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The published version is available via https://doi.org/10.1016/j.bbrc.2017.01.001

# Type II cGMP-dependent protein kinase negatively regulates fibroblast growth factor signaling by phosphorylating Raf-1 at serine 43 in rat chondrosarcoma cells

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**Abstract:**

Although type II cGMP-dependent protein kinase (PKGII) is a major downstream effector of cGMP in chondrocytes and attenuates the FGF receptor 3/ERK signaling pathway, its direct target proteins have not been fully explored. In the present study, we attempted to identify PKGII-targeted proteins, which are associated with the inhibition of FGF-induced MAPK activation. Although FGF2 stimulation induced the phosphorylation of ERK1/2, MEK1/2, and Raf-1 at Ser-338 in rat chondrosarcoma cells, pretreatment with a cell-permeable cGMP analog strongly inhibited their phosphorylation. On the other hand, Ser-43 of Raf-1 was phosphorylated by cGMP in a dose-dependent manner. Therefore, we examined the direct phosphorylation of Raf-1 by PKGII. Wild-type PKGII phosphorylated Raf-1 at Ser-43 in a cGMP-dependent manner, but a PKGII D412A/R415A mutant, which has a low affinity for cGMP, did not. Finally, we found that a phospho-mimic mutant, Raf-1 S43D, suppressed FGF2-induced MAPK pathway. These results suggest that PKGII counters FGF-induced MEK/ERK activation through the phosphorylation of Raf-1 at Ser-43 in chondrocytes.

Keywords: PKGII; Raf-1; chondrocytes; FGF

**Abbreviations:** PKG, cGMP-dependent protein kinase; MAPK, mitogen activated protein kinase; ERK, extracellular signal regulated kinase; MEK, mitogen-activated protein kinase kinase; PKA, cAMP-dependent protein kinase; RCS, rat chondrosarcoma; FGF, fibroblast growth factor; CNP, C-type natriuretic peptide.

## 1. Introduction

Endochondral bone formation is regulated by many hormones and growth factors. Fibroblast growth factors (FGFs) play a critical role in the regulation of bone growth. FGFs binding to their receptors (FGFR1-4), which belong to the tyrosine kinase receptor family, induce receptor dimerization and autophosphorylation on tyrosine residues, triggering of a signaling cascade involving Ras, Raf, MEK, and ERK [1]. FGFR3 is expressed in proliferating and prehypertrophic chondrocytes in growth plates [2], and missense mutations in human *FGFR3* lead to several skeletal dysplasias, including hypochondroplasia and achondroplasia [3, 4]. In addition, FGFR3$^{-/-}$ mice show an expanded proliferating and hypertrophic chondrocyte zone along with skeletal overgrowth, whereas mice overexpressing an activated form of FGFR3 develop skeletal dwarfism. This indicates that FGFR3 signaling negatively regulates chondrocyte proliferation and differentiation [5-7].

C-natriuretic peptide (CNP) is a peptide hormone that plays an important role in the progression of endochondral ossification. CNP-deficient mice show dwarfism, with a significantly reduced length of endochondral bones [8]. A similar phenotype has been observed in mice deficient for guanylyl cyclase/natriuretic peptide receptor-B (GC-B/NPRB), a specific receptor of CNP [9]. The binding of CNP to GC-B increases the levels of intracellular cGMP, followed by the activation of cGMP-dependent protein kinases (PKGs). Two types of PKGs, cytosolic PKGI and membrane-bound PKGII, have been identified to date. PKGI-deficient mice show vascular, intestinal, and erectile dysfunction [10, 11], whereas PKGII-deficient mice show dwarfism due to impaired endochondral ossification [12-14] These findings indicate that PKGII is essential for CNP/cGMP-mediated endochondral ossification. Importantly, CNP has been shown to reverse the FGFR-3-mediated inhibition of endochondral ossification in achondroplasia [15]. CNP and cGMP markedly reduce the FGF-induced phosphorylation of ERK1/2 [16]. Although a previous report has shown that PKGII promotes chondrocyte hypertrophy and skeletal growth through the phosphorylation and inactivation of glycogen synthase kinase-3β [17], the mechanism underlying the CNP/cGMP/PKGII-mediated inhibition of the FGF-induced MAPK pathway still remains unclear.

