

# The MAP3K2-ERK5 pathway upregulates cyclin D1 expression through histone H2A (Thr120) phosphorylation by VRK1

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VRK1 plays a pivotal role in the upregulation of cyclin D1 (also known as CCND1) by phosphorylating histone H2A (Thr120) around its promoter region. However, the pathways or proteins that regulate this activity upstream of VRK1 remain unknown. It has been confirmed that after serum stimulation VRK1 is recruited to the cyclin D1 promoter region, and phosphorylation of the neighboring histone H2A (Thr120) is elevated. To clarify the upstream signals that regulate VRK1, we knocked down mitogen-activated protein kinases (MAPKs), including ERK5, MAP3K2, ERK1/2, JNK1/2, JNK3, and p38 in HeLa cells. We found that ERK5 knockdown decreases cyclin D1 expression, and this dependence on ERK5 was confirmed with the ERK5 inhibitors, BIX02188 and XMD8-92. Knockdown of MAP3K2, which is a well-known kinase acting upstream of the MEK5-ERK5 pathway, also reduced cyclin D1 expression, signifying the importance of the MAP3K2-ERK5 axis in regulating the expression of this gene. Next, evidence from chromatin immunoprecipitation qPCR (ChIP-qPCR) assays indicated that ERK5 or MAP3K2 knockdown reduces the recruitment of VRK1 and phosphorylation of H2A (Thr120) around the cyclin D1 promoter. Moreover, public microarray analysis of HeLa cells treated with either EGF or a DNA-damaging agent showed that ERK5 and cyclin D1 expression levels were significantly correlated in both treatments. Pathway analysis using the Ingenuity database supported our experimental observation that the MAP3K2-ERK5 pathway promotes cyclin D1 expression upstream of the VRK1 phosphorylation of H2A (Thr120). Finally, we showed that H2A (Thr120) phosphorylation is correlated with cyclin D1 expression in clinical tissue analysis. These results suggest that the MAP3K2-ERK5 pathway elevates cyclin D1 expression by recruiting VRK1 and elevating H2A (Thr120) phosphorylation in the cyclin D1 promoter region, which may be involved in dysregulated cell proliferation and cancer progression.

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## Introduction

An accumulation of evidence suggests that VRK1 is involved in tumor development in multiple types of cancers. For example, higher expression of VRK1 is reported in a variety of clinical cancers, including hepatocellular carcinoma, breast cancer, and multiple myeloma [1-4]. In addition, this overexpression is correlated with poor prognosis in these

cancers, indicating that it contributes to tumor progression [5]. Furthermore, the VRK1 expression level is positively correlated with cellular proliferation markers, such as Ki67, also suggesting that VRK1 is involved in the accelerated cell cycle in cancer cells [2,5,6].

VRK1 is activated by mitogenic stimuli, which is followed by histone H2A phosphorylation, cyclin D1 (also known as CCND1) overexpression, and several sequential molecular

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processes related to cell proliferation [7,8]. In addition to its established role of phosphorylating histone H2A at the onset of the G1/S cell cycle phase, VRK1 also phosphorylates BAF1, which controls nuclear envelope formation [9]. Another role of VRK1 is in the response to DNA damage, such as from UV irradiation or treatment with doxorubicin [10,11]. One of the mechanisms underlying VRK1-mediated DNA repair is the specific phosphorylation of p53, resulting in activation of the G1 cell cycle checkpoint [12]. VRK1 is also known to upregulate the mRNA expression of cyclin D1 [7], which functions in DNA repair processes by interacting with RAD51 as well as BRCA2, which is increased in response to DNA damage [13].

Mitogen-activated protein kinases (MAPKs) mediate the transduction of extracellular signaling stimulation in the nucleus [14,15]. These cell signaling proteins are activated by various stimuli, such as serum, growth factors, cytokines, or environmental stress, and the resulting phosphorylation signal is transmitted by a three-step series of kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. The MAPK family is functionally classified into four groups: ERK1/2, JNK, p38, and ERK5. ERK1/2 is mainly involved in the response to Ras and receptor tyrosine kinases, while JNK and p38 are responsible for the responses to stresses such as UV and DNA damage. ERK5 has been reported to be activated via the MAP3K2-MEK5 pathway by various stimuli, such as serum, EGF, NGF, and cytokines. While ERK5 phosphorylates several transcription factors and kinases [16–20], its physiological significance remains unclear. On the other hand, it has been speculated that certain kinases of the MAPK family regulate VRK1 and subsequent nuclear events.

We previously found that VRK1 phosphorylates histone H2A and induces the expression of cyclin D1, suggesting one of the mechanisms of VRK1-mediated oncogenic transformation [7]. In the present study we determined that the MAP3K2–ERK5 pathway elevates cyclin D1 expression, possibly by recruiting VRK1 and elevating H2A (Thr120) phosphorylation in the cyclin D1 promoter region, which might be involved in dysregulated cell proliferation and cancer progression.

## Materials and methods

### Cell lines and reagents

HeLa and HEK293 cells were obtained from the Health Science Research Resources Bank (HSRRB, Japan) and maintained in RPMI-1640 medium (Wako Pure Chemical,

Japan) supplemented with 10% fetal bovine serum (MP Bio, Japan). Both cell lines were authenticated by short tandem repeat analysis. siRNAs were purchased from the following suppliers: VRK1 (cat. no. HSS111308), ERK5 (cat. no. HSS140815), and MAP3K2 (cat. no. VHS40663) siRNAs from Thermo Fisher Scientific (Waltham, MA, USA); cyclin D1 (cat. no. 12962S), ERK1/2 (cat. no. 6560S), JNK1/2 (cat. no. 6232S), and p38 (cat. no. 6564S) siRNAs from Cell Signaling Technology (Danvers, MA, USA); and negative control (cat. no. 4390844) and JNK3 (cat. no. S11161) siRNAs from Ambion (Austin, TX, USA). BIX02188 and XMD8-92 were purchased from Selleck Chemicals (Houston, TX, USA).

