such as *r*H2AX and cleaved caspase3. (Figure 4C) These consequences suggest that depletion of TP53RK causes a reduction of DNA stability and activity of replication initiating factors. All such changes of phosphorylation status were rescued when overexpression vector including single nucleotide alteration was infected. (Figure 4E)

Additionally, we investigated the effect of TP53RK knock out on cell cycle dynamics. Even though there were a little distinction between cell lines, population entering S-G2/M phase was about the same in control group and TP53RK knock out group. (Figure 4F) It means that under TP53RK deficiency condition, cells did not completely arrest in specific phase despite alteration of phosphorylation status described above. In this context, we would expect that deficiency of TP53RK causes "badly-controlled" DNA replication on the basis of "unsettled fuel" accumulating various damages, and consequently induces apoptosis in colon cancer cell lines.

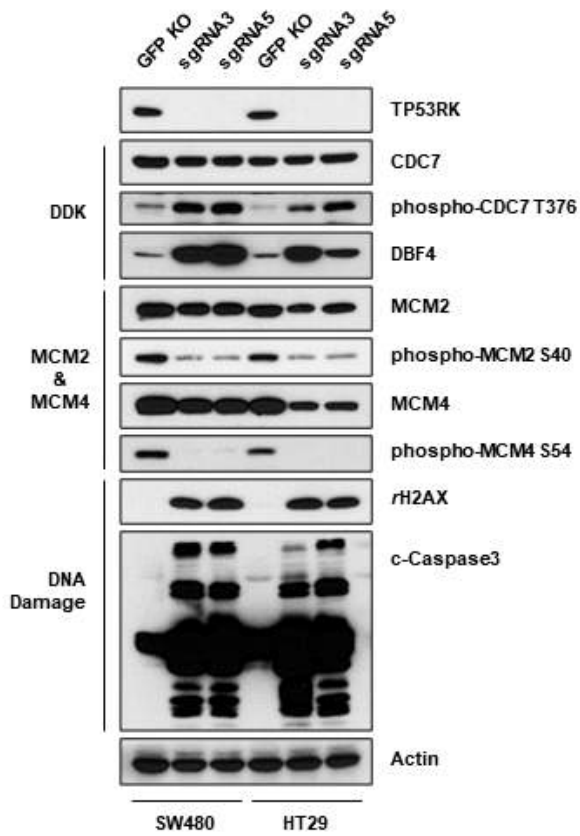
Supplementally, to determine effect of CDC7 depletion on its downstream substrate and DNA stability, we conducted CDC7 knock out. (Figure 4G) Same as the previous result, phosphorylation of

MCM2 and MCM4 was decreased and expression of DNA damage markers was increased. Cell death also proceeded very rapidly. Interestingly, DBF4 showed aspect of increase, it is thought to be related to protein stability or feedback mechanism.

Taken together, these finding strongly implied that TP53RK is involved in DNA replication mechanism by regulating phosphorylation of CDC7, and that there was obvious correlation between TP53RK and CDC7 during that event.


A
phospho-CDC7
TP53RK
DAPI
Merge
B
Nucleus
Cytosol
Fractionation
TP53RK
CDC7
DBF4
Histone H3
SW480
HT29
Input
Anti-IgG
Anti-HA
Input
Anti-IgG
Anti-HA
IP: HA
WB: HA
WB: CDC7
pBABE Puro TP53RK-HA