In the present study, we identified Raf-1 as a target protein of PKGII, which is associated with the inhibition of FGF-induced MAPK activation in rat chondrosarcoma (RCS) cells. Raf-1, a

serine/threonine kinase, is activated by binding to the GTP-activated form of Ras, followed by the activation of the downstream protein kinases MEK and ERK [18, 19]. Raf-1 activity is tightly regulated by multiple mechanisms, including protein interactions and phosphorylation events. Ser-43 of Raf-1 was phosphorylated in response to cGMP in RCS cells. The forced expression of a phospho-mimic mutant of Raf-1 at Ser-43 (S43D) almost completely blocked the activation of the MAPK pathway by FGF2 in RCS cells. These findings indicate that cGMP/PKGII negatively regulates FGF-induced MAPK activation via the phosphorylation of Raf-1 at Ser-43 in chondrocytes.

## 2. Materials and methods

### *2.1. Cell culture and transfection*

Rat chondrosarcoma (RCS) cells were donated from Dr. Karen B. King (University of Colorado School of Medicine). RCS and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in 5% $CO_2$ [20]. Transfections of HEK293T and RCS cells were performed according to the manufacturer’s instructions using Lipofectamine 2000 and Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific), respectively.

### *2.2. Immunoblot analysis*

HEK293T and RCS cells were transfected with an expression plasmid encoding either the C-terminal FLAG-tagged human PKGII (pcDNA3.1-hPKGII WT-FLAG) or its mutant (pcDNA3.1-hPKGII D412A/R415A-FLAG) [21]. After 24 hours, the cells were placed into a serum-free medium for an additional 16 hours. After treatment with 8-*p*CPT-cGMP (50 μM or 250 μM) (Wako Pure Chemical Industries), 8-bromo-cGMP (50 μM) (Sigma-Aldrich), or forskolin (10 μM or 50 μM) for 30 min, the cells were stimulated with 1 nM FGF2 (PeproTech) for 30 min and were then lysed in an ice-cold lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM EDTA] supplemented with proteases inhibitors (10 μg/ml leupeptin and 10 μg/ml aprotinin) and a phosphatase inhibitor mixture (Nacalai Tesque, Japan). The lysates were centrifuged at 10,000 x g for 10 min, and the supernatants were analyzed by immunoblot analysis using antibodies against

ERK1/2, phospho-ERK1/2, MEK1/2, phospho-MEK1/2, phospho-Raf-1 (Ser-259), phospho-Raf-1 (Ser-338) (Cell Signaling Technology), phospho-Raf-1 (Ser-43) (Abgent), and Raf-1 (BD Transduction Laboratories). Immunoblot band intensities were quantified using the Image J software (NIH).

*2.3. Luciferase reporter assay.*

RCS cells were plated at a density of 2 x $10^4$ cells in a 24-well plate. After 16 hours, the cells were transfected with pFLAG-Raf-1 wild type or pFLAG-Raf-1 S43D together with pFR-Luc, pFA2-Elk1, and pCMV-β-gal in an Opti-MEM I reduced serum medium (Gibco; Thermo Fisher Scientific). Twenty-four hours after transfection, the cells were treated with 50 μM 8-bromo-cGMP for 30 min, followed by stimulation with 1 nM FGF2 for an additional 8 hours. After stimulation, the cells were lysed and subjected to luciferase reporter assay. The luciferase and β-galactosidase assays were performed as previously described [20]. Luciferase activity was normalized to β-galactosidase activity.

*2.4. Statistical analysis*

All experiments were performed multiple times to confirm their reproducibility. One representative data set is shown in the figures. Data were expressed as mean ± standard deviation, and the statistical significance of the difference in the mean values was assessed by two-way analysis of variance (ANOVA) and Tukey's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

## 3. Results

*3.1. cGMP signaling inhibits FGF2-induced ERK1/2 activation in chondrosarcoma cells*

The MEK/ERK signaling pathway activated by FGF has been shown to be attenuated by cGMP signaling in chondrocytes [16]. First, we examined whether a low concentration of FGF2 could induce the phosphorylation of ERK1/2 in RCS cells, which show the characteristics of pre-expansion cartilage cells and express type II collagen, but not type X collagen. Moreover, we have previously shown that RCS cells highly express PKGII [20]. As shown in Fig. 1A, stimulation with 1 nM FGF2

resulted in the high phosphorylation of ERK1/2 between 15 to 30 min, and then, the level of phosphorylation gradually decreased. Therefore, in further studies, we decided to treat RCS cells with 1 nM FGF2 for 30 min.

