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Novel homeodomain-interacting protein kinase family member, HIPK4, phosphorylates human p53 at serine 9

Shigeki Arai[a], Akio Matsushita[a], Kun Du[a], Ken Yagi[b], Yasushi Okazaki[b], Riki Kurokawa[a,*]

[a] *Division of Gene Structure and Function, Research Center for Genomic Medicine, Saitama Medical University, Japan*
[b] *Division of Functional Genomics and Systems Medicine, Research Center for Genomic Medicine, Saitama Medical University, Japan*

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Abstract **We describe here the cloning and characterization of a novel mouse homeodomain-interacting protein kinase (HIPK)-like gene, Hipk4. Hipk4 is expressed in lung and in white adipose tissue and encodes a 616 amino acid protein that includes a serine/threonine kinase domain. We demonstrate that HIPK4 could phosphorylate human p53 protein at serine 9, both in vitro and in vivo. Among known p53-responsive promoters, activity of the human *survivin* promoter, which is repressed by p53, was decreased by HIPK4 in p53 functional A549 cells. Human *BCL2-associated X protein*-promoter activity was not affected. These findings suggest that phosphorylation of p53 at serine 9 is important for p53 mediated transcriptional repression.**
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Keywords: Kinase; Transcription; Tumor suppressor; p53; Apoptosis

1. Introduction

The mammalian genome encodes ~500 protein kinase-like domain containing proteins that putatively phosphorylate serine, threonine or tyrosine residues of target proteins [1]. Proteins that become phosphorylated may alter characteristics, including enzymatic activity, half-life, localization, and partner binding [2]. Mammalian homeodomain-interacting protein kinases (HIPKs), HIPK1, 2 and 3, are members of the nuclear serine/threonine kinase family [3,4]. HIPKs are localized in nuclear speckles and phosphorylate several proteins, including homeodomain transcription factor NKx-1.2, androgen receptor and tumor suppressor p53 [3–5]. p53 is phosphorylated at several serine and threonine residues including 6, 9, 15, 20, 33, 37, 46, and 392 in response to various signals, such as genotoxic stress (e.g., ultraviolet (UV), ionizing radiation and anti-cancer agents) and non-genotoxic stress (e.g., hypoxia, senescence, and osmotic shock) [6–8]. Site-specific phosphorylation of p53 regulates its activity. For example, HIPK2 phosphorylates human p53 at Ser46 and co-repressor CtBP at Ser422, which induces apoptotic cell death [5,9]. DNA damage induces phosphorylation of p53 at Ser15 and Ser37 and inhibits interaction of p53 and its negative regulator, oncoprotein mouse double minute 2 homolog (MDM2) [10]. Human p53 is rapidly phosphorylated at Ser9 after exposure to γ-rays and to UV light. Several kinases, such as casein kinase 1δ (CK1δ), CK1ε, and DNA-PK have the ability to phosphorylate p53 at Ser9, in addition to several other serine residues, but the detailed role of phospho-p53 (Ser9) is not clear [11]. Recently, Cordenonsi et al. reported that human p53, phosphorylated at Ser9, and injected into *Xenopus* embryos, serves as an activator of mesoderm marker genes and that phospho-p53 (Ser9) promotes TGF-β cytostasis in human cells [12]. In the latter case, phospho-p53 (Ser9) induces the formation of a complex with Smad2 and activates transcription of several TGF-β target genes, such as p21Waf1 and p15INK4b.

We show here that HIPK4, the product of a previously uncharacterized kinase-like gene [13], has direct kinase activity on human p53 at serine 9 in vitro and in vivo. We observed that over-expression of Hipk4 in A549 cells decreased the promoter activity of human *survivin*, which is an anti-apoptotic gene and which is directly repressed by p53 at the transcriptional level [14,15]. Interestingly, the human *BCL2-associated X protein* (*BAX*) promoter, which is a pro-apoptotic, and direct-target gene of p53 [16,17], was not affected by HIPK4. These results will further the understanding of cell proliferation and of differentiation pathways that are under the control of the p53 network, especially in circumstances where p53 phosphorylation at serine 9 is tightly regulated.

2. Materials and methods

2.1. Materials

Cultured cell lines were obtained from Cell Resource Center for Biomedical Research, Tohoku University. Oligonucleotides, for PCR primers, were synthesized by Hokkaido System Science (Hokkaido, Japan). *Pwo* SuperYield DNA Polymerase (Roche, Mannheim, Germany) was used for PCR experiments. Reagents for RNA isolation and for cDNA synthesis were purchased from Invitrogen (Carlsbad, CA). Antibodies were purchased from Cell Signaling Technology (Beverly, MA). Myelin basic protein (MBP) and purified histone proteins were obtained from Sigma–Aldrich (St. Louis, MO) and from Roche, respectively.

2.2. Cloning, sequencing and PCR

The open reading frame of Hipk4 was predicted from mouse genomic DNA clone (NC_000073.5). Primer pairs, HIPK4-fw 5′-atggccaccatccagtcaggacactg-3′ and HIPK4-rv 5′-tcagtggtgccctccaacatgctg-3′,

*Corresponding author. Fax: +81 42 985 7347.
E-mail address: rkurokaw@saitama-med.ac.jp (R. Kurokawa).

Abbreviations: HIPK, homeodomain-interacting protein kinase; UV, ultraviolet; MBP, myelin basic protein; GFP, green fluorescent protein; NCBI, national center for biotechnology information; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDM2, mouse double minute 2 homolog; BAX, BCL2-associated X protein

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which included the putative start ATG site and the stop codon were used for cloning of the Hipk4 cDNA. PCR products from NIH/3T3 cDNA and mouse lung cDNA were sequenced using the ABI-377 sequencer using the BigDye Terminator 3.1 Kit (Applied Biosystems, Foster City, CA). For RT-PCR experiments, we used the following oligonucleotide pairs. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification, primer sets were: fw-primer 5′-catcaccatcttccaggagcg-3′ and rv-primer 5′-caccaccttcttgatgtcatc-3′. For human and mouse HIPK4 amplification, primer sets were: fw-primer 5′-accagattgagacagtgaatggtgg-3′ and rv-primer 5′-caytctccaggacagctgctggg-3′ (y indicates c and t mixture).