C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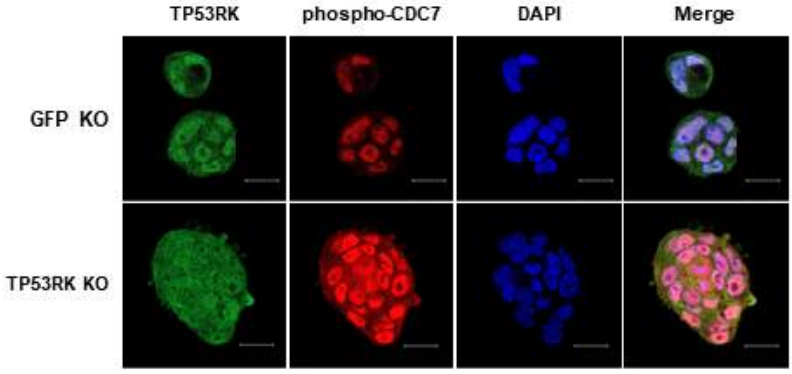
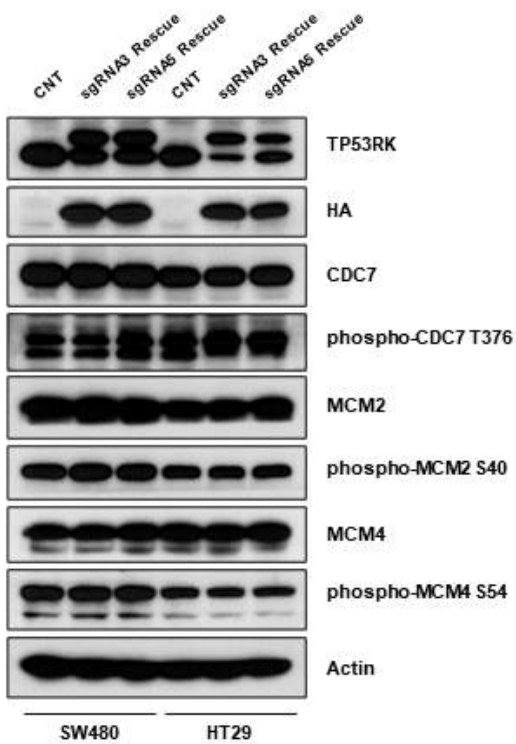
sgRNA3
sgRNA5
GFP KO
sgRNA3
sgRNA5
TP53RK
CDC7
phospho-CDC7 T376
DBF4
MCM2
phospho-MCM2 S40
MCM4
phospho-MCM4 S54
rH2AX
c-Caspase3
Actin
DDK
MCM2 & MCM4
DNA Damage
SW480
HT29

D


TP53RK
phospho-CDC7
DAPI
Merge
GFP KO
TP53RK KO

E


CNT
sgRNA3 Rescue
sgRNA5 Rescue
CNT
sgRNA3 Rescue
sgRNA5 Rescue
TP53RK
HA
CDC7
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MCM2
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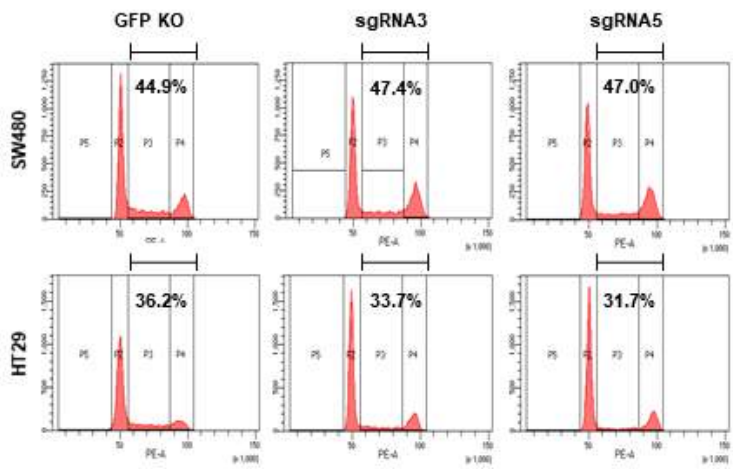
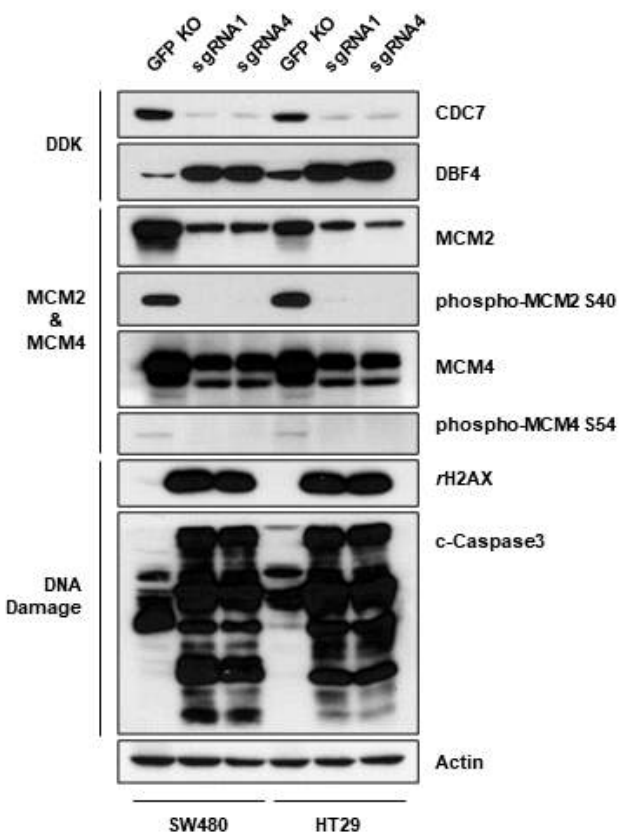
MCM4
phospho-MCM4 S54
Actin
SW480
HT29