Next, we investigated the inhibitory effect of cGMP signaling on FGF2-induced ERK activation. RCS cells were pretreated with a cell permeable cGMP analog, 8-*p*CPT-cGMP (50 or 250 μM), followed by stimulation for 30 min with 1nM FGF2. As shown in Fig. 1B, FGF2 enhanced the phosphorylation of ERK1/2 and its upstream kinase, MEK1/2, and the extent of their phosphorylation was completely blocked by pretreatment with 50 μM 8-*p*CPT-cGMP. Furthermore, changes in the phosphorylation state of Raf-1, an upstream activator of the MEK/ERK pathway, were examined. Because Raf-1 is regulated through multiple phosphorylation sites, we performed immunoblotting analysis using site-specific phospho-antibodies against Ser-43, Ser-259, and Ser-338 to determine the phosphorylation level of Raf-1. FGF2 significantly induced Raf-1 phosphorylation at Ser-338, which is required for Raf-1 kinase activation [22], in addition to MEK and ERK phosphorylation, and its induction was suppressed by cGMP treatment in a dose-dependent manner. On the other hand, Ser-43, a negative regulatory phosphorylation site [23], was phosphorylated in response to 8-*p*CPT-cGMP, but was not affected by FGF2 stimulation. Another inhibitory phosphorylation site of Raf-1 (Ser-259) was not phosphorylated under these conditions.

A previous study suggested that high levels of cGMP cross-activate PKA [24]. In addition, PKA is known to phosphorylate Raf-1 at Ser-43 and Ser-259 and negatively regulate the MEK/ERK signaling pathway [25]. To eliminate the possibility that the inhibitory effect of 8-*p*CPT-cGMP on FGF2-induced ERK activation is dependent on PKA activation, we also examined the effect of cAMP signaling on the FGF2-induced activation of the Raf/MEK/ERK pathway in RCS cells, and compared the differences between the two cyclic nucleotide signaling systems (cAMP and cGMP). Forskolin, an adenylyl cyclase activator, was used to activate the cAMP signaling pathway. As shown in Fig. 2, forskolin (at 10 and 50 μM) as well as 8-*p*CPT-cGMP inhibited FGF2-induced phosphorylation of ERK1/2, MEK1/2, and Raf-1 at Ser-338. On the other hand, forskolin stimulated the phosphorylation of Raf-1 at Ser-259 in addition to Ser-43 in a dose-dependent manner. These results suggest that 8-*p*CPT-cGMP at concentration used in our experiments, does not activate PKA, and that treatment

with 8-*p*CPT-cGMP stimulates the phosphorylation of Raf-1 at Ser-43 through the activation of PKG.

*3.2. PKGII phosphorylates Raf-1 at Ser-43 in a cGMP-dependent manner.*

To examine the phosphorylation of Raf-1 at Ser-43 by PKGII, PKGII WT-FLAG and FLAG-Raf-1 were ectopically expressed in HEK293T cells owing to the poor expression of PKGII, after which the cells were treated with a cell-permeable cGMP analog. Immunoblot analysis using the anti-phospho-Raf-1 (Ser-43) antibody showed that wild-type PKGII phosphorylated Raf-1 at Ser-43 in a cGMP-dependent manner (Fig. 3A and B). On the other hand, the Raf-1 S43A mutant, in which Ser-43 is mutated to Ala, failed to be phosphorylated by PKGII (Fig. 3A). Furthermore, the phosphorylation of Raf-1 at Ser-43 was examined using the PKGII D412A/R415A mutant, which shows a low affinity for cGMP [21]. As shown in Fig. 3B, PKGII D412A/R415A failed to phosphorylate Raf-1 even in the presence of 50 μM 8-bromo-cGMP, indicating that PKGII directly phosphorylates Raf-1 at Ser-43.

*3.3. Phosphorylation of Raf-1 at Ser-43 suppresses FGF2-induced MAPK activation.*

Finally, we examined whether Raf-1 phosphorylation at Ser-43 affects FGF-induced MAPK activation in RCS cells. The phospho-mimic mutant Raf-1 S43D (in which Ser-43 is replaced by Asp) was generated. In RCS cells expressing wild-type Raf-1, FGF2 stimulation induced ERK1/2 activation, and pretreatment with 8-bromo-cGMP almost completely blocked it (Fig. 4A). On the other hand, in cells expressing Raf-1 S43D, FGF2 failed to increase the phosphorylation of ERK1/2. Furthermore, we assessed the effect of the phosphorylation of Raf-1 at Ser-43 on MAPK activation using the GAL4/Elk-1 reporter system, in which Elk-1 is fused to the DNA-binding domain of GAL4, in combination with a 5x upstream activating sequence-luciferase reporter. FGF2 stimulated an approximately 4-fold increase in the Elk-1-dependent luciferase activity in cells expressing wild-type Raf-1, and its increase was suppressed by cGMP (Fig. 4B). However, Elk-1-dependent luciferase activity was not increased by FGF2 in Raf-1 S43D-transfected cells. These results suggest that the phosphorylation of Raf-1 at Ser-43, but not at Ser-259, is sufficient to suppress FGF2-induced MAPK activation in chondrocytes.

## 4. Discussion

Although the importance of PKGII has been recognized in CNP-mediated endochondral ossification [12-14], little is known about the detailed molecular mechanisms of PKGII. A previous study identified GSK-3β as a target protein of PKGII, which is associated with enhanced hypertrophic differentiation [17]. PKGII directly phosphorylates GSK-3β at Ser-9, which is known to inactivate GSK-3β kinase activity. The phosphorylation of GSK-3β is necessary for inducing chondrocyte hypertrophy by PKGII. On the other hand, CNP signaling has been shown to counter the abnormal FGFR3 signaling in endochondral ossification [15]. CNP and cGMP markedly reduce the FGF-2-induced phosphorylation of ERK1/2 in RCS cells, suggesting that activated PKG leads to the suppression of the MAPK pathway [16]. In the present study, we identified Raf-1 as a target protein of PKGII, which is responsible for MAPK inactivation in RCS cells. FGF2 stimulation induced the phosphorylation of Raf-1 at Ser-338, which is required for Raf-1 activation and is used as its indicator. Pretreatment with the cell-permeable cGMP analog and forskolin inhibited Ser-338 phosphorylation in a dose-dependent manner. In addition, we found that 8-*p*CPT-cGMP treatment increases the phosphorylation of Raf-1 at Ser-43, but not at Ser-259, whereas forskolin triggered phosphorylation at both Ser-43 and Ser-259. Differences in Raf-1 phosphorylation patterns between 8-*p*CPT-cGMP and forskolin suggest that 8-*p*CPT-cGMP specifically activates PKG in RCS cells, but not PKA. We also showed that exogenously expressed PKGII phosphorylates Raf-1 at Ser-43 in a cGMP-dependent manner. The consensus phosphorylation sequences for PKA and PKG are R/K-$X_{1\text{-}2}$-S/T and R-R/K-X-S/T (X represents any amino acid), respectively [26]. Although Ser-43 (RRA$^{43}$S) is located within the PKA/PKG consensus phosphorylation site, the sequence around Ser-259 (RST$^{259}$S) matches only the PKA consensus sequence. Taken together, we conclude that cGMP stimulates the specific phosphorylation of Raf-1 at Ser-43 through PKGII activation.

A previous report showed that PKA phosphorylates Raf-1 at Ser-43, Ser-259, and Ser-621 [27]. The phosphorylation of these three serine residues negatively regulates the kinase activation of Raf-1. Phosphorylated Ser-259 and Ser-621 serve as binding sites for 14-3-3 proteins, which recognize and bind to phosphorylated serine/threonine residues of the target proteins at specific positions [28, 29].

The bivalent binding of 14-3-3 to Raf-1 prevents Raf-1 recruitment to the plasma membrane and suppresses its binding with Ras, resulting in the inhibition of the MAPK pathway. In addition, phosphorylation at Ser-43 by PKA has been shown to diminish the affinity for Ras and to suppress Ras-mediated activation [23]. However, it has also been reported that the mutation of Ser-43 to alanine does not overcome the effects of cAMP *in vivo* [30]. Therefore, we showed that the phospho-mimic mutant of Raf-1 at Ser-43, Raf-1 S43D, diminished FGF2-induced ERK1/2 phosphorylation and Elk-1 transcriptional activity in RCS cells. These results indicate that phosphorylation at Ser-43, but not at Ser-259, may be sufficient to suppress the MAPK pathway. Other studies have previously reported that PKGII inhibits the EGF- or FGF-triggered MAPK pathway in gastric cancer cells and glioma cells, but direct target proteins for PKGII have not yet been identified [31, 32]. In these cells as well as in chondrocytes, Raf-1 may be a target protein for PKGII, and Ser-43 phosphorylation may be involved in MAPK inactivation.

In conclusion, we revealed that PKGII inhibits FGF-induced MAPK activation via the phosphorylation of Raf-1 at Ser-43. Mutations in *FGFR3* lead to abnormalities in chondrocyte proliferation and terminal differentiation, resulting in skeletal dysplasias, such as hypochondroplasia and achondroplasia. Our findings will provide new insights for the therapy of these diseases.

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**Figure legends**

**Fig. 1.** cGMP signaling inhibits FGF2-induced MEK/ERK activation in RCS cells. (A) After changing to serum-free medium, RCS cells were stimulated with 1 nM FGF2. At the indicated times, the cells were lysed and analyzed by immunoblotting using anti-phospho-ERK1/2 and total ERK1/2 antibodies. Band intensities were quantified using the Image J software. The levels of phosphorylated ERK1/2 were normalized to total ERK1/2 levels. The results are expressed as means ± S.E. of three separate experiments. (B) RCS cells cultured in serum-free medium were treated with 50 or 250 μM 8-*p*CPT-cGMP for 30 min, followed by 1 nM FGF2 for 30 min. The cell lysates were subjected to immunoblotting analysis using the indicated antibodies. The levels of phosphorylation of Raf-1 at Ser-43, Ser-259 and Ser-338 were quantitated. The levels of phosphorylated form were normalized to the levels of total proteins. The results are expressed as means ± S.E. of three separate experiments. *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

**Fig. 2.** Forskolin inhibits FGF-induced MAPK activation in RCS cells. Serum-starved cells were pretreated with forskolin for 30 min, followed by stimulation with FGF2 for 30 min. The cells were lysed and analyzed by immunoblotting with antibodies against phospho-ERK1/2, phospho-MEK1/2, or phospho-Raf-1. The levels of phosphorylation of Raf-1 at Ser-43, Ser-259 and Ser-338 were quantitated. Phospho-specific signals were normalized to the total amount of Raf-1. The results are expressed as means ± S.E. of three separate experiments. ** $p < 0.01$ and *** $p < 0.001$.

**Fig. 3.** PKGII phosphorylates Raf-1 at Ser-43. (A) HEK293T cells were transiently cotransfected with pcDNA3.1-PKGII WT-FLAG together with pFLAG-Raf-1 wild type or pFLAG-Raf-1 S43A mutant. After changing to serum-free medium, the cells were treated with 100 μM 8-*p*CPT-cGMP for 30 min. The cell lysates were subjected to immunoblotting analysis using anti-phospho-Raf-1 (Ser-43) or anti-FLAG antibodies. (B) HEK293T cells cotransfected with pFLAG-Raf-1 wild type together with pcDNA3.1-PKGII WT-FLAG or pcDNA3.1-PKGII D412A/R415A-FLAG were treated with 50 μM 8-bromo-cGMP for 30 min. The cell lysates were subjected to immunoblotting analysis using anti-phospho-Raf-1 (Ser-43) or anti-FLAG antibodies. The levels of phosphorylated Raf-1 (Ser-43)

were normalized to the levels of total FLAG-Raf-1 proteins. The results are expressed as means ± S.E. of three separate experiments. *** $p < 0.001$.

**Fig. 4.** Phosphorylation of Raf-1 at Ser-43 suppresses FGF2-induced MAPK activation. (A) RCS cells were transiently transfected with either pFLAG-Raf-1 WT or pFLAG-Raf-1 S43D in Opti-MEM medium. After pretreatment with 50 μM 8-bromo-cGMP, the cells were stimulated with 1 nM FGF2 for 30 min. The cell lysates were examined by immunoblotting with anti-phospho-ERK1/2 and total ERK1/2 antibodies. The levels of phosphorylated form were normalized to the levels of total proteins. The results are expressed as means ± S.E. of three separate experiments. *** $p < 0.001$. (B) RCS cells were transfected with either pFLAG-Raf-1 WT or pFLAG-Raf-1 S43D together with a GAL4-responsive luciferase reporter plasmid, a GAL4-Elk-1 expression plasmid, and pCMV-β-gal in Opti-MEM medium. After 24 hours, the cells were pretreated with 8-bromo-cGMP (50 μM), followed by stimulation with FGF2 (1 nM) for another 8 hours. Luciferase and β-galactosidase activities were measured, and the luciferase activity was normalized to the β-galactosidase activity. The results are presented as a mean of the luciferase activity ± S.E. of at least three independent experiments.

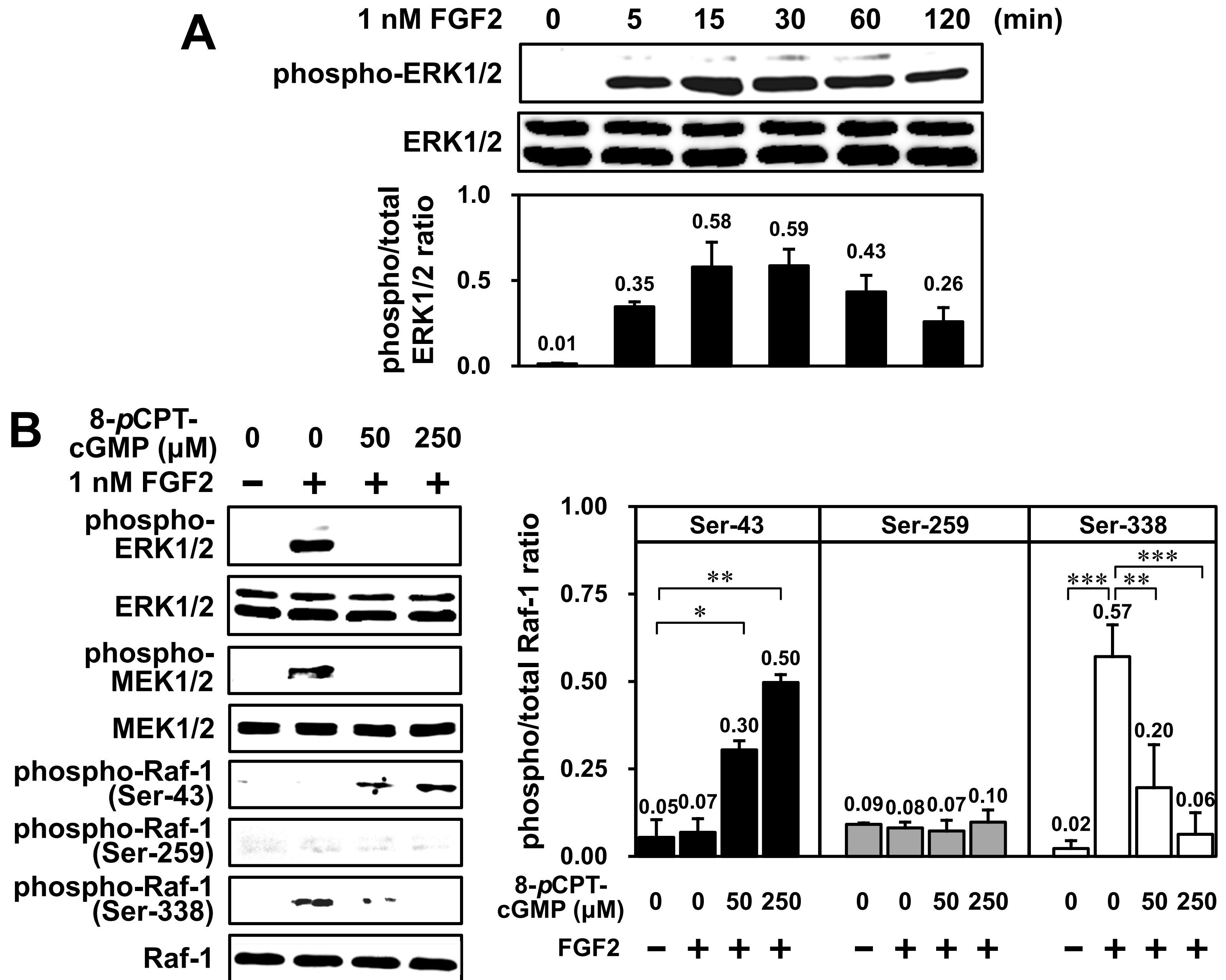
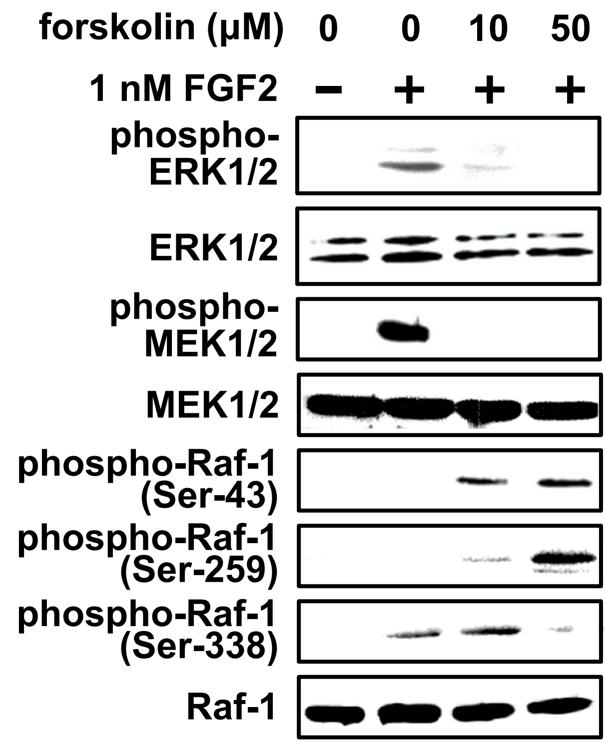


Fig.1. Kamemura et al. (2016)



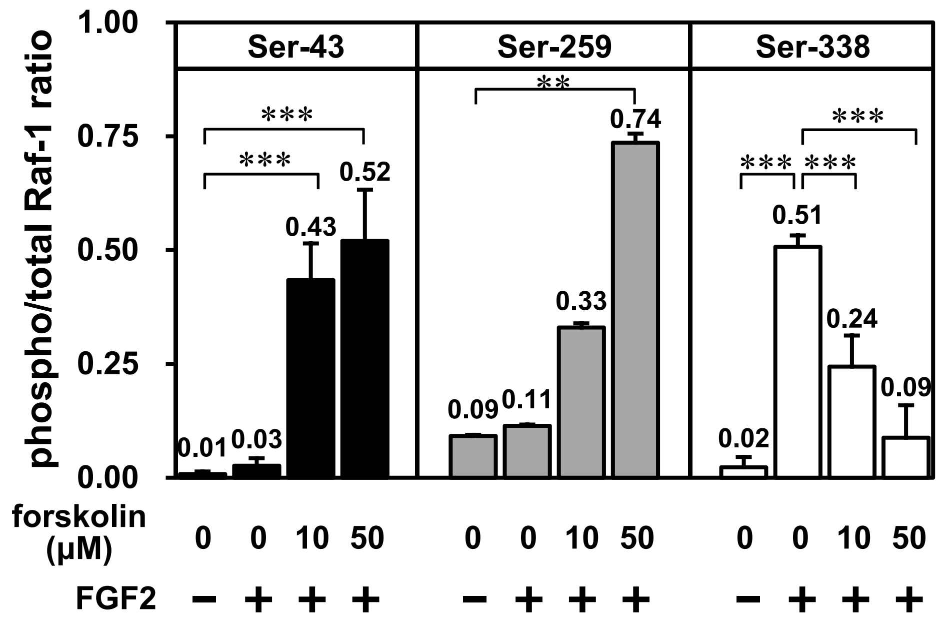
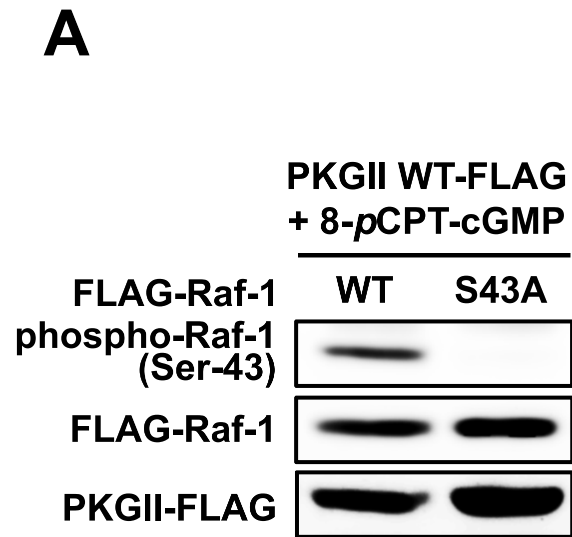




Fig.2. Kamemura et al. (2016)



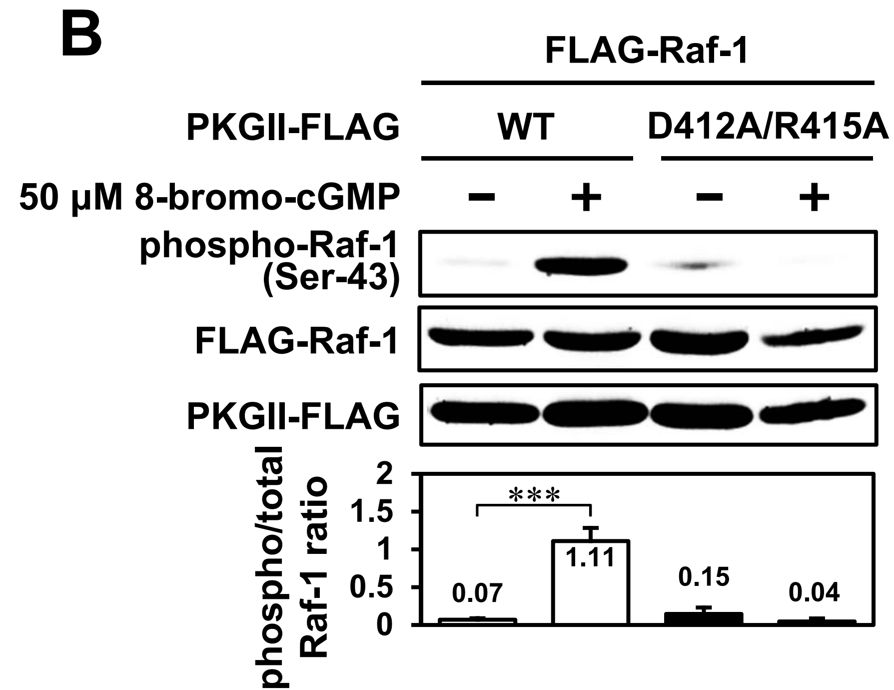
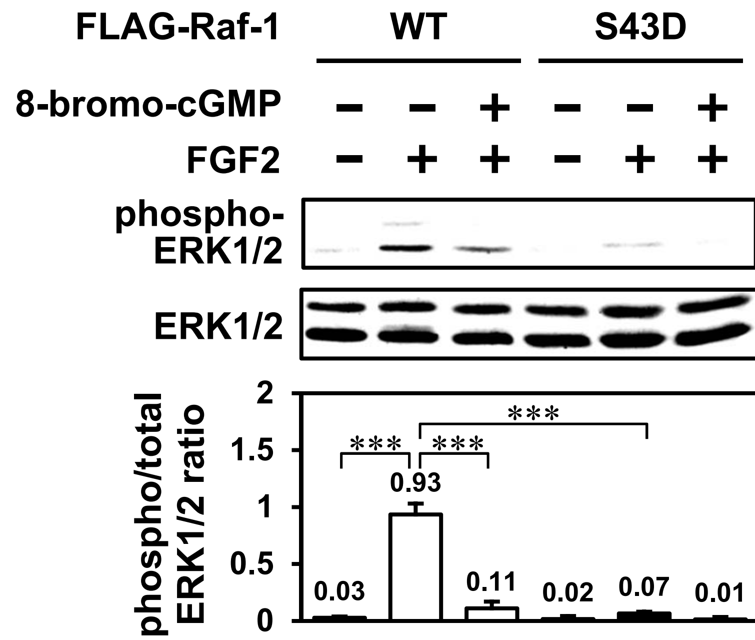




Fig.3. Kamemura et al. (2016)



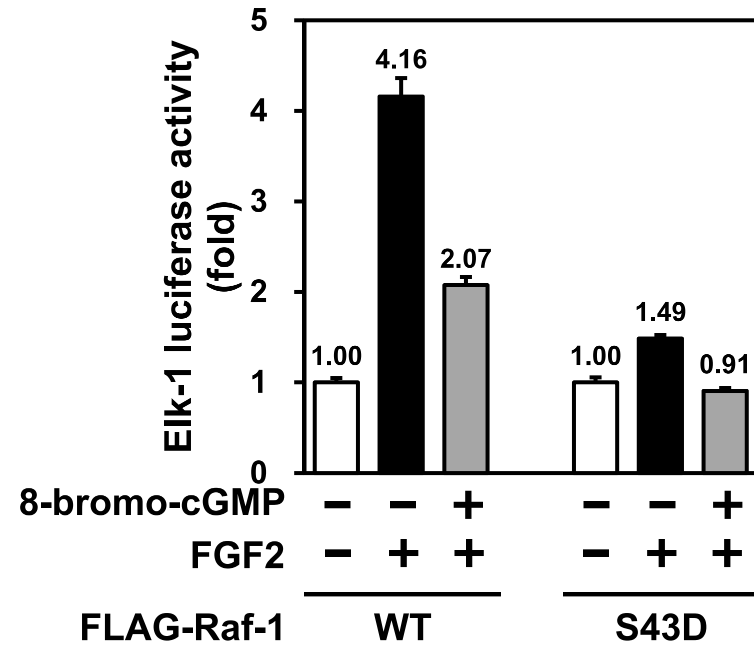




Fig.4. Kamemura et al. (2016)