2.3. Western blotting

Western blotting was performed following standard protocols using nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA). Protein samples of cultured cells were extracted in WCE buffer [25 mM HEPES (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.05% Triton-X100, 10% glycerol] by sonication, followed by centrifugation at $14000 \times g$ for 10 min at 4 °C. Polyclonal anti-HIPK4 antibody was raised in rabbit by injection of purified $6 \times$His-HIPK4 (162–553 aa) antigen. Subcutaneous injection was performed four times using Complete Freund's Adjuvant (DIFCO LABORATORIES, Detroit, MI) at day 0, and using Incomplete Freund's Adjuvant (DIFCO LABORATORIES) at days 10, 20, and 30. Serum was isolated at day 40. This anti-serum was used, at a dilution of 1:1000, in PBS [137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4] containing 0.05% Tween 20 and 2% skimmed milk, overnight at 4 °C. HRP-linked anti-rabbit IgG (Cell Signaling Technology) was incubated, at a dilution of 1:4000, in PBS containing 0.05% Tween 20 and 2% skimmed milk for 1 h at room temperature. For protein detection, we used Supersignal West-Pico System (Pierce, Rockford, IL) and Hyperfilm MP (GE Healthcare, Piscataway, NJ). In vitro translated HIPK4 protein was prepared using TNT quick-coupled transcription/translation systems (Promega, Madison, WI).

2.4. Cell fractionation

Sub-confluent cells, grown on a 15-cm plate, were dounced in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF) until at least 95% of the cells were disrupted. The cytoplasmic fraction was obtained as supernatant after centrifugation at $3300 \times g$ for 15 min. The pellet was solubilized with SDS sample buffer, yielding the nuclear fraction. Both cytoplasmic and nuclear fractions were analyzed by Western blotting.

2.5. Protein expression and purification

Full-length mouse Hipk4 cDNA, containing the FLAG epitope sequence at the amino-terminal, was cloned into pFastBac1 vector (Invitrogen). Protein production was performed according to the Bac-to-Bac system (Invitrogen). Protein was extracted from HIPK4 expressing *Sf9* cells in WCE buffer by sonication, and by centrifugation at $14000 \times g$ for 10 min at 4 °C. The soluble fraction was stored at −80 °C until use.

2.6. In vitro kinase assay

For kinase assays, 100 μl of HIPK4 lysate was mixed with 15 μl of anti-FLAG M2 agarose conjugated antibody (Sigma–Aldrich) for 30 min at 4 °C. The beads were then washed 4 times with kinase reaction buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.001% Tween 20]. Antibody bound proteins were eluted in kinase buffer, containing a final concentration of 0.4 mg/ml FLAG peptide (Sigma–Aldrich), for 10 min on ice, and separated by centrifugation at $1000 \times g$ for 1 min at 4 °C. Enzymatic reactions were composed as follows: 50 ng of HIPK4, 1 μg of substrate protein, 10 μM ATP, 0.5 Ci/mmol [γ-^{32}P]ATP (GE Healthcare), 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.001% Tween 20 in a total volume of 40 μl. Incubation was performed at 30 °C for 20 min. Reactions were terminated by the addition of $4 \times$ Sample buffer: 200 mM Tris–HCl (pH 6.8), 86 mg/ml SDS, 0.2 ml/ml 2-mercaptethanol, 0.28 ml/ml glycerol, 0.1 mg/ml bromophenol blue. These samples were separated by 10% SDS–PAGE (for p53 protein) or 16% SDS–PAGE (for MBP and histones) and were detected by autoradiography. Polypeptides for p53 phosphorylation assays were expressed in *Escherichia coli*, which were transformed with pASK-IBA5-human p53 or with pGEX6p-1-mouse p53 (1–40 aa). Affinity-purification of these samples was performed using Strep-Tactin Sepharose (IBA, Gottingen, Germany) or Glutathione-Agarose (Sigma–Aldrich). Mutant protein of human p53 Ser9Ala (p53-S9A) was constructed using KOD-Plus-mutagenesis Kit (TOYOBO, Osaka, Japan).

2.7. Cellular localization analysis

A Green fluorescent protein (GFP)-HIPK4 expression vector was engineered by the insertion of Hipk4 into vector pAcGFP1-C3 (Clontech, Palo Alto, CA). Transient transfection was performed using FuGene HD reagent (Roche), following the manufacturers instructions. COS-1 cells, into which the GFP-HIPK4 expression plasmid had been introduced, were cultured for 48 h in DMEM medium (Invitrogen) containing 10% fetal bovine serum (invitrogen), and were then fixed with 4% formaldehyde in PBS for 30 min on ice. For the staining of DNA, Hoechest 33258 (Nacalai Tesque, Kyoto, Japan) was added at a final concentration of 1 mM. The IX71 system (Olympus, Tokyo, Japan) was used for fluorescent microscopy analysis.

2.8. Transient transfection and reporter assay

We performed transient transfection assays using the Lipofectamine 2000 reagent (Invitrogen) following the supplier's protocol. Briefly, 3×10^5 A549 cells were seeded on a 12-well plate 24 h before transfection. To each well were added 1.6 μg of DNA and 4 μl of Lipofectamine 2000. Six hundred nanograms of expression vector; either pcDNA3.1 mock vector, pcDNA3.1-FLAG-HIPK4 or pcDNA3.1-FLAG-HIPK4-K40S, was added with 400 ng of pRL-TK vector (Promega) (as an internal control) and 600 ng of luciferase expression vector ($2 \times$ p53-RE-tk-luc, tk-luc, human *BAX*-luc or human *survivin*-luc) (to assess p53 activity). Human *BAX*- and *survivin*-luc vectors were amplified from human genomic DNA using the following PCR primer sets. For human *BAX* promoter (−174/+109 bp) amplification, primer sets were: fw-primer 5′-gacgcgtagctcatgcctgtaatcccagc-3′ and rv-primer 5′-gagatctaatgccttcatttatccaggc-3′. For human *survivin* promoter (−148/+105 bp) amplification, primer sets were: fw-primer 5′-gacgcgttctttgaaagcagtcgagg-3′ and rv-primer 5′-tagatctgccaacgggtcccgcgattc-3′. The amplified DNA fragments were inserted into MluI and BglII sites of pGL3-Basic vector (Promega). The activities of *Firefly* luciferase and *Renilla* luciferase were measured by the Dual Luciferase reporter assay system (Promega). Each data point was assayed in triplicate. Experiments were repeated at least three times.