F


GFP KO
sgRNA3
sgRNA5
SW480
HT29
44.9%
47.4%
47.0%
36.2%
33.7%
31.7%

G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sgRNA1
sgRNA4
GFP KO
sgRNA1
sgRNA4
DDK
CDC7
DBF4
MCM2
&
MCM4
MCM2
phospho-MCM2 S40
MCM4
phospho-MCM4 S54
DNA
Damage
γH2AX
c-Caspase3
Actin
SW480
HT29

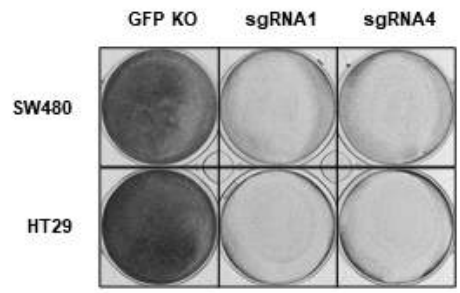
GFP KO
sgRNA1
sgRNA4
SW480
HT29

Figure 4. TP53RK depletion causes hypophophorylation of CDC7 leading to DNA replication disorder

(A) Confocal immunofluorescence images of basal HT29 stained for the different antibody as indicated. Pictures are representative of biological triplicate trial. Scale bars, 20 ㎛.

(B) Western blot after fractionation for verifying location of TP53RK, CDC7, DBF4 (left) and after nuclear extracted immunoprecipitation using TP53RK overexpression vector. (right) Immunoprecipitation with an anti-HA antibody was performed.

(C) Western blot after TP53RK knock out in SW480 and HT29. Cells were harvested 9 or 12 days after sgRNA infection. sgRNA targeting GFP was used as a negative control.

(D) Confocal immunofluorescence images of basal HT29 stained for the different antibody as indicated. Pictures are representative of 2 different experiments reproduced conducting biological triplicate trial. Scale bars, 20 ㎛.

(E) Western blot after infection of TP53RK-HA overexpression vector including single nucleotide alteration and sgRNA targeting TP53RK.

(F) Cell cycle profiles after TP53RK knock out. Cells were harvested 12 days after sgRNA infection.

(G) Western blot and CFA after CDC7 knock out in SW480 and HT29. Cells were harvested at least 4 days after sgRNA infection. CFA staining was performed for 3 days from 1 day after sgRNA infection.