### siRNAs and inhibitors treatment

HeLa cells were seeded at 1,000 cells/well into 96-well plates for quantitative PCR, or at 40,000 cells/well into 6-well plates for western blotting. After overnight incubation, the cells were transfected with various siRNAs at 25 nM using Lipofectamine RNAi MAX reagents (Thermo Fisher Scientific) or were treated with various concentrations of BIX2188 and XMD8-92. After 48 hours, the cells were collected for further analysis.

### Mitogenic stimulation

HeLa cells were seeded at 100,000 cells/well into 6-well plates. For serum and EGF stimulation, after overnight incubation the cells were washed twice with FBS-free media and cultured for 24 hours. To the starved cells, 10% serum or 100 ng/mL EGF was added, and the cells were then incubated for 24 or 48 hours. For SN-38 treatment, after overnight culture following seeding, the cells were treated with various concentrations of SN-38 for 8 hours. The cells were harvested and lysed in a buffer containing 250 mM Tris-HCl, 0.5 mM EDTA, 2% lithium dodecyl sulfate, and 10% glycerol and then sonicated to reduce viscosity.

### Quantitative PCR

The cells were then rinsed with ice-cold PBS and lysed using the SuperPrep Cell Lysis & RT Kit for qPCR (TOYOBO, Japan). cDNA synthesis was performed according to the manufacturer's instructions. qPCR was performed using the standard TaqMan method. Briefly, reaction mixtures consisting of cDNA samples, TaqMan probe (*CCND1*, assay ID Hs00765553\_m1; *GAPDH*, assay ID Hs99999905\_m1), and TaqPath qPCR Master Mix (Thermo Fisher Scientific) were

processed and analyzed using a QuantStudio 7 instrument (Thermo Fisher Scientific). Relative cyclin D1 mRNA expression was calculated from the Ct value of each sample in comparison with that of GAPDH as an endogenous control.

### ChIP-qPCR assay

ChIP-qPCR was performed as previously described [7]. Briefly, siRNA-treated cells were fixed with 1% formaldehyde for 10 min and then neutralized with glycine. The cells were washed with PBS and lysed with lysis buffer (50 mM HEPES-KOH, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100). Chromatin was prepared using micrococcal nuclease (Sigma-Aldrich, St. Louis, MO, USA) digestion and brief sonication. Chromatin (20  $\mu$ g), Protein A magnetic beads (New England Biolabs, Ipswich, MA, USA), and anti-hH2A (phospho-Thr120) antibody [7], anti-hVRK1 antibody [7], or normal rabbit IgG (2729S, Cell Signaling Technology) were used for immunoprecipitation. The immune complexes were treated with RNase and eluted with 1% SDS at 65°C for 12 hours. Following proteinase K digestion, gDNA was purified using phenol–chloroform extraction and ethanol precipitation. qPCR was performed using the SYBR green method, and primer sequences for the transcription start site of the cyclin D1 gene were as follows: forward, 5'-CTC TGC CGG GCT TTG ATC TTT GC-3'; reverse, 5'-CTG CGC CGA CAG CCC TCT G-3'. The percent input was calculated from the Ct values of input gDNA and the samples.

### Western blotting

HEK293 cells were seeded into 6-well plates at 200,000 cells/well. After overnight incubation, the cells were transfected with 3  $\mu$ g of a HaloTag–MAP3K2 expression vector (FHC01733, Promega KK, Japan) using ViaFect (Promega, Madison, WI, USA) or 25 nM siRNA using Lipofectamine RNAi MAX reagents and incubated for 48 hours. The cells were lysed with 1x SDS-PAGE sample buffer (Nacalai Tesque, Japan) and sonicated to reduce viscosity.

Equal amounts of sample were run on SDS-PAGE (4–20% Criterion TGX gel, Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes (Trans-Blot Turbo system, Bio-Rad). The membranes were blocked with 10% skim milk/TBS-T and incubated with the following antibodies: anti-cyclin D1 (ab134175, Abcam), anti-ERK5 antibody (ab40809, Abcam), anti-hH2A (phospho-Thr120) antibody [7], anti-HaloTag antibody (G9281, Promega), and anti- $\beta$ -actin antibody (8457L, Cell Signaling Technology). Anti-

rabbit-HRP antibody (7074S, Cell Signaling Technology) together with SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific) were used to visualize specific protein bands.

### Analysis of public microarray data

Expression profiling data from the NCBI Gene Expression Omnibus (GEO) were obtained for HeLa (cervical carcinoma) cells treated with EGF (dataset GSE6783) and for HeLa cells treated with the topoisomerase inhibitor camptothecin (dataset GSE1417). The signal intensities of the probe sets were normalized using the robust multi-array average (RMA) method, and the relative expression levels compared with control were calculated.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were obtained from 5 mesothelioma cases, 9 breast cancer cases, 7 lung cancer cases, 4 pancreatic cancer cases, 7 stomach cancer cases, and 6 colon cancer cases. Tissue sections (4-  $\mu$  m thick) were prepared from formalin-fixed, paraffin-embedded tissues and mounted on slides. After deparaffinization and rehydration through a graded series of ethanol–water solutions, endogenous peroxidase activity was quenched by incubation with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes. The streptavidin–biotin–peroxidase complex technique was then carried out. Sections were incubated overnight at 4°C with anti-hH2A (phospho-Thr120) antibody [7] or anti-cyclin D1 antibody (clone no. ZY-7D3, Zymed). After visualization of the hH2A (phospho-Thr120) with 3, 3'-diaminobenzidine, the sections were counter-stained with hematoxylin and eosin (H&E) and the slides observed by light microscopy. The positively stained cells were counted in high-power fields (x400), and the positive labeling index was scored as the number of positively stained nuclei per total nuclei in each field. Adjacent sections were subjected to H&E staining to confirm histology. The tissue samples were retrieved from the archive of the Department of Pathology at Hiroshima University Graduate School of Biomedical and Health Sciences. The collection of tissue specimens for this study was carried out in accordance with the Ethics Guidelines for Human Genome/Gene Research enacted by the Japanese Government. Ethical approval was obtained from the institutional ethics review committee (Hiroshima University E-974). All experimental procedures were in accordance with the ethical guidelines.