2.9. Statistical analysis

Data were compared using the paired Student's *t*-test. *P*-values <0.05 were considered significant.

3. Results and discussion

3.1. Cloning of Hipk4 cDNA

Numerous kinds of extra- and intra-cellular stimuli activate protein kinase pathways, and coordinate the phosphorylation status of transcription factors and of nucleosomal proteins. To discover a novel kinase that affects the gene-regulation network, we searched for candidate proteins in the National Center for Biotechnology Information (NCBI) database with the search term Cd00180 (a serine/threonine kinase domain). In the Homologene Database (http://www.ncbi.nlm.nih.gov/HomoloGene), we found an uncharacterized kinase-like protein, deposited as HIPK4. We focused on this protein, because HIPK family members are localized in the nucleus and regulate the transcriptional activities of several transcription factors. Human HIPK4 was originally cloned by the NIH full-length cDNA project, but its expression level in tissues and its kinase activity were not characterized [13]. Because the complete coding sequence of the mouse orthologue, Hipk4, had not been determined, we tried to clone the full-length Hipk4 cDNA using RT-PCR. We successfully amplified full

length Hipk4 from mouse lung and from NIH/3T3 cells. Hipk4 encodes a 616 amino acid protein, including a serine/threonine kinase domain in its amino-terminal region (supplementary Fig. 1A). Hipk4 also has a predicted PEST sequence in the carboxy-terminal region, like other HIPK family members (supplementary Fig. 1B). The PEST sequence, which is a Pro/Glu/Ser/Thr-rich region, flanked by two Lys/Arg residues, is related to protein stability [18]. Fig. 1A illustrates the conservation between kinase domains of HIPK4 and of known mouse HIPK family members. Over 85% of residues within the kinase domain were identical between HIPK1, 2 and 3, whereas the similarity of HIPK4 to HIPK1-3 was slightly lower. About 50% of residues in the HIPK4-kinase domain were identical to those of HIPK1-3. Compared with known proteins, HIPK4 shows the highest similarity to HIPK1, however, it is possible that HIPK4 is a member of an independent protein kinase family because of its moderate homology to HIPK1-3. However, the important amino acids for kinase function were conserved in all HIPK members.

3.2. Tissue and cell line distribution of HIPK4 mRNA and protein

We performed RT-PCR to characterize the distribution of human and mouse HIPK4 mRNAs. We designed the primer pairs to be on different exons to distinguish the size of products from cDNA and genomic DNA. Hipk4 was expressed moderately in mouse lung and white adipose tissue, and was weakly expressed in brain and liver (Fig. 1B). Hipk4 expression was detected in NIH/3T3 and C2C12 cells, which are derived from mouse embryo and from myoblasts, respectively. Human HIPK4 mRNA was detected in A549 (lung cancer), Jurkat (T cell leukemia), PC-3 (prostate cancer) and THP-1 (monocytic leukemia) cells. To confirm the expression of HIPK4 protein, we performed Western blot analysis using rabbit polyclonal anti-HIPK4 antibody. Endogenous HIPK4 of NIH/3T3 cells was detected, corresponding to the size (approximate molecular mass of 80 kDa) of in vitro translated HIPK4 (Fig. 2B). This indicated that the Hipk4 cDNA contained the full-length open reading frame.

A

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                                    K40S
HIPK4  YDIIEVLGKGTFGEVAKGWRRSTGEMVAIKILKNDAYRSRIIKNELKLLRCVRGLDPDEAHVIRFLEFFHDALKF   85
HIPK1  YEVLEFLGRGTFGQVAKCWKRSTKEIVAIKILKNHPSYARQGQIEVSILSRLSSENADEYNFVRSYECFQHKNHT  264
HIPK2  YEVLEFLGRGTFGQVVKCWKRGTNEIVAIKILKNHPSYARQGQIEVSILARLSTESADDYNFVRAYECFQHKNHT  266
HIPK3  YEVLDFLGRGTFGQVVKCWKRGTNEIVAIKILKNHPSYARQGQIEVSILARLSTENADEYNFVRAYECFQHRNHT  271
       *::::.**:****:*.* *:*.* *:*********..  :*  : *:.:*  :   ..*: :.:*  * *:.  :

HIPK4  YLVFELLEQNLFEFQKENNFAPLPARHIRTVTLQVLRALARLKELAIIHADLKPENIMLVDQTRCPFRVKVIDFG  160
HIPK1  CLVFEMLEQNLYDFLKQNKFSPLPLKYIRPILQQVATALMKLKSLGLIHADLKPENIMLVDPVRQPYRVKVIDFG  339
HIPK2  CLVFEMLEQNLYDFLKQNKFSPLPLKYIRPVLQQVATALMKLKSLGLIHADLKPENIMLVDPSRQPYRVKVIDFG  341
HIPK3  CLVFEMLEQNLYDFLKQNKFSPLPLKVIRPVLQQVATALKKLKSLGLIHADLKPENIMLVDPVRQPYRVKVIDFG  346
        ****:*****::* *:*:*:*** : **.:  **  ** :**.*.:*************  * *:********