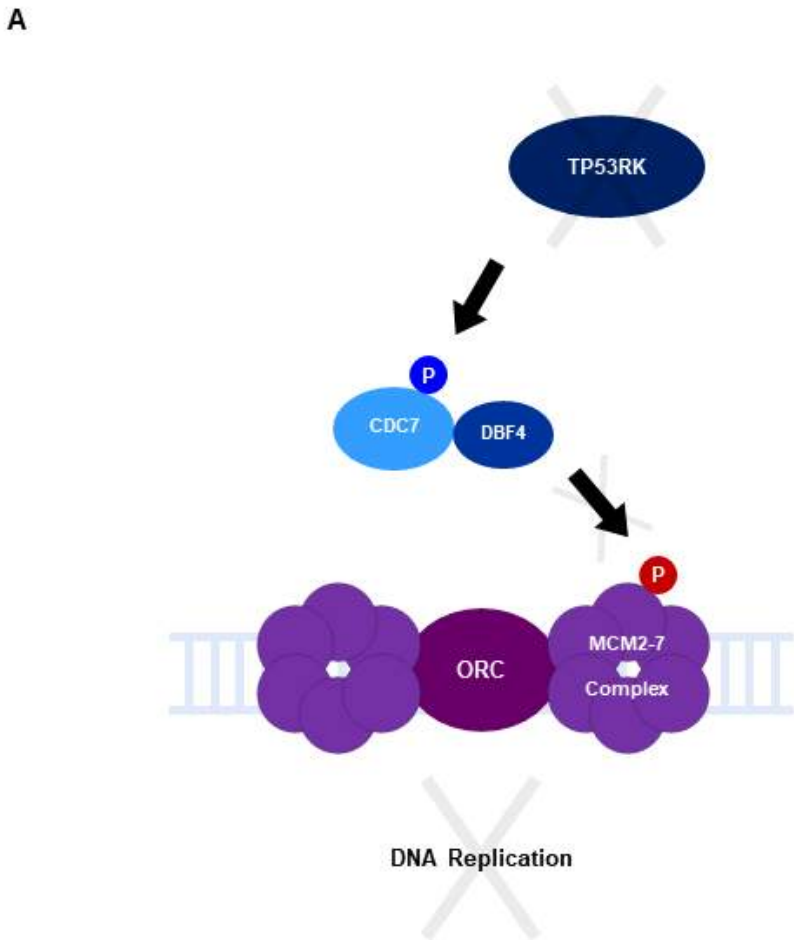

Figure 5. A mechanism of TP53RK-mediated DNA replication via DDK dependent pathway

When TP53RK was abrogated, phosphorylation of CDC7 was increased. This result causes hypophosphorylation of MCM complex, consequently, induces disruption of DNA replication.

Discussion

KRAS, APC and p53 mutations are known to be the most frequently occurring genetic alteration in colorectal cancer. So far, several evidences have indicated that these mutations crucially correlated with pathogenesis of malignancy and therapeutic approach, but in terms of precision oncology, there are still realistic limitations for its popularization. In this study, we performed CRISPR library screening and proteomic analysis and identified TP53RK as a novel candidates for druggable target to colorectal cancer. During this work we have attempted to validate oncogenic properties of TP53RK through the CRISPR knock out and investigated mechanism of how TP53RK expression change affects cell viability. Depletion of TP53RK induces hyperphosphorylation of CDC7, as a result, interrupts the DNA replication by causing reduction of MCM2 and MCM4 phosphorylation. (Figure 5) Notably, this process is irrelevant to presence or absence of p53. (Figure 2B)

Further studies are required to clarify the TP53RK-mediated DNA replication mechanism, such as which TP53RK domain plays an important role in binding with CDC7. And it is necessary to verify in which phase the interaction between two molecules occurs for cell cycle. As of now, that is also unclear whether binding efficiency

of origin related protein, for instance Sld3 and CDC45, is reduced owing to hypophosphorylation of MCM2/4 by TP53RK loss. This course is expected to be confirmed by conducting ChIP assay or chromatin fraction immunoblotting. Interestingly, we observed that protein expression of DBF4 strikingly increases when TP53RK is inhibited and same phenomenon was showed after CDC7 knock out too. (Figure 4B and G) That could be due to protein accumulation, occurring in "flawed cell cycle". Although the level of DBF4 should be fluctuate with each phase of cell cycle, upon lack of TP53RK, protein is not degraded properly because of "incomplete arrest" described above. It would be revealed through cell cycle profiling using synchronized cells after knock out.