## Statistical analysis

All experiments were independently repeated at least twice. All data were presented as the mean  $\pm$  standard deviation, which was calculated from triplicate measurements at each data point. Data were analyzed by Student's t-test for comparison between two groups and Dunnett's multiple comparison test for multiple comparisons using SAS version 9.2 (SAS Institute Japan, Tokyo, Japan). A p-value of  $< 0.05$  was considered statistically significant.

## Results

### MAP3K2-ERK5 knockdown represses cyclin D1 expression

Although we previously showed that VRK1 enhances cyclin D1 expression [7], the identity of the upstream factors that regulate VRK1 remain elusive. Before searching for these factors, we initially confirmed that serum stimulation increased recruitment of both H2A (phospho-Thr120) and VRK1 to the transcription start site (TSS) of the cyclin D1 promoter and increased cyclin D1 mRNA expression in HeLa cells (Fig. 1A, B), consistent with our previous observations [7]. Since the VRK family of proteins is expected to be involved in the signal transduction of mitogen stimulation, we then attempted to examine the silencing effects of several ERK-family genes by RNA interference to discover these upstream factors. VRK1 knockdown repressed cyclin D1 expression, consistent with our previous report (Fig. 1C, lane 3). ERK5 knockdown completely reduced cyclin D1 expression, unlike other MAPKs, including ERK1/2, JNK1/2, JNK3, and p38 (Fig. 1C, lanes 4–8). It was previously shown that MAP3K2 phosphorylates MEK5, leading to ERK5 activation. Thus, it remained to be determined whether MAP3K2 knockdown also downregulates cyclin D1, and we indeed found this to be the case (Fig. 1C, lanes 11, 12). Cyclin D1 reduction with ERK5 and MAP3K2 siRNAs was confirmed not only at the mRNA level but also at the protein level (Fig. 1D).

### MAP3K2 and ERK5 knockdown reduces recruitment of VRK1 and H2A (Thr120) phosphorylation around the cyclin D1 promoter

To corroborate the regulation of cyclin D1 expression by ERK5, two of its inhibitors, XMD8-92 and BIX02188, were tested for their ability to repress cyclin D1 expression, and it was found that both inhibitors do so in a dose-dependent manner (Fig. 2A, B). These results, together with the above-

mentioned findings that knockdown of ERK5 and its upstream kinase MAP3K2 decrease cyclin D1 expression, led us to hypothesize that the MAP3K2-ERK5 pathway is involved in transcriptional activation of cyclin D1, possibly by modulating VRK1 activity and H2A (Thr120) phosphorylation around its promoter. We next conducted a ChIP assay in the cyclin D1 promoter TSS region using antibodies against phosphorylated H2A (Thr120). Both ERK5 and MAP3K2 knockdown significantly reduced H2A (Thr120) phosphorylation in the promoter region (Fig. 2C). In order to further confirm the relationship between the MAP3K2-ERK5 axis and cyclin D1 expression, we also performed a ChIP assay using an anti-VRK1 antibody. Both ERK5 and MAP3K2 knockdown reduced VRK1 recruitment around the cyclin D1 promoter (Fig. 2C). The results of both of the ChIP assays suggest that the MAP3K2-ERK5 axis regulates cyclin D1 expression by the recruitment of VRK1 for H2A (Thr120) phosphorylation around the cyclin D1 promoter.

### ERK5 expression is correlated with cyclin D1 expression

To further confirm the relationship between ERK5 and cyclin D1, the correlation of mRNA expression between the two genes was evaluated using microarray data in the public domain. Global mRNA gene expression data from the NCBI (dataset GSE6783) contains expression profiling data of HeLa cells treated with EGF as mitogenic stimulation. After EGF treatment, both cyclin D1 and ERK5 (also known as mitogen-activated protein kinase 7, MAPK7) mRNA levels were increased at 24 and 48 hours (Fig. 3A). The expression of MAPK1, however, was not increased at either time point, indicating that transcription of MAPK mRNAs was not induced by the stimulus. The ERK5 pathway is also known to be activated by several types of cellular stress [21,22]. Therefore, we also examined microarray data (dataset GSE1417) for HeLa cells treated with a DNA-damaging agent. Similar to the results for EGF stimulation, camptothecin increased transcription of both ERK5 and cyclin D1 mRNAs in HeLa cells (Fig. 3B). However, no change was observed in MAPK1 mRNA expression. Cyclin D1 expression is known to increase in response to various upstream regulators, and we have shown that one of the mechanisms is by phosphorylation of histone H2A (Thr120) via VRK1. On the other hand, it has been reported that SMAD4 [23] and oxidative phosphorylation in mitochondria [24] are involved in ERK5 expression, but there has been little reported about the regulation of ERK5 expression at the protein level. Therefore, the effect of MAP3K2 and ERK5 on the phosphorylation level of histone H2A was evaluated to confirm the involve-

ment of the MAP3K2-ERK5 pathway in regulating cyclin D1 expression via the VRK1 axis. EGF stimulation and SN-38 (an active metabolite of a camptothecin analog) treatment induced cyclin D1 and ERK5 expression corresponding to the phosphorylation of H2A (Thr120) (Fig. 3C, D). It was reported previously that cyclin D1 plays a role in the DNA-damage response in addition to its role in cell cycle progression [13,25]. Therefore our finding that SN-38 stimulates elevated H2A phosphorylation as well as upregulation of cyclin D1 and ERK5 expression might reflect the DNA-damage response.

In addition, HEK-293 cells were transfected with an expression vector containing MAP3K2 cDNA, and phosphorylation of H2A was measured by western blotting. Compared with empty vector-transfected HEK293 cells, overexpression of MAP3K2 increased histone H2A phosphorylation at Thr120 (Fig. 3E). We next examined the effect of ERK5 knockdown on the phosphorylation of H2A (Thr120). As expected, the H2A phosphorylation level was decreased by ERK5 siRNA as well as by VRK1 siRNA in HEK293 cells (Fig. 3F). These results suggest that MAP3K2 and ERK5 function upstream of, and regulate, H2A phosphorylation in HEK293 cells.

### **Ingenuity pathway analysis supports the association of ERK1 and cyclin D1**

Ingenuity pathway analysis (IPA) is a human-curated database that displays networks of proteins and genes. To test the plausibility of our experimental finding that ERK5 regulates cyclin D1 expression, we assessed the association of ERK5, cyclin D1, MAP3K2, and VRK1 using IPA. The analysis was performed with these input molecules, and potential pathways to connect them were hypothesized. Very short paths were found to connect all the input molecules, as shown in Figure 3G. ERK5 was shown to be involved in phosphorylation of RB1 in response to growth factor stimulation, and VRK1 was identified as one of the RB1-E2F pathway target genes in a previous study [26,27]. These findings suggest that the ERK5 regulation of VRK1 and cyclin D1 expression found in our experiment is indeed plausible.

### **Core histone H2A (Thr120) is strongly phosphorylated in gastrointestinal cancer and is associated with cyclin D1 expression**

To investigate the clinical importance of histone H2A phosphorylation and cyclin D1 expression, we examined 74 miscellaneous primary cancer tissues. Immunostaining of representative cancers and adjacent normal tissues showed

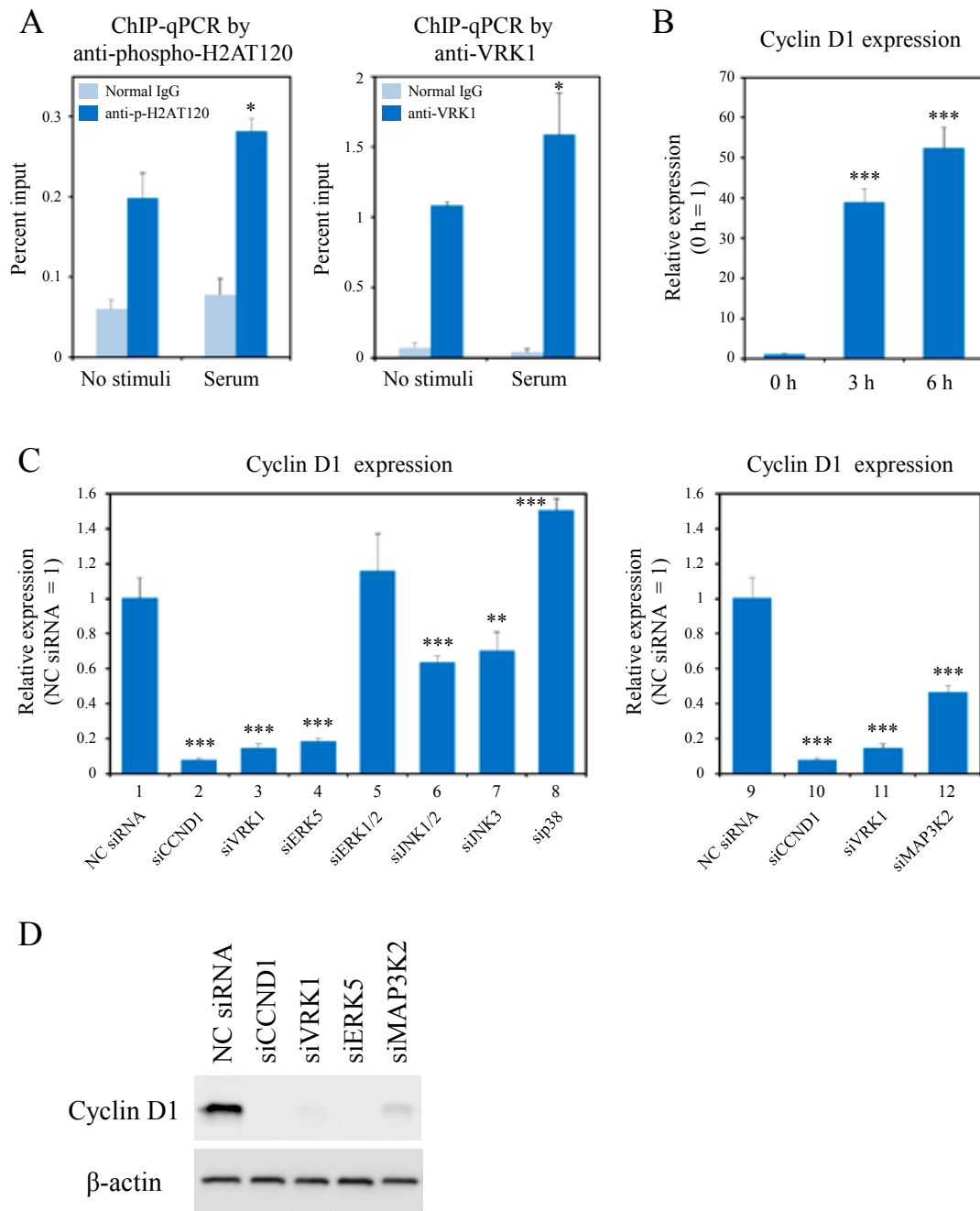
few cells with H2A (phospho-Thr120) in normal colonic tissues (Fig. 4E) or gastric tissue (Fig. 4K, Q). By contrast, there were cells with strong phosphorylation of H2A (Thr120) in colon (Fig. 4B) and gastric cancer tissues (Fig. 4H, N). On the other hand, strong cyclin D1 expression was observed in cells having H2A (phospho-Thr120) (Fig. 4C, I, O). The labeling index (L.I.) of H2A (phospho-Thr120) in cancers from different tissues indicates that phosphorylation is more likely to be present in colon and gastric cancers than in breast cancer ( $p < 0.001$ , Fig. 5A, B).

For the 10 cases of breast cancers, 14 cases of colon cancers, and 18 cases of gastric cancers for which there was both H2A (Thr120) phosphorylation and cyclin D1 immunostaining data, the two data sets were plotted against each other, and Pearson's correlation coefficients calculated (Fig 5C-E). The p-values for breast cancers, colon cancers, and gastric cancers were 0.45, 0.036, and 0.86, respectively. Therefore, while the Pearson's correlation coefficient of  $r=0.56$  between H2A (Thr120) phosphorylation and cyclin D1 expression in colon cancer tissues indicates a significant correlation ( $p = 0.036 < 0.05$ ), it was not significant for the other cancer tissues, suggesting that cyclin D1 overexpression due to phosphorylation of H2A (Thr120) is involved only in colon cancer development. These results lead to the speculation that H2A (Thr120) hyperphosphorylation promotes cell growth by increasing the expression of key genes, including cyclin D1, in colon cancers. Analysis of five cases of mesothelioma was attempted; however, the sample preservation was inadequate for immunohistochemistry, and therefore this data was omitted from the study.

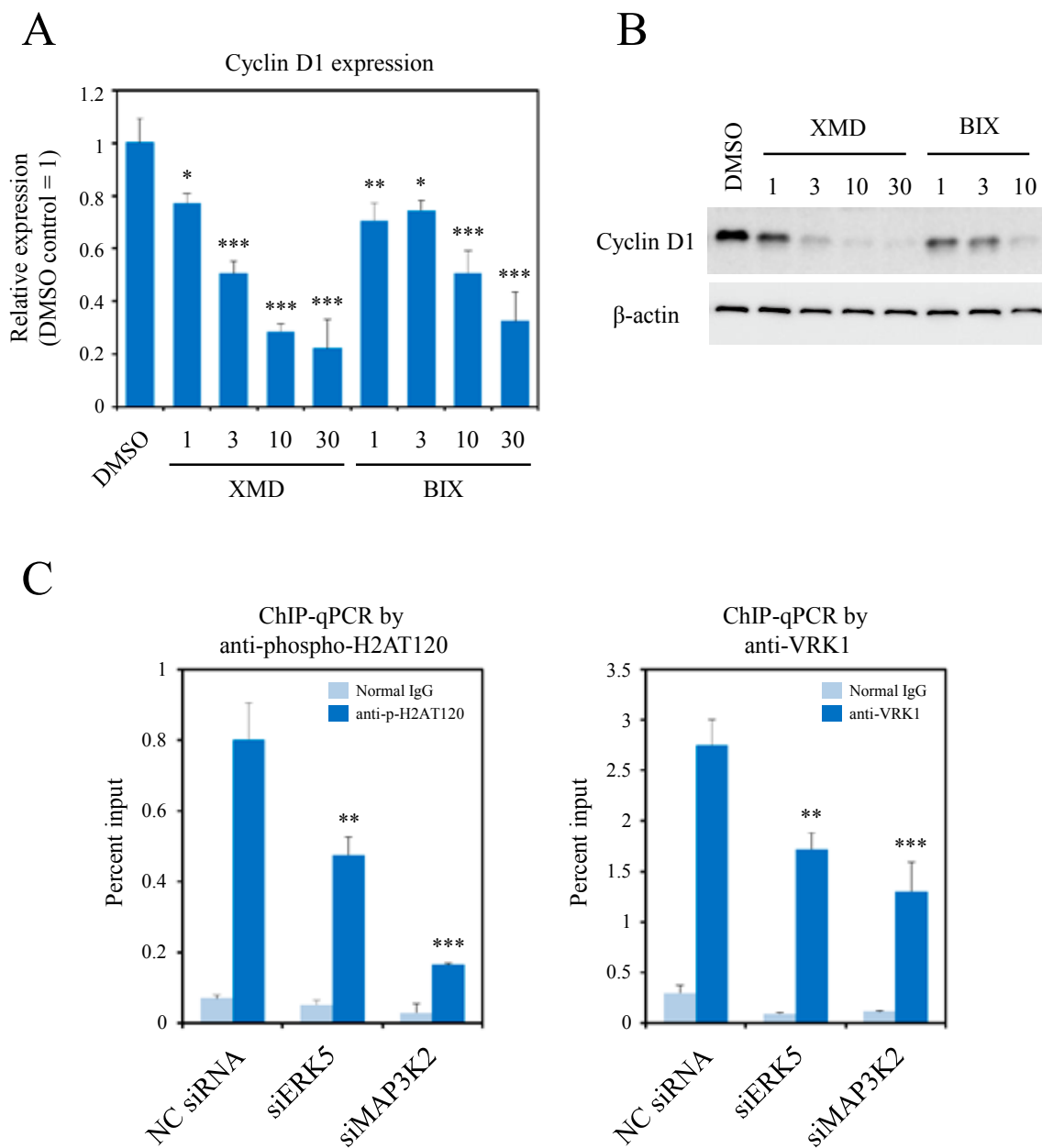
## **Discussion**

### **The expected role of the ERK5–VRK1–cyclin D1 pathway**

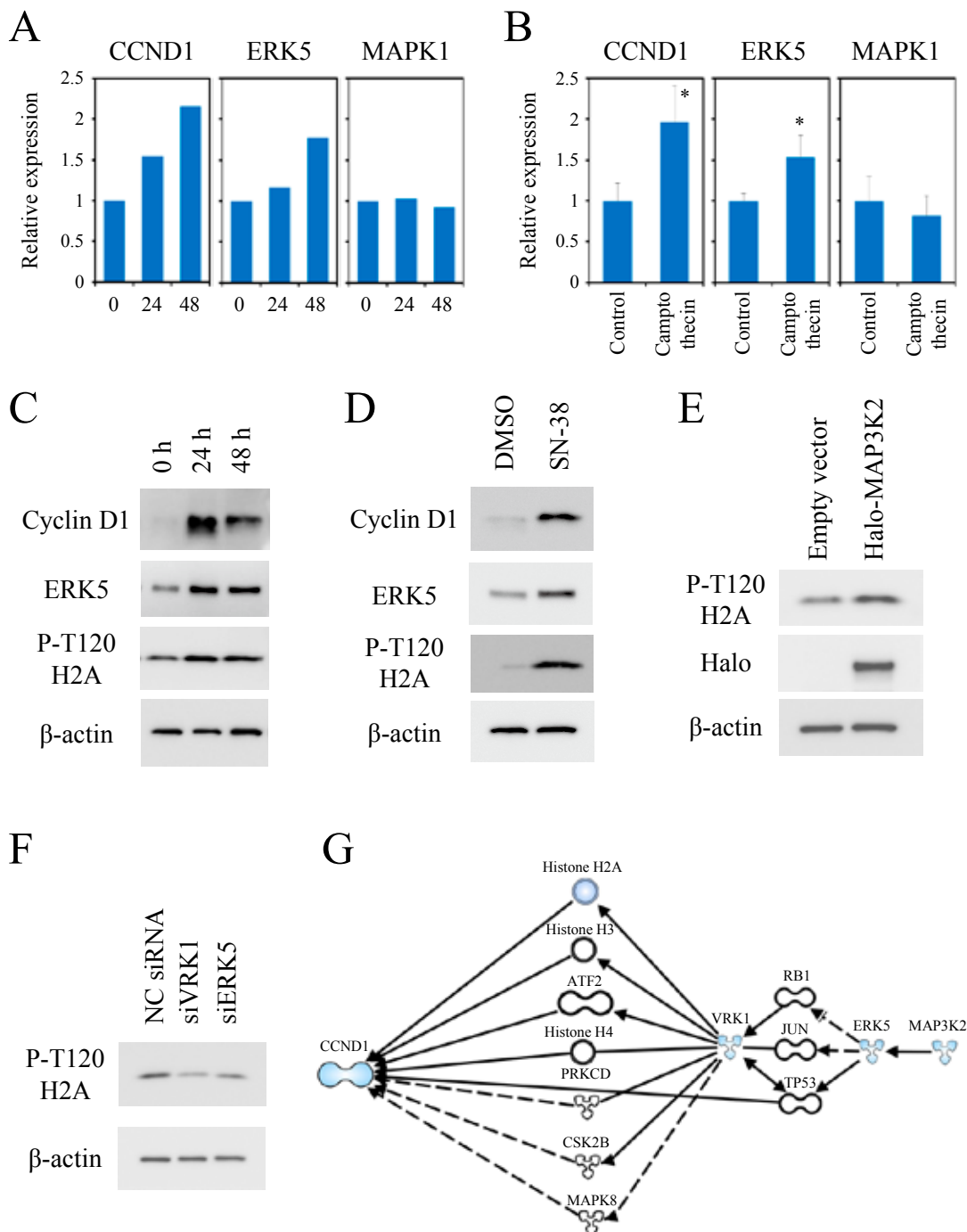
In response to various stimuli, ERK5 is activated via phosphorylation of MAP3K2. These stimuli include growth factors, such as EGF, G-CSF, and NGF, as well as cytokines, including LIF and CT1 [16-18,20,28]. Since most of the stimuli upstream of ERK5 promote cellular proliferation, it is reasonable that the VRK1-mediated increase of cyclin D1 expression is facilitated by ERK5, as found in the present study. Results of MAP3K2 silencing/overexpression also provided independent confirmation of the involvement of VRK1-histone H2A interactions in facilitating cyclin D1 expression. This finding is supported by global gene expression profiling of HeLa cells, which was conducted independently of our study, showing that EGF treatment increased both ERK5 and cyclin D1 expression in a similar way. In



**Figure 1. ERK5 and MAP3K2 knockdown by siRNA reduces the mRNA and protein expression of cyclin D1.** (A) HeLa cells were cultured without serum for 24 hrs and then stimulated with serum. ChIP-qPCR assays for the region around the cyclin D1 promoter in HeLa cells, with or without serum stimulation, were carried out using an anti-H2A (phospho-Thr120) antibody (left), an anti-VRK1 antibody (right), or normal IgG. Bars indicate the mean  $\pm$  SD (n = 3, \* $p$  < 0.05, compared with no stimulus). (B) HeLa cells were treated with serum for 3 hrs or 6 hrs, and the mRNA expression for cyclin D1 was measured by real-time RT-PCR. Bars indicate the mean  $\pm$  SD (n = 3, \*\*\* $p$  < 0.001, compared with 0 hr). (C) HeLa cells were treated with siRNAs targeting the designated genes, and the mRNA for cyclin D1 was measured in each sample by quantitative RT-PCR. Bars indicate the mean  $\pm$  SD (n = 3, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, compared with negative control siRNA). (D) HeLa cells were treated with the indicated siRNAs, and cyclin D1 expression was analyzed by western blotting, with  $\beta$ -actin as loading control.

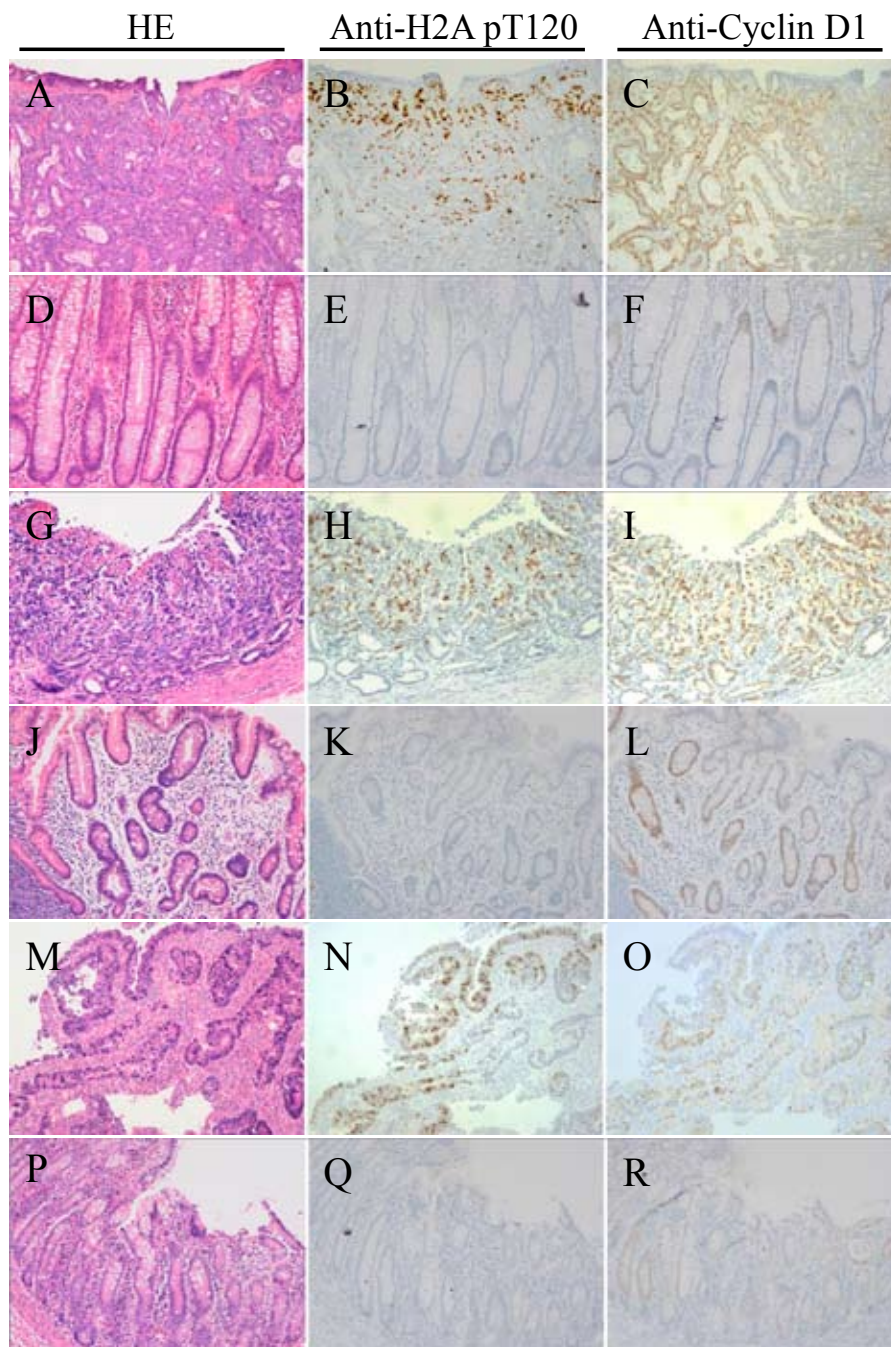


**Figure 2. Both ERK5 and MAP3K2 regulate recruitment of VRK1 and H2A at the chromatin locus of the cyclin D1 promoter.** HeLa cells were treated with increasing concentrations of the ERK5 inhibitors, BIX02188 and XMD8-92, and the expression of cyclin D1 mRNA was measured by RT-PCR (A) and western blotting (B). Bars indicate the mean  $\pm$  SD (n = 3, \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, compared with DMSO control). (C) ChIP-qPCR assays in HeLa cells treated with siRNAs for control, ERK5, or MAP3K2 using anti-H2A (phosphor-Thr120), anti-VRK1 antibodies, or normal IgG. Bars indicate the mean  $\pm$  SD (n = 3, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, compared with negative control siRNA).

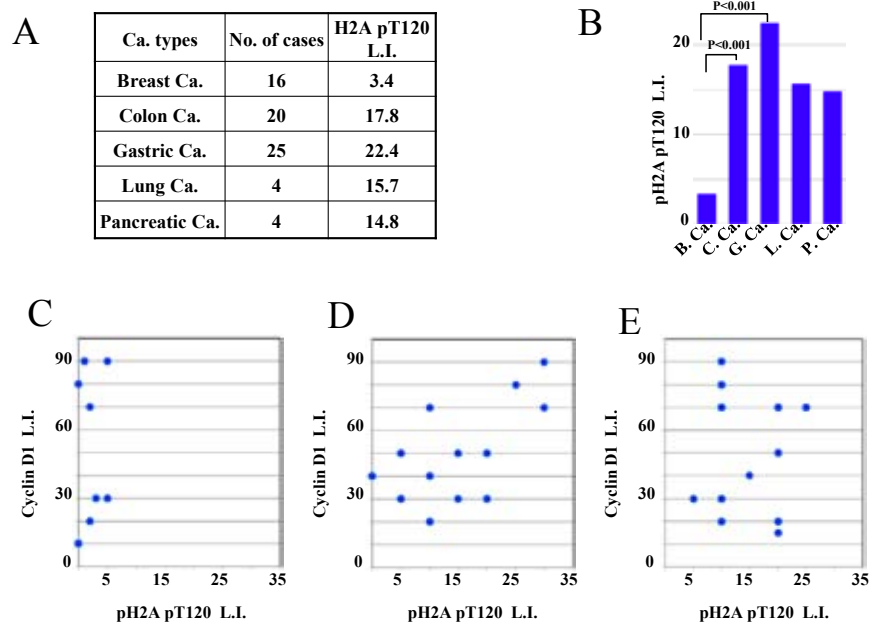


**Figure 3. ERK5 is the upstream regulator of cyclin D1 expression by the VRK1-H2A phosphorylation axis.** (A) Publicly available global expression profiling data for HeLa cells treated with EGF were analyzed for cyclin D1, ERK5, and MAPK1 expression. The expression level was normalized to the untreated control (0 h). (B) The publicly available global expression profile for HeLa cells treated with camptothecin was analyzed for cyclin D1, ERK5, and MAPK1. The expression level was normalized to the untreated control. Bars indicate the mean  $\pm$  SD ( $n = 3-4$ ,  $*p < 0.05$ ). HeLa cells were stimulated with EGF for 0, 24, or 48 hrs (C) or with SN-38 for 8 hrs (D), and the cell lysates were subjected to western blotting. (E) HEK293 cells were transfected with empty vector or a HaloTag–MAP3K2 expression vector. Phospho-H2A and the HaloTag were detected with specific antibodies by western blotting, and  $\beta$ -actin was used as a loading control. (F) HEK293 cells were transfected with VRK1 or ERK5 siRNA. H2A (phospho-Thr120) was detected with a specific antibody by western blotting, and  $\beta$ -actin was used as a loading control. (G) Ingenuity pathway analysis (IPA) was carried out by pathway exploration to examine the validity of the ERK5-VRK1-histone H2A-cyclin D1 relationship.





**Figure 4. H2A (Thr120) hyperphosphorylation and cyclin D1 expression are observed in some primary cancer tissues.** Colonic cancer (A–C) and gastric cancer (G–I, M–O) tissues were analyzed by immunohistochemistry together with their adjacent normal colon (D–F) and normal gastric (J–L, P–R) tissues using anti-H2A (phospho-Thr120) and anti-cyclin D1 antibodies together with hematoxylin and eosin (HE) staining, as indicated.



**Figure 5. H2A (Thr120) hyperphosphorylation is correlated with cyclin D1 expression in colonic cancer tissues.**

(A, B) Summary of staining with an anti-H2A (phospho-Thr120) antibody using 69 cancer tissues, including breast cancer (B.Ca.), colon cancer (C.Ca.), gastric cancer (G.Ca.), lung cancer (L.Ca.), and pancreatic cancer (P.Ca.). The labeling index based on immunostaining of H2A (phospho-Thr120) indicated that both colon and gastric cancers are strongly phosphorylated compared with breast cancers ( $p < 0.001$ , as analyzed by Student's *t*-test). The labeling index is based on the immunostaining of H2A (phospho-Thr120) and cyclin D1 in 10 breast cancer (C), 14 colon cancer (D), and 18 gastric cancer cases (E). A Pearson correlation test shows a correlation ( $r = 0.56$ ,  $p < 0.05$ ) between H2A (phospho-Thr120) and cyclin D1 expression in colon cancer tissues.

addition to biological stimuli, certain stresses, including DNA damage, also activate VRK1, as found in a study of breast cancer cells treated with doxorubicin [11]. Our results indicating ERK5 and cyclin D1 induction in HeLa cells by camptothecin is consistent with this previous report, suggesting that the ERK5-VRK1 interaction exerts its function in response to DNA damage. Again, to our knowledge, this is the first report to show that ERK5 increases cyclin D1 expression by facilitating formation of a chromatin complex with VRK1 in the promoter region of cyclin D1.

Multiple studies have shown that various types of cancers overexpress both ERK5 and VRK [2,5,29-31]. However, their molecular functions and relationship in cancer development are unknown. The ERK5-VRK1-cyclin D1 axis observed in the present research might play an important role in tumor initiation and progression in addition to well-known ERK1/2 signaling pathways. Importantly, several other reports also found that the ERK5 pathway is activated in specific cellular or oncogenic processes, such as the epithelial-to-mesenchyme (EM) transition, in which ERK1/2 is

not involved [21,22]. Examples include the EM transition in bladder cancer caused by the carcinogen benzidine [21]. In another example, it was shown that cyclin D1 expression is regulated by ERK5, but not ERK1/2, in CCL39 cells [22]. These reports, together with our current results, indicate the important role of ERK5, in addition to ERK1/2, in cancer development in a context-dependent manner.

The experiments in our previous report [7] and the current results using HeLa cells were a part of preclinical research analyzing cancer cell lines. Therefore, it is of great importance that the findings be confirmed by assessing clinical samples. The correlation of H2A (phospho-Thr120) and cyclin D1 expression shown by immunohistochemistry using clinical cancer samples (Fig. 4) was important in showing that the VRK1-mediated stimulation of cyclin D1 expression also contributes to tumor progression in a clinical setting. This implies that H2A and the cyclin D1 pathway could be targeted by investigational anti-cancer therapeutic drugs for a subset of cancers with coactivated histone H2A and cyclin D1. In the future, we expect that there will be additional research

examining the ERK5-VRK1-pH2A-cyclin D1 axis in clinical samples.

In general, MAPK proteins exert their function as intracellular signal transduction factors [32,33]. On the other hand, ERK family proteins are also known to be recruited to chromatin [34]. Several lines of evidence have shown that MAPK proteins, including ERK5, bind to chromatin in the promoter region of genes related to cellular proliferation by the unique NLS and TAD domains [35,36]. ERK5 induces transcriptional activation of c-jun by inducing transcriptional activation of MEF2C [37]. Another study showed that ERK5 promotes c-MYC-mediated transcriptional activation [38,39]. These reports are again consistent with our finding that the MAP3K2-ERK5 pathway contributes to the expression of cyclin D1 by modulating the VRK1-H2A (phospho-Thr120) chromatin complex.

In summary, according to the results of our experiments employing siRNA knockdown and overexpression of ERK5 and MAP3K2, we conclude that VRK1, which phosphorylates histone H2A and thereby induces cyclin D1 expression, is regulated by ERK5. The chromatin immunoprecipitation results strongly support our proposal that the ERK5-MAP3K2 pathway is an upstream regulator of VRK1. Since VRK1 and ERK5 activation or overexpression are associated with several cancers, these findings provide further insight into the mechanisms underlying cancer development and clues for developing oncology drugs targeting the ERK5-VRK1 axis.

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## Conflict of interest

The authors declare that they have no conflicts of interest.

## Reference

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