HIPK4  SASIFSEVRYVKEPYIQSRFYRAPEILLGLPFCEKVDVWSLGCVMAELHLGWPLYPGNNEYDQVRYICETQGLPK  235
HIPK1  SASHVSKA--VCSTYLQSRYYRAPEIILGLPFCEAIDMWSLGCVIAELFLGWPLYPGASEYDQIRYISQTQGLPA  412
HIPK2  SASHVSKA--VCSTYLQSRYYRAPEIILGLPFCEAIDMWSLGCVIAELFLGWPLYPGASEYDQIRYISQTQGLPA  414
HIPK3  SASHVSKT--VCSTYLQSRYYRAPEIILGLPFCEAIDMWSLGCVIAELFLGWPLYPGALEHDQIRYISQTQGLPG  419
       *** .*:.  * ..*:***:******:******* :*:******:***.********  *:**:***.:*****

HIPK4  PHLLHAARKAHHFFKRNPHPDATNP-WQLKSSADYLAETKVRPLERRKYMLKSLDQIETVNGGGAVSRLSFPDRE  309
HIPK1  EYLLSAGTKTTRFFNRD--PNLGYPLWRLKTPEEHELETGIKSKEARKYIFNCLDDMAQVN-----MSTDLEGTD  480
HIPK2  EYLLSAGTKTTRFFNRD--TDSPYPLWRLKTPDDHEAETGTKSKEARKYIFNCLDDMAQGN-----MTTDLEGSD  482
HIPK3  EQLLNVGTKSTRFFCRE--TDMSHSGWRLKTLEEHEAETGMKSKEARKYIFNSLDDIVHVN-----TVMDLEGGD  487
         ** .. *: :** *:  .:   . *:**:  ::  **  :. * ***:::.**::   *        .: . :

HIPK4  ALAEHADLKSMVELIKRMLTWESHERISPSAALRHPFV   347
HIPK1  MLAEKADRREYIDLLKKMLTIDADKRITPLKTLNHQFV   518
HIPK2  MLVEKADRREFIDLLKKMLTIDADKRVTPIETLNHPFV   520
HIPK3  LLAEKADRREFVNLLKKMLLIDADLRITPIETLNHPFV   525
        *.*:** :. ::*:*:**  ::. *::*  :*.* **
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B

mouse normal tissues: marker, brain, liver, lung, kidney, skeltal muscle, heart, white adipose

mouse cell lines: marker, NIH/3T3, C2C12, dH_2O

human cell lines: marker, A549, Jurkat, PC-3, THP-1, dH_2O

HIPK4

GAPDH

Fig. 1. Mouse HIPK4 is a novel homeodomain-interacting protein kinase like protein. (A) Amino acid alignment of serine/threonine kinase domains from mouse HIPK family members. Alignment was calculated by the MAFFT program. GenBank Accession Numbers, used for alignment, were HIPK1 (NP_034562.1), HIPK2 (NP_034563.1) and HIPK3 (NP_034564.1). The symbols (*), (:) and (.) indicate amino acid residues that were identical, very similar or similar, respectively. A kinase-dead mutant protein was engineered by introducing the point-mutation lysine 40 to serine (boxed). Nucleotide sequence of mouse Hipk4 is deposited in GenBank, Accession Number AB354583. (B) Tissue and cell line distribution of Hipk4 mRNA. Results of RT-PCR experiments for HIPK4 and for GAPDH of mouse tissues and of mouse and human cell lines are shown. The number of amplification cycles required to detect HIPK4 and GAPDH were 32 cycles and 20 cycles, respectively.

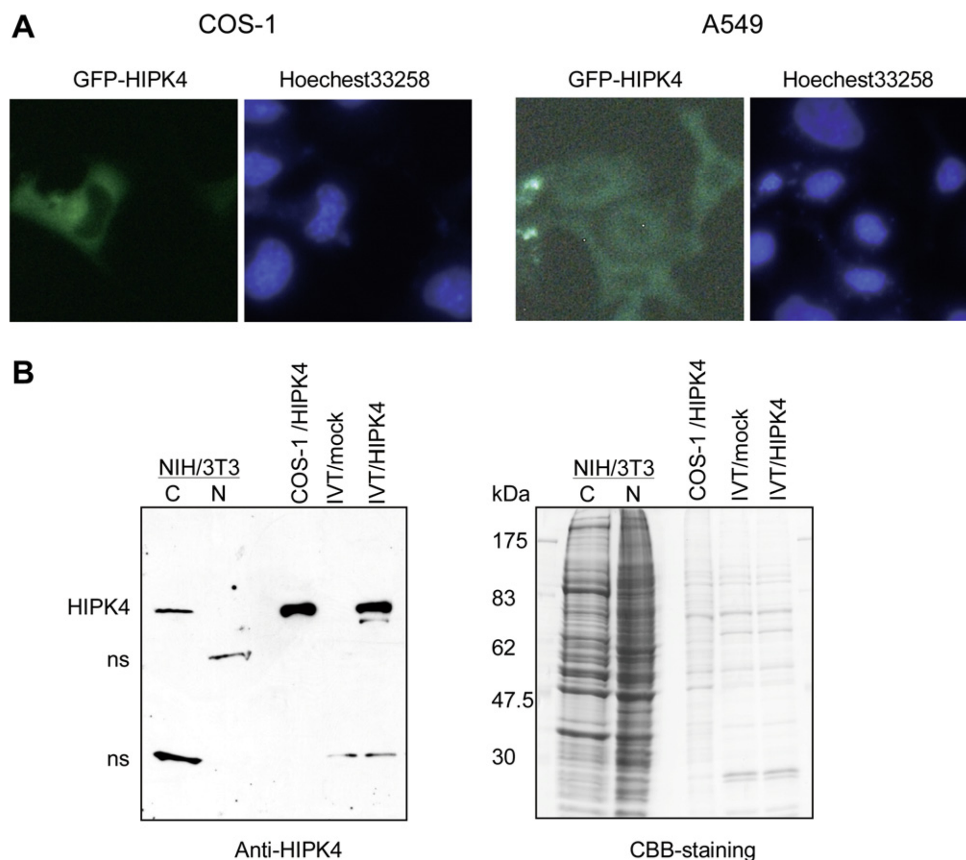

Fig. 2. HIPK4 is mainly localized in the cytoplasm. (A) GFP-HIPK4 expression vectors were introduced into COS-1 cells (left panel) and into A549 cells (right panel) by FuGENE HD reagent. After culture for 48 h, cells were fixed with 4% formaldehyde, and analyzed by fluorescent microscopy. One mM (final) of Hoechst 33258 was used to stain DNA. (B) Detection of endogenous HIPK4 in NIH/3T3 cells. Protein fractions of cytoplasmic (C) and of nuclear (N) extract of NIH/3T3 cells, whole cell extract of mHIPK4-transfected COS-1 cells, and in vitro translated (IVT) mHIPK4 were separated by 10% SDS–PAGE, and transferred onto nitrocellulose membrane, followed by immunoblot analysis using rabbit polyclonal anti-HIPK4 antibody. For the IVT reaction, pcDNA3.1(−) (as negative control) and pcDNA3.1(−)-mHIPK4 were used.

3.3. Subcellular localization of HIPK4

HIPKs are localized in the nucleus, especially in nuclear speckles [3,4,19]. Nuclear speckles are complexes containing protein factors for transcription, splicing factors and factors for the nuclear export of mRNA. We examined the cellular localization of HIPK4, by the transient transfection of a GFP-HIPK4 fusion protein into A549 and COS-1 cells. GFP-HIPK4 signal was observed throughout the cytoplasm and weakly in nucleus (Fig. 2A). This result is reflected in that HIPK4 does not have a typical nuclear localization signal (NLS). As expected, from the localization of GFP-HIPK4, endogenous HIPK4 in NIH/3T3 cells was detected in the cytoplasmic fraction by Western blot analysis (Fig. 2B). Recently, Taira et al. reported that dual-specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) localizes to the nucleus in response to genotoxic stress and phosphorylates human p53 at serine 46 [19]. This raises the possibility that HIPK4 localizes to nucleus in response to certain stimuli.

3.4. Catalytic activity of HIPK4

Members of the HIPK family can phosphorylate several transcription factors and are capable of auto-phosphorylation [4,20]. We performed kinase assays using recombinant mouse HIPK4 expressed in *Sf9* insect cells (HIPK4 produced in *E. coli* had very weak auto-phosphorylation activity (data not shown) and was, therefore, not used). We made two kinds of HIPK4 proteins containing the FLAG-sequence; one was wild-type (wt) and the other carried a point mutation resulting in the substitution of lysine 40 to serine (K40S). These two proteins were produced at similar levels, confirmed by Coomassie-staining and by Western blot analysis (Fig. 3A). Then, these proteins were assayed for kinase activity using MBP, histone H1, H2A, H2B, H3 and H4 as substrates [21,22]. We detected significant phosphorylation activity for MBP and for histone H3, but not for histone H1, H2A, H2B or H4 (Fig. 3B). As with other HIPK family members, auto-phosphorylation activity was observed for HIPK4-wt (Fig. 3B). HIPK4-K40S mutant protein did not exhibit kinase activity, either to itself or to any of the substrate proteins (Fig. 3B). These results suggest that HIPK4 has an intrinsic kinase activity, and that there was no contamination with any other kinase. Post-translational modification of histone H3 influences cellular responses, such as gene regulation and cell division. Several kinases including MSK1/2, Aurora kinase B, DLK/ZIP and HASPIN have phosphorylation activity to Histone H3 and regulate chromosome condensation, metaphase chromosome alignment and cytokinesis [23–27]. Although the amount of HIPK4 in the nucleus was low, it would be interesting to investigate the possibility that H3 is a target of HIPK4 in vivo.

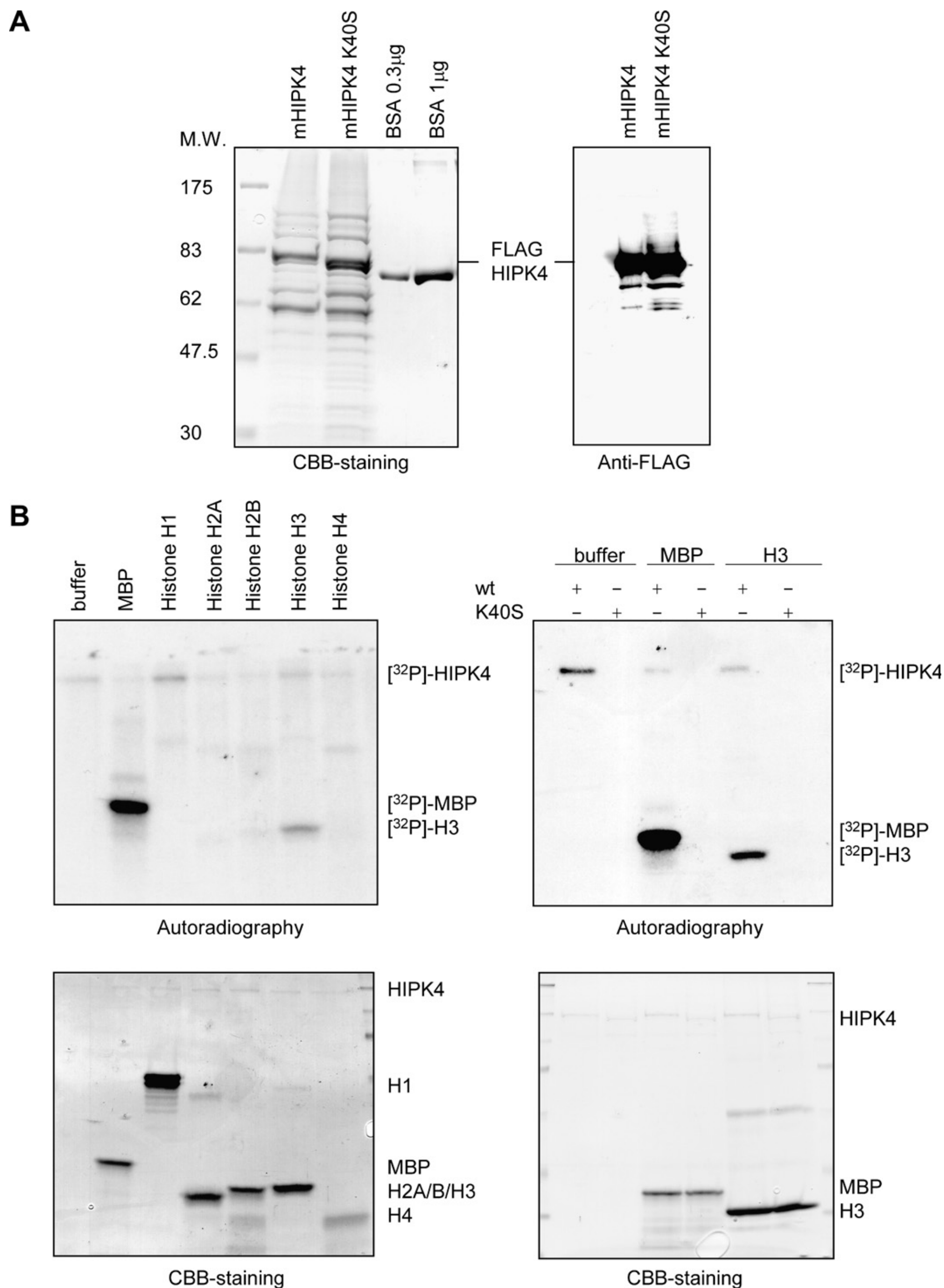

Fig. 3. HIPK4 possesses direct kinase activity towards MBP and Histone H3 in vitro. (A) Purification of recombinant HIPK4 protein and of the mutant, HIPK4-K40S. The affinity-purified samples of recombinant HIPK4 proteins, produced in insect cells, were separated by 10% of SDS–PAGE and stained by Coomassie Brilliant Blue (left panel). Immunoblot analysis using anti-FLAG M2 antibody is shown in the right panel. (B) Phosphorylation of MBP and of histone H3 by HIPK4. The kinase property of HIPK4 was characterized by an in vitro kinase assay using substrate proteins, as indicated. [^{32}P]-labeled proteins were detected by autoradiography (left panel). The kinase properties of wild-type (wt) HIPK4 and of point mutant (K40S) HIPK4 were characterized by an in vitro kinase assay using MBP and histone H3 (right panel).

3.5. HIPK4 phosphorylates p53 in vitro

HIPK2 phosphorylates human p53 at serine 46 and induces apoptotic responsive genes [5]. We, therefore, investigated the possibility of p53 phosphorylation mediated by HIPK4. We detected substantial phosphorylation of human p53 by HIPK4 in an in vitro kinase assay (Fig. 4A). There are a number of reported phosphorylation sites in human p53, such as at serine 6, 9, 15, 20, 33, 37, 46, 392 and threonine 18 [5–8]. We, therefore, further investigated the residues of human p53 targeted by HIPK4 by using anti-p53 antibodies that recognize site-specific phosphorylation. We detected phosphorylation of p53 at serine 9, but not at 6, 15, 20, 37, or 46 (Fig. 4B and supplementary Fig. 2A). We performed further in vitro kinase assays using the mutant protein, human p53 Ser9Ala, to confirm the target site

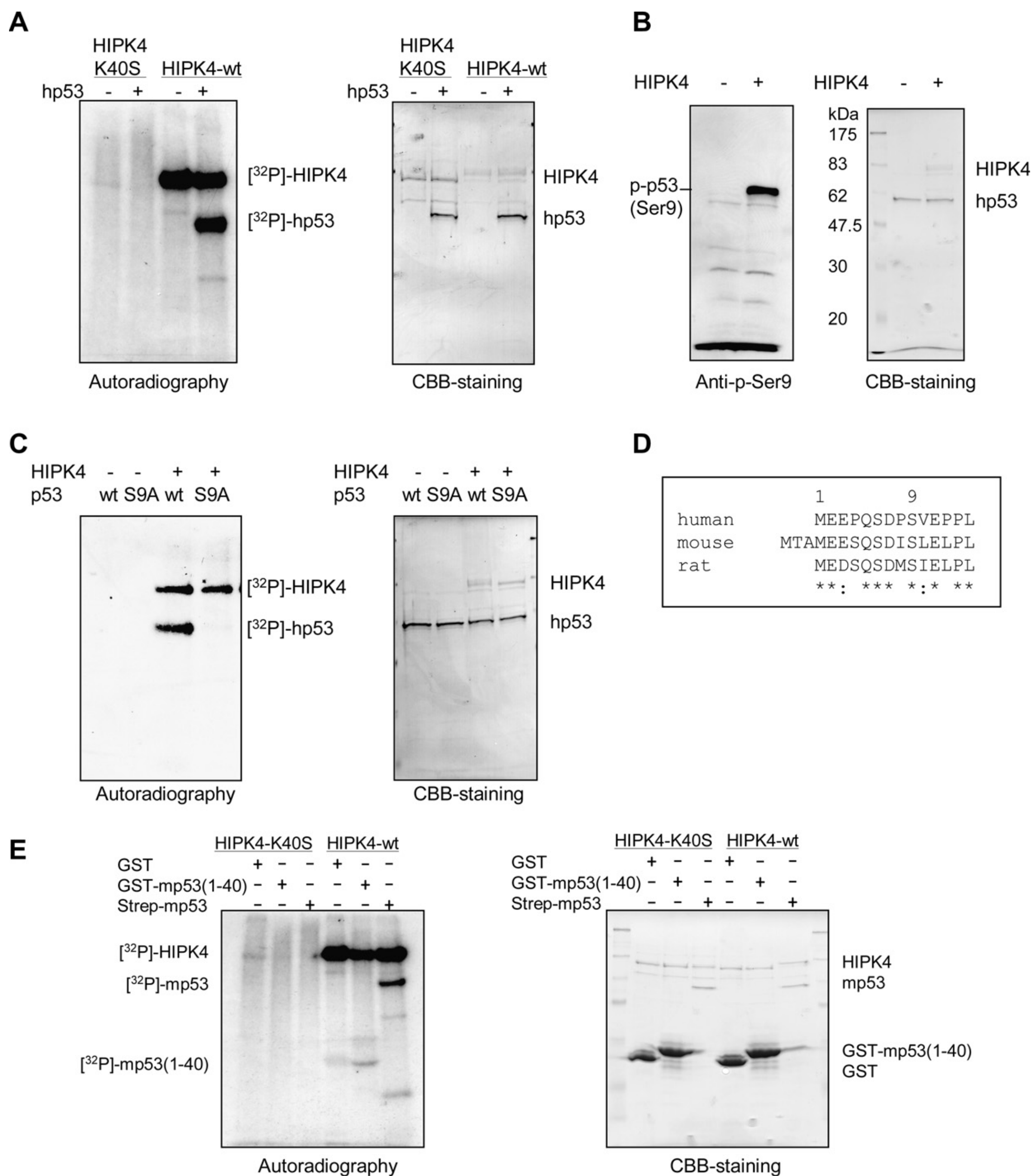

Fig. 4. HIPK4 phosphorylates human p53 at serine 9. (A) Phosphorylation of human p53 by HIPK4 in vitro. Affinity-purified, full-length human p53 was incubated with or without HIPK4-wt or HIPK4-K40S, for 20 min at 30 °C. [^{32}P]-labeled proteins were detected by autoradiography. Right panel shows the loading control of left panel. (B) HIPK4 phosphorylates human p53 at serine 9. Affinity-purified full-length human p53 protein was phosphorylated in vitro with or without HIPK4, and 10% SDS–PAGE was performed. After these samples were transferred onto membrane, immunoblot analysis was performed using rabbit polyclonal anti-phospho-human p53 (Ser9) antibody. (C) Affinity-purified full-length wild-type (wt) human p53 and its point mutant serine 9 to alanine (S9A) were incubated, with or without HIPK4-wt, for 20 min at 30 °C. [^{32}P]-labeled proteins were detected by autoradiography. Right panel shows the loading control of left panel. (D) Alignment of the N-terminal sequences of human, mouse and rat p53 proteins. These sequences were derived from GenBank Accession Numbers: NP_000537.2 (human), NP_035770.1 (mouse) and NP_112251.1 (rat). (E) Phosphorylation of mouse p53 by HIPK4. Affinity-purified GST-mouse p53 (1–40 aa) and full-length mouse p53 protein was incubated with or without HIPK4 for 20 min at 30 °C. [^{32}P]-labeled-proteins were detected by autoradiography.

of HIPK4 and the specificity of the anti-p53 Ser9 antibody (Fig. 4C and supplementary Fig. 2B). The amount of phospho-human p53 (Ser9) was dramatically decreased by the Ser9Ala mutation, therefore, we concluded that HIPK4 preferentially phosphorylates human p53 at serine 9. The sequence of p53 (Ser9) is conserved in mouse, rat and human, but the surrounding sequence is different (Fig. 4D). This finding indicates that this serine residue is important in transcriptional control mediated by HIPK4. We, therefore, performed in vitro kinase assays using full-length mouse p53 and

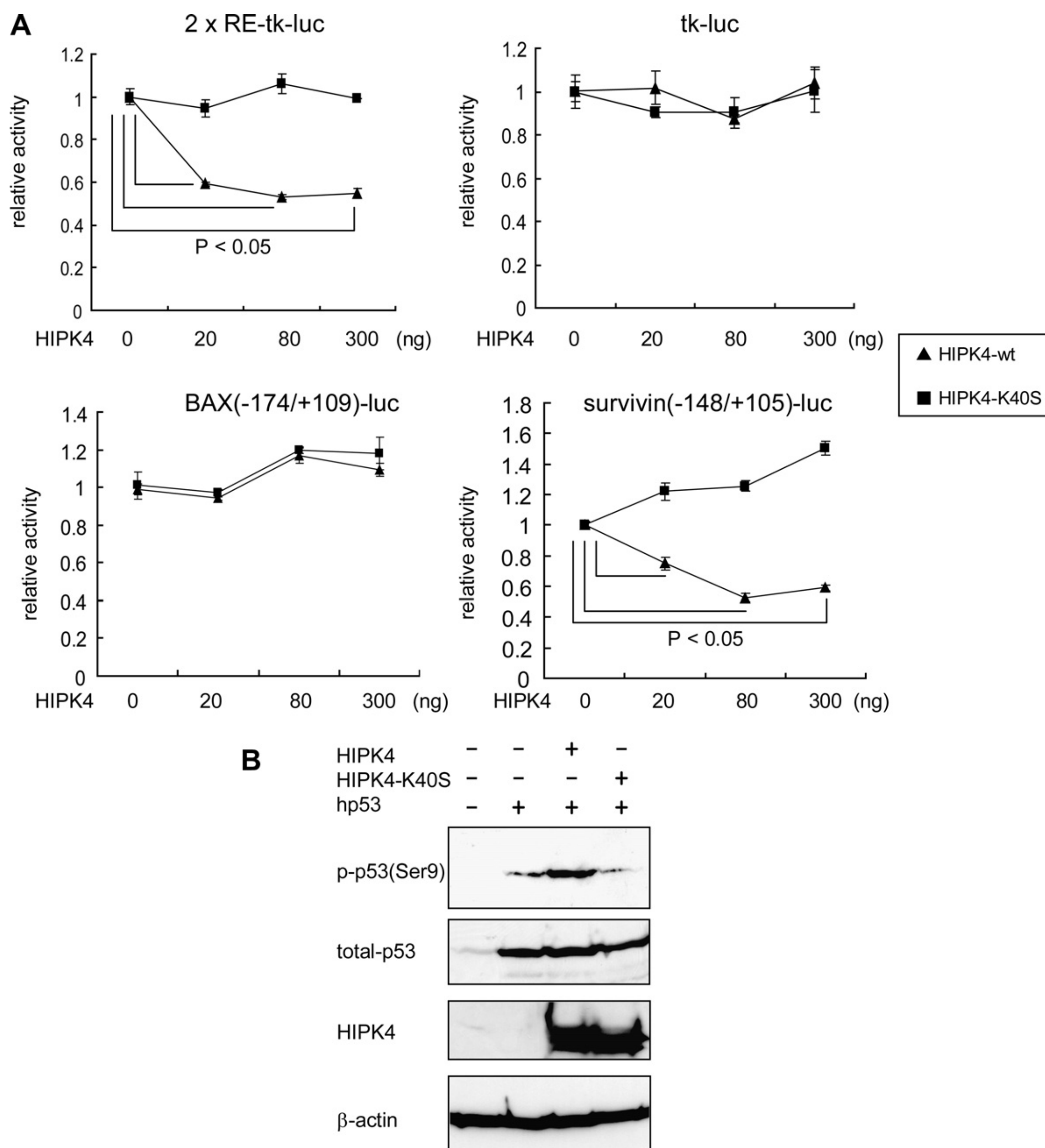

Fig. 5. Over-expression of HIPK4 decreases human *survivin* gene promoter activity. (A) Effect on p53-responsive promoter activity by HIPK4. Expression vector (HIPK4-wt or HIPK4-K40S) and reporter vector, as indicated, were transiently introduced into human, lung cancer derived, A549 cells. *Firefly* luciferase activities, after normalization by *Renilla* luciferase, are shown. Each experiment was repeated at least three times and error bars represent S.D. values of triplicated samples. (B) Phosphorylation status of human p53 in HIPK4 over-expressing cells. Protein samples of COS-1 cells were prepared 24 h after the transfection of a p53 expression vector, with or without, co-transfection of a HIPK4 expression vector. These samples were separated by 10% SDS–PAGE and transferred onto PVDF membrane. Phosphorylation levels of p53 were analyzed using anti-phospho-p53 (Ser9) antibody. Protein levels of p53, HIPK4 and β-actin were assayed using respective antibodies.

GST-mouse p53 (1–40 aa) as substrates (Fig. 4E). Both peptides were phosphorylated by HIPK4, hence, HIPK4 might have the ability to phosphorylate mouse p53 (Ser12) which corresponds to human p53 (Ser9). Casein kinase 1δ (CK1δ) can phosphorylate human p53 at Ser6 and Ser9 in vitro, but prior phosphorylation of Ser6 is necessary for Ser9 phosphorylation [6]. As phosphorylation of human p53 (Ser9) by HIPK4 occurred in the absence of phospho-p53 (Ser6), HIPK4 could be the first p53 (Ser9) principal kinase to be identified, and could be important for the rapid phosphorylation of p53 (Ser9).

3.6. HIPK4 represses survivin gene promoter activity

Properties of p53, such as localization and stability, are affected by phosphorylation. Although phospho-p53 (Ser9) has been observed in several types of cells in response to UV, H_2O_2, adriamycin and TGF-β [6,8,12,28], there is little information about the function of Ser9 phosphorylation. We investigated the effect of HIPK4 over-expression on a synthetic p53 responsive element containing the promoter (2 × RE-tk-luc) and on two kinds of human p53-dependent gene promoter (*BAX*-luc and *survivin*-luc), using a luciferase assay in A549 cells, which express functional p53. We selected the human *BAX*

promoter as a pro-apoptotic, p53-dependent inducible promoter [14,15]. The human *survivin* promoter was investigated as an anti-apoptotic, p53-dependent repressive promoter [16,17]. A549 cells, transfected with HIPK4, showed a remarkable reduction of luciferase activity in 2 × RE-tk-luc and in human *survivin*-luc transfected cells, compared with control cells (Fig. 5A). The kinase mutant, HIPK4-K40S, did not have such an effect on 2 × RE-tk-luc transfected cells. HIPK4-K40S slightly increased the human *survivin* promoter activity. Thus, these repression effects on 2 × RE-tk-luc and human *survivin* promoter were HIPK4 kinase activity dependent. Interestingly, HIPK4 did not affect the activity of the *BAX* promoter. Although further experiments using various gene promoters are necessary, we presume that HIPK4 primarily affects the activity of p53 repressive promoters. The phosphorylation status of endogenous p53 could not be characterized because the sensitivity of the anti-phospho-p53 (Ser9) antibody was too low. Therefore, we introduced human p53 and mouse HIPK4 expression vectors into COS-1 cells, and analyzed the phosphorylation status of exogenous human p53. We detected the phosphorylation of human p53 at serine 9 mediated by HIPK4 in vivo (Fig. 5B). Thus, phospho-p53 (Ser9), produced by HIPK4, increases the repression activity of p53 at p53 repressive promoters.

There are number of reports that document the site-specific phosphorylation of p53, such as at serine 15, 20, and 46, to be involved in the activation of transcription [5,8,10]. Notably, phospho-p53 (Ser46) has been reported to selectively induce apoptotic genes. There is little evidence that p53-phosphorylation correlates with gene repression. Ando et al. reported that polo-like kinase 1 (PLK1) inhibited p53 transcriptonal activity [29]. Recently, Chen et al. reported that this inhibition was due to the activation of cdc25c phosphatase by PLK1, not due to direct phosphorylation of p53 [30]. There are several possibilities, including a direct or an indirect effect of HIPK4, with regard to the inhibition of activity of selective gene promoters by p53. Several factors, such as DNMT1 and Sin3, had been reported as co-repressors of p53 in the human *survivin* promoter system [14,31]. Therefore, we speculate that phospho-p53 (Ser9) enhances the binding affinity of these factor(s). Further analysis is necessary to elucidate the precise mechanism of p53-repression.

We have described here that HIPK4, which is expressed in lung and white adipose tissue, can directly phosphorylate human p53 (Ser9) both in vitro and in vivo. We also demonstrated the possibility that the kinase activity of HIPK4 is important for the repressive function of p53. Additional studies of HIPK4 would provide further understanding of the cellular events that are regulated by p53, such as apoptosis, DNA repair and cell-cycle progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.11.022.

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