In addition, there is a need for clinical approach based on our results but there are no FDA-approved chemotherapeutic agents as a inhibitor of TP53RK and CDC7 yet. Nevertheless, anticancer effect with the regulation of TP53RK is certainly expected. Additional experiments, such as *in vivo* test and treatment with CDC7 inhibitor on TP53RK overexpressed cell lines will need to be done. Through these processes, we aim to solidify the possibility of TP53RK as a novel colorectal cancer specific biomarker and therapeutic target.

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국문 초록

현대에 이르러, 대장암은 가장 높은 발병률과 사망률을 나타내는 암종 가운데 하나로 알려져 있다. 지난 수십 년간 대장암을 유발하는 유전학적/후성유전학적 요인에 대한 연구가 활발히 진행되어 왔으나 대다수 암종이 그러하듯 질환의 발병 기전에 대한 이해와 항암 요법의 효과적인 적용을 위해서는 보다 심도 깊은 접근이 필요한 시점이다. 이 같은 맥락에서, 환자 개개인의 환경적/생물학적 정보가 반영된 정밀 의료(Precision medicine) 또는 맞춤 의료(Personalized medicine) 대중화의 핵심 기술로 자리잡은 것이 차세대 염기서열 분석(Next generation sequencing)과 바이오 마커(Biomarker)를 이용한 표적 치료(Targeted therapy) 전략이다.

새로운 대장암 특이적 바이오 마커 발굴을 목적으로, 본 논문에서는 약 700여개 유전자가 포함된 타이로신 인산화효소 수용체(Receptor tyrosine kinase) 패널 기반의 크리스퍼 라이브러리 스크리닝(CRISPR library screening)을 진행했다. 총 6개의 대장암 세포주를 사용한 녹 아웃(Knock out) 데이터 분석 결과, 발현 감소시 모든 세포주에서 가장 높은 수준의 세포 성장 억제 효율을 나타내는 유전자로 TP53RK(TP53 regulating kinase, PRPK)가 랭크되었다. TP53RK는 다발골수종과 피부암을 비롯한 다수 암종에서 과발현 양상을 나타내는 것으로 보고되어 왔으나 유전자의 종양 형성 관련 기능은 명확히 밝혀진 바 없다. 이에 본 논문은 TP53RK 녹 아웃 후 질량 분석(Mass spectrometry) 기반의 단백체 분석(Proteome analysis)을 진행함으로써 TP53RK의 발현 저하로

인해 유도되는 세포 사멸 기전을 규명하고자 하였다. 실제로, 녹 아웃을 통한 TP53RK 억제시 대장암 세포주의 성장 저해 및 DNA 복제 개시에 관여하는 CDC7(Cell division cycle 7) 키네이스(Kinase)와 DNA 나선 효소(Helicase)의 중심 인자이자 CDC7의 기질로 알려진 MCM2/4 인산화 양상의 변화를 확인할 수 있었으며 이러한 결과는 TP53RK가 CDC7과 CDC7의 조절 소단위인 DBF4(Dumbbell former 4 protein) 의존적 신호 전달 경로를 거쳐 DNA 복제 과정의 조절자로 기능한다는 점을 시사하는 것이다.

주 요 어: TP53RK/PRPK; CDC7; DBF4; DDK; MCM 복합체; DNA 복제; 크리스퍼 라이브러리 스크리닝; 대장암;

학 번: 2018-27373