

The signaling pathway leading to ERK5 activation via G-proteins and ERK5-dependent neurotrophic effects

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Extracellular signal-regulated kinase (ERK); mitogen-activated protein kinase (MAPK); epidermal growth factor (EGF); nerve growth factor (NGF); G-protein-coupled receptor (GPCR); protein kinase A (PKA); brain-derived neurotrophic factor (BDNF); lysophosphatidic acid (LPA); A-kinase anchoring protein (AKAP); GTPase-activating protein (GAP); protein kinase C (PKC); dorsal root ganglia (DRG); cAMP response element-binding protein (CREB); p90 ribosomal S6 kinase (RSK); myocyte enhancer factor (MEF); fibroblasts growth factor (FGF).

Abstract

Extracellular signal-regulated kinases (ERKs) or mitogen-activated protein kinases (MAPKs) are involved in cellular proliferation, differentiation, migration and gene expression. The MAPK family includes ERK1/2, c-Jun N-terminus kinase 1/2/3, p38MAPK $\epsilon/\epsilon/\epsilon/\epsilon$ and ERK5 as classical MAPKs, and ERK3, ERK4 NLK and ERK7 as atypical MAPKs. Like other MAPKs, ERK5 is activated by variety of stimuli including growth factors, G-protein-coupled receptor (GPCR) agonists, cytokines and stress. However, the signaling pathway leading to ERK5 activation is not well understood as compared to the other classical MAPKs. For example, the pharmacological reagents that induce second messengers cAMP and Ca^{2+} downstream of GPCRs do not activate ERK5 in neuronal cells. Also, conflicting results have come from studies examining the involvement of small G-proteins in ERK5 activation by growth factors, and the details of the signaling pathway remain controversial. In addition, the physiological roles of ERK5 in neuronal cells have not been clarified. One reason was the lack of a selective ERK5 pharmacological inhibitor, until the novel selective MEK5/ERK5 inhibitors, BIX02188 and BIX02189, were reported last year. Another reason is that the use of interfering mutants is limited in neuronal cells since the transfection efficiency is low. Despite these difficulties, recent studies suggest that ERK5 mediates the promotion of neuronal survival and neuronal differentiation *in vitro* and *in vivo*. In this review, the signaling pathway leading to ERK5 activation through heterotrimeric and small G-proteins and the physiological roles of ERK5 in neuronal cells are summarized and discussed.

Introduction

Extracellular signal-regulated kinases (ERKs) or mitogen-activated protein kinases (MAPKs) are involved in cellular proliferation, differentiation, migration and gene expression. The MAPK family includes ERK1/2, c-Jun N-terminus kinase 1/2/3, p38MAPK $\epsilon/\epsilon/\epsilon/\epsilon$ and ERK5 as classical MAPKs, and ERK3, ERK4 NLK and ERK7 as atypical MAPKs (Coulombe and Meloche, 2007). Threonine and tyrosine activation motifs (T-X-Y) are conserved among classical MAPKs and ERK7, whereas the atypical MAPKs lack these motifs. The most well-studied MAPK family member, ERK1/2, is activated by variety of stimuli, and the signaling pathway leading to ERK1/2 activation has been better characterized than that to ERK5 activation (Goldsmith and Dhanasekaran, 2007). ERK5 is approximately twice the molecular size of ERK1/2. The kinase domain is encoded by its amino-terminal half and shares approximately 50% of homology with ERK1/2, while its unique carboxy-terminus encodes two proline-rich regions and a nuclear localization signal (Nishimoto and Nishida, 2006; Wang and Tournier, 2006). Recently, it was reported that the autophosphorylated carboxy-terminus of ERK5 plays a critical role in activating transcription (Morimoto et al., 2007). The

threonine and tyrosine residues on ERK5 are phosphorylated by MEK5, but not MEK1/2. In contrast, ERK1/2 is not phosphorylated by MEK5, but is phosphorylated by MEK1/2 (Fig.1) (English et al., 1995; Zhou et al., 1995).

Growth factors such as epidermal growth factor (EGF) and nerve growth factor (NGF) activate not only ERK1/2, but also ERK5 in many cell types (Kamakura et al., 1999; Kato et al., 1998; Obara et al., 2008; Obara et al., 2009; Watson et al., 2001). However, although it is well-known that small G-proteins such as Ras and Rap1 mediate ERK1/2 activation upon ligand binding to tyrosine kinase receptors (York et al., 1998), the involvement of these small G-proteins in ERK5 activation remains unclear. In addition, heterotrimeric G-proteins activate or inhibit ERK1/2 through a variety of signaling pathways (Goldsmith and Dhanasekaran, 2007). However, few reports have described the mechanisms of ERK5 activation through heterotrimeric G-proteins.

ERK1/2 plays critical roles in neuronal survival, differentiation and regeneration in many types of neurons. However, the number of studies concerning the role of ERK5 in neuronal cells is much less than those examining ERK1/2. One reason is that a pharmacological inhibitor that is selective for ERK5 was not available, until the

novel selective MEK5/ERK5 inhibitors, BIX02188 and BIX02189, were reported last year (Tatake et al., 2008). In addition, the use of interfering mutants of MEK5 or ERK5 is limited in neuronal cells, because the transfection efficiency is low. Regardless of these obstacles, progress in the study of ERK5-dependent effects on neuronal survival or differentiation has been reported. This review examines the signaling pathway leading to ERK5 activation through heterotrimeric and small G-proteins and the physiological roles of ERK5 in neuronal cells.

Signaling Pathways of ERK5 activation

Regulation of ERK5 activity by G-protein-coupled receptors (GPCRs)/heterotrimeric G-proteins

Mechanisms of ERK1/2 activation by heterotrimeric G-protein have been better characterized (Goldsmith and Dhanasekaran, 2007). For examples, cAMP produced by G_{ϵ} /adenylyl cyclases either activates or inhibits ERK1/2 activity. In B-Raf-positive cells, small G-protein Rap1 activated by cAMP can lead to ERK1/2 activation via B-Raf/MEK1/2. In contrast, Rap1 or protein kinase A (PKA) negatively regulates Raf-1, suppressing ERK1/2 in B-Raf-negative cells. In case of G_q , Ca^{2+} -sensitive tyrosine kinase, Pyk2, can regulate upstream of Ras, or Rap1 is activated by Ca^{2+} and diacylglycerol-dependent GEF for Rap1. Moreover, PKC can regulate Raf-1 activity. However, the activation of ERK5 is less understood. Figure 2a summarizes the reported GPCR signaling pathways leading to ERK5 activation. ERK5 kinase activity was increased by carbachol and thrombin in Cos7 cells overexpressing their respective receptors M_1 and Par_1 (Fukuhara et al., 2000). In that study, G_q , G_{12} and G_{13} , but not G_s , G_i or $G_{\epsilon\epsilon}$ were capable of activating ERK5, as determined using constitutively active

G-protein mutants and chimeric G-proteins. However, since the Ca^{2+} influx induced by glutamate or depolarization by high KCl did not induce ERK5 activation in cortical neurons (Cavanaugh et al., 2001), an increase in intracellular Ca^{2+} concentration through G_q /phospholipase C may not be sufficient for ERK5 activation. As shown in Figure 1, ERK5 is activated through MEKK2,3/MEK5 pathway, it is assumed that these heterotrimeric G-proteins activate this kinase cascade. Although these heterotrimeric G-proteins often utilize small G-proteins of Ras or Rho families to activate ERK1/2 pathway, none of the small G-proteins including Ras, Rho, Rac and Cdc42 mediated the signaling of ERK5 activation by G_q , G_{12} or G_{13} in the study (Fukuhara et al., 2000).

In many cell types including neuronal cells that express abundant B-Raf isoforms, GPCR agonists or pharmacological compounds that promote cAMP elevation can activate ERK1/2, while cAMP suppresses ERK activity in other cells such as fibroblasts (Goldsmith and Dhanasekaran, 2007; Stork and Schmitt, 2002). In studies of ERK5, forskolin and prostaglandin E_2 inhibited ERK5 activation induced by EGF via PKA (Pearson et al., 2006). In addition, we demonstrated that cholera

toxin and forskolin negatively regulated ERK5 activity through PKA in PC12 cells (Obara et al., 2008). Furthermore, although forskolin induced sustained ERK1/2 phosphorylation in cortical neurons, ERK5 activity was not altered (Cavanaugh et al., 2001). In that study, it was not determined whether cAMP showed the inhibitory effect on ERK5 activation by brain-derived neurotrophic factor (BDNF) or other growth factors, as described above. These results suggest that cAMP accumulated by G_{ϵ_s} activation has a negative role in ERK5 activation. Studies in other cells are necessary to examine whether these phenomena are universal events. Concerning the inhibitory mechanism, PKA was required for ERK5 inhibition by G_{ϵ_s} since this inhibitory effect was reversed by PKA inhibitor. The phosphorylation of MEKK2 by PKA did not reduce MEKK2 activity. Therefore, it has been suggested that phosphorylation of MEKK2 by PKA blocks the connection of MEKK2 with its upstream regulatory mechanism (Pearson et al., 2006).

Concerning pertussis toxin-sensitive $G_{i/o}$ proteins, we have shown that these G-proteins suppress ERK5 activation by EGF and NGF in PC12 cells (Obara et al., 2008). While the stimulation of lysophosphatidic acid (LPA)₁ receptor that couples to

$G_{i/o}$ promoted ERK1/2 activation, ERK5 activation was reduced in a pertussis toxin-sensitive manner. $G_{\epsilon\epsilon}$ overexpression also blocked EGF-induced ERK5 activation, and LPA-induced inhibition of ERK5 was reversed by the overexpression of the c-terminus of GPCR kinase2, which inhibits the function of $G_{\epsilon\epsilon}$ subunits. Thus, we concluded that the $\epsilon\epsilon$ subunits of $G_{i/o}$ negatively regulated ERK5 signaling. In contrast, LPA activation of ERK1/2 was largely blocked by the overexpression of GPCR kinase2 c-terminus and a dominant-negative RasN17 mutant, suggesting the involvement of $G_{\epsilon\epsilon}$ subunits and Ras. Concerning the mechanism of ERK5 inhibition by $\epsilon\epsilon$ subunits of $G_{i/o}$, the $\epsilon\epsilon$ subunits bind to EGF receptor in response to stimulation and partial inhibition of tyrosine autophosphorylation levels on EGF receptor was observed. Therefore, it is assumed that $G_{\epsilon\epsilon}$ inhibits interaction of MEKK2/3 with the receptor tyrosine kinase (Obara et al., 2008).

Regulation of ERK5 by growth factors/neurotrophic factors via small G-proteins

It is well-known that growth factors, such as EGF, or neurotrophins, such as NGF and BDNF, cause ERK5 activation (Cavanaugh, 2004; Cavanaugh et al., 2001;

Kamakura et al., 1999; Kato et al., 1998; Obara et al., 2008; Obara et al., 2009; Watson et al., 2001). However, the mechanisms of ERK5 activation, especially the involvement of small G-proteins, by these factors remain controversial (Fig. 2b). Growth factors utilize Ras/Raf/MEK1/2 or Rap1/B-Raf/MEK1/2 pathways to increase the activity of ERK1/2 (Obara et al., 2004; York et al., 1998), resulting in cellular proliferation, differentiation and survival. In contrast, the roles of Ras in ERK5 activation are controversial. For example, overexpression of the oncogenic mutant of Ras, RasV12, increased ERK5 kinase activity in PC12 cells, and ERK5 activation by EGF and NGF was blocked by the overexpression of a dominant-negative mutant of Ras, RasN17, in PC12 cells or Cos7 cells although MEKK2/3 activation by Ras has not been shown in the study (Kamakura et al., 1999). However, conflicting results have been reported RasV12 did not activate ERK5 despite its activation of ERK1/2, and RasN17 did not affect ERK5 activation by EGF in HeLa cells (Kato et al., 1998). RasV12 was not sufficient to activate ERK5 in Cos7 cells, as mentioned in the above section (Fukuhara et al., 2000). Furthermore, involvement of Ras in ERK5 activation by BDNF was excluded in cortical neurons

(Wang et al., 2006). We have shown that RasV12 was not sufficient for the activation of ERK5, and that RasN17 did not block ERK5 activation by NGF and EGF in PC12 cells, while RasN17 largely blocked ERK1/2 activation (Obara et al., 2008; Obara et al., 2009).

Rap1, another member of the Ras-family is activated by neurotrophins including NGF and BDNF, followed by ERK1/2 activation via B-Raf and MEK1/2 (Obara et al., 2007; Obara et al., 2004; York et al., 1998). The internalization of BDNF TrkB receptor and Rap1 activation are essential for ERK5 activation by BDNF in cortical neurons (Wang et al., 2006). In that study, a consistently active Rap1 mutant activated ERK5, and dominant-negative Rap1, but not Ras, blocked ERK5 activation by BDNF. It was clearly demonstrated that MEKK2 activation by Rap1 was essential for ERK5 activation. In addition, this Rap1-mediated ERK5 signaling is required for neuronal survival after serum-deprivation. In contrast, Rap1, when is activated by Epac, inactivated MEKK/MEK5/ERK5 pathway in A-kinase anchoring protein (AKAP)-associated pools (Dodge-Kafka et al., 2005). In that study, the local ERK5 activity in AKAP-associated pools after AKAP immunoprecipitation was examined

rather than that in the total cell fraction, therefore, their results differ from the results of Wang et al. In our previous studies, we found that cAMP strongly activated Rap1 through PKA and Src tyrosine kinase in PC12 cells (Obara et al., 2004). However, Rap1 activated by forskolin did not induce ERK5 phosphorylation (Obara et al., 2008). In addition, ERK5 was not activated by a cAMP analog capable of activating Rap1 (Obara et al., 2009). Moreover, EGF activated ERK5 in PC12 cells (Obara et al., 2008; Obara et al., 2009) although EGF did not increase Rap1 activity in PC12 cells, as determined by a pull-down assay (Obara et al., 2004). In PC12 cells that overexpress Rap1GTPase-activating protein (GAP) 1, an inactivator of Rap1, ERK5 activation by NGF was not blocked. The siRNA knock-down of C3G guanine nucleotide exchange factor, which mediates the activation of Rap1 by NGF (Hisata et al., 2007; York et al., 1998), did not affect ERK5 activity (Obara et al., 2009). These results strongly suggest that Rap1 is not a major transducer of ERK5 activation in PC12 cells, and that the Rap1-dependent ERK5 activation observed in cortical neurons (Wang et al., 2006) may be cell type-specific. This may depend on different expression levels in unidentified components that link Rap1 and ERK5, such as

scaffolding proteins, or that inhibit the signaling transduction between Rap1 and ERK5.

As mentioned above, Rho family G-proteins including RhoA, Rac1 and Cdc42 were not required for ERK5 activation by stimulation of muscarinic receptor 1 or thrombin Par1 receptor (Fukuhara et al., 2000). A NOMA-GAP, which belongs to a new family of multiadaptor proteins with Rho GAP activity, was essential for sustained ERK5 activation and neurite extension by NGF in PC12 cells (Rosario et al., 2007). Although NOMA-GAP negatively regulates Cdc42 and p21 activated kinase pathways down-stream of NGF, the relationship between Cdc42 and ERK5 remains unclear.

ERK5 activity is often measured by a kinase assay or the detection of ERK5 phosphorylation, as determined by band shift due to its autophosphorylation or by using a phospho-ERK5 antibody. Different techniques used in different laboratories may be a reason for such diverse results. For example, a number of reports that refer to an ERK5 kinase assay was performed using an immunoprecipitated ERK5 and a substrate such as myelin basic protein. However, myelin basic protein can be a good

substrate for other kinases such as ERK1/2, and the possibility that other kinases are contained in the ERK5 immunoprecipitates was not ruled out. Hence, the results obtained from the kinase assay must be carefully considered as to whether they reflect pure ERK5 activity. In addition, the ERK5 band shift determined by Western blotting is assumed to be a result of autophosphorylation at the ERK5 C-terminus as ERK5 kinase-dead mutant lacks the mobility shift. However, the ERK5 C-terminus may be transphosphorylated by other kinases. Thus, the requirement of small G proteins for ERK5 regulation in various cells should be clarified in more detail.

Regulation of ERK5 by other than G-proteins

The mechanisms of ERK5 activation have also been examined focusing on other than heterotrimeric or small G-proteins (Fig. 2c). An atypical PKC-interacting protein, p62/ZIP, associates with the NGF TrkA receptor, and p62/ZIP is required for TrkA internalization into the endosomal compartment and activation of ERK5 in PC12 cells (Geetha and Wooten, 2003). p62/ZIP interacts with not only TrkA, but also TrkB and TrkC, which are primary receptors for BDNF and neurotrophin-3,

respectively. However, it remains unclear whether atypical protein kinase C (PKC) activity is required for ERK5 activation. In murine bone marrow-derived mast cells, stimulation of FcεR1 with IgE leads to ERK5 activation. This ERK5 activation was enhanced by the overexpression of PKCε and ε isoforms, and was completely inhibited in cells lacking MEKK2 (Li et al., 2005). These results suggest that the PKC/MEKK2 pathway is involved in FcεRI-mediated ERK5 activation.

In the mink lung epithelial cell line, CCL64, EGF activation of ERK5 was mediated by an interaction between adaptor protein Lad and MEKK2 (Sun et al., 2003). In addition, Src tyrosine kinase activity was essential for ERK5 activation since its pharmacological inhibitor, PP1, and a dominant-negative mutant of Src blocked both tyrosine phosphorylation of MEKK2 and ERK5 activation by EGF.

Physiological roles of ERK5 in neuronal cells

Representative examples of reported ERK5-dependent effects in neurons are summarized in Table 1. After NGF binds to the TrkA receptor at the distal axon, the NGF-TrkA complex is internalized followed by retrograde transport to the cell body (Zweifel et al., 2005). In dorsal root ganglia (DRG), ERK5 was activated by NGF during retrograde transport of TrkA, and this action prevented apoptosis (Watson et al., 2001). Surprisingly, ERK1/2 was only activated at the distal axon and not in the cell body. In that study, a transcription factor, cAMP response element-binding protein (CREB), activated by the ERK5/p90 ribosomal S6 kinase (Rsk) pathway played an essential role in the promotion of neuronal survival. Recently, the same group demonstrated that gene expression of retrograde response genes including anti-apoptotic *bcl-2* family members, such as *bcl-w* and *mef2d*, were enhanced to promote the survival of DRG sensory neurons (Pazyra-Murphy et al., 2009). The expression of these genes is known to be regulated by ERK5/myocyte enhancer factor (MEF) 2D that was activated by target-derived neurotrophin and the retrograde signaling pathway. Surprisingly, *bcl-w* and *mef2d* genes were not induced following

selective neurotrophin stimulation at the cell body, despite the fact that both ERK5 and ERK1/2 were activated. ERK5 activation from target-derived retrograde transport seems essential for neuronal survival, but a hypothesis of “Signaling endosomes” during retrograde transport has not been established, as alternative non-vesicular models have been suggested (Ginty and Segal, 2002; MacInnis and Campenot, 2002).

Because cortical progenitor cells can differentiate towards neurons, astrocytes and oligodendrocytes, a role of ERK5 in this differentiation process was investigated by Xia’s laboratory (Liu L, 2006). In their study, dominant-negative ERK5 and MEK5 mutants block the differentiation of cortical progenitor cells toward neurons, while constitutively active MEK5 promotes differentiation into neurons. Thus, ERK5 activity is necessary and sufficient for specifying the cell fate. In addition, the same group has published reports showing that ERK5 plays a critical role in the survival of cortical neurons by BDNF, and that ERK5 activation of a transcription factor MEF2-regulated gene expression mediates this effect. This observation was more apparent in developing embryonic cortical neurons that express abundant ERK5 (Liu

et al., 2003; Wang et al., 2006). In addition, dominant-negative ERK5 and MEK1 mutants decreased basal survival in MN9D cells, a model of a dopaminergic cell line. Constitutively active MEK5 and MEF2C increased basal survival in MN9D cells (Cavanaugh et al., 2006). By using a novel model of sympathetic neurons where *erk5* gene can be deleted in vitro, it was demonstrated that ERK5 is required to mediate the survival response of neurons by NGF (Finegan et al., 2009). ERK5 suppressed the transcription of genes responsible for apoptosis such as *bad* via Rsk and CREB. In addition, Akt (or PKB) phosphorylation by ERK5 inactivated Foxo3a and the following gene expression of *Bim* (Finegan et al., 2009).

Xiao et al. (2008) examined a role of ERK5 activation in peripheral inflammation in the spinal cord and DRG, and whether this activation is involved in the heat and mechanical hyperalgesia response. (Xiao et al., 2008). In their study, sustained ERK5 activation was observed in the DRG and spinal dorsal horn during inflammation induced by the injection of complete Freund's adjuvant into a hindpaw, and the hyperalgesia response was suppressed by ERK5 knockdown by antisense oligonucleotides accompanied by a reduction in the numbers of phospho-CREB and

c-Fos positive neurons. These results suggest that ERK5 may be a trigger for inflammation accompanied by the enhancement of gene expression.

We have demonstrated that dominant-negative ERK5 and MEK5 mutants and novel pharmacological reagents, including BIX02188 and BIX02189 that block MEK5/ERK5 signaling selectively, inhibited neurite outgrowth induced by NGF. Hence, ERK5 activity is required for neurite outgrowth in PC12 cells (Fig. 3) (Obara et al., 2009). Tyrosine hydroxylase, a rate-limiting enzyme for catecholamine synthesis, is an indicator of functional differentiation. When both ERK1/2 and ERK5 signaling were suppressed, up-regulation of tyrosine hydroxylase proteins by NGF was abolished (Obara et al., 2009). One reason to explain this phenomenon is that ERK5 stabilizes ERK5 protein by an unidentified mechanism. In general, ERK1/2 promotes neuronal differentiation by enhancing gene expression through various transcription factors (Markus et al., 2002). Therefore, ERK5-dependent neuronal differentiation may be mediated by additional gene expression, as seen in case of ERK1/2.

A few reports have examined the physiological effect of ERK5 on neuronal

development *in vivo*. For example, neuronal differentiation was reduced by ERK5 knock-down using antisense morpholino oligonucleotides in *Xenopus laevis* (Nishimoto et al., 2005). In their study, it was assumed that ERK5 regulates neuronal differentiation downstream of SoxD and upstream of Xngnr1. Because gene expression of fibroblasts growth factor (FGF) 13 was increased by SoxD and antisense morpholino oligonucleotides of FGF13 inhibited the activation of MEK5-ERK5 pathways, the authors concluded that FGF13 was involved in the activation of ERK5 (Nishimoto and Nishida, 2007). In animal models, knockout of the ERK5 gene was lethal at E9.5-10.5 due to cardiovascular defects, indicating the involvement of ERK5 in heart development (Hayashi and Lee, 2004). Whereas specific ERK5 gene knockout in cardiomyocytes showed normal development, conditional knockout of ERK5 in endothelial cells displayed similar cardiovascular defects, suggesting these are a consequence of abnormal vasculogenesis and angiogenesis (Hayashi et al., 2004). Although ERK5 played critical roles in neuronal survival and development *in vitro* as described above, conditional ERK5 knockout mice in neurons where Cre-recombinase expression is driven under nestin and

synapsin-I promoters developed normally and survived similarly to control mice (Hayashi and Lee, 2004). This surprising observation may be due to alternative intracellular pathways to compensate for the loss of the ERK5 gene in neuronal cells.

Future direction

In addition to examining signaling pathways leading to ERK5 activation in more detail, the downstream effectors of ERK5 need to be better understood as well. Transcription factors including activator protein-1, CREB, MEF2, c-Myc and nuclear factor of activated T cells are activated by ERK5 directly or indirectly. Gene expression profiles or novel ERK5 binding partners should be investigated by microarray or proteome analysis. To investigate the ERK5-specific effects, a combination of novel pharmacological inhibitors of ERK5 signaling (BIX02188/02189) (Fig. 3b), siRNA approach and biochemical tools such as interfering mutants of MEK5 and ERK5 will be helpful to examine ERK5-specific effects to compare these results to those obtained using either ERK1/2 knockout or conditional ERK5 knockout mice. In addition, although normal neuronal survival and development has been reported in neuronal cells

of conditional ERK5 knockouts, more functional analysis such as that of memory and learning is essential, as determined by electric physiological and behavioral pharmacological analyses.

References

- Cavanaugh JE (2004) Role of extracellular signal regulated kinase 5 in neuronal survival. *Eur J Biochem* **271**(11):2056-2059.
- Cavanaugh JE, Ham J, Hetman M, Poser S, Yan C and Xia Z (2001) Differential regulation of mitogen-activated protein kinases ERK1/2 and ERK5 by neurotrophins, neuronal activity, and cAMP in neurons. *J Neurosci* **21**(2):434-443.
- Cavanaugh JE, Jaumotte JD, Lakoski JM and Zigmond MJ (2006) Neuroprotective role of ERK1/2 and ERK5 in a dopaminergic cell line under basal conditions and in response to oxidative stress. *J Neurosci Res* **84**(6):1367-1375.
- Coulombe P and Meloche S (2007) Atypical mitogen-activated protein kinase: Structure, regulation and functions. *Biochim Biophys Acta* **1773**:1376-1387.
- Dodge-Kafka KL, Soughayer J, Pare GC, Carlisle Michel JJ, Langeberg LK, Kapiloff MS and Scott JD (2005) The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. *Nature* **437**(7058):574-578.
- English JM, Vanderbilt CA, Xu S, Marcus S and Cobb MH (1995) Isolation of MEK5

and differential expression of alternatively spliced forms. *J Biol Chem* **270**(48):28897-28902.

Finegan KG, Wang X, Lee EJ, Robinson AC and Tournier C (2009) Regulation of neuronal survival by the extracellular signal-regulated protein kinase 5. *Cell Death Differ* **16**(5):674-683.

Fukuhara S, Marinissen MJ, Chiariello M and Gutkind JS (2000) Signaling from G protein-coupled receptors to ERK5/Big MAPK 1 involves Galpha q and Galpha 12/13 families of heterotrimeric G proteins. Evidence for the existence of a novel Ras AND Rho-independent pathway. *J Biol Chem* **275**(28):21730-21736.

Geetha T and Wooten MW (2003) Association of the atypical protein kinase C-interacting protein p62/ZIP with nerve growth factor receptor TrkA regulates receptor trafficking and Erk5 signaling. *J Biol Chem* **278**(7):4730-4739.

Ginty DD and Segal RA (2002) Retrograde neurotrophin signaling: Trk-ing along the axon. *Curr Opin Neurobiol* **12**(3):268-274.

Goldsmith ZG and Dhanasekaran DN (2007) G Protein regulation of MAPK networks. *Oncogene* **26**(22):3122-3142.

Hayashi M, Kim SW, Imanaka-Yoshida K, Yoshida T, Abel ED, Eliceiri B, Yang Y,

Ulevitch RJ and Lee JD (2004) Targeted deletion of BMK1/ERK5 in adult mice
perturbs vascular integrity and leads to endothelial failure. *J Clin Invest*
113(8):1138-1148.

Hayashi M and Lee JD (2004) Role of the BMK1/ERK5 signaling pathway: lessons
from knockout mice. *J Mol Med* **82**(12):800-808.

Hisata S, Sakisaka T, Baba T, Yamada T, Aoki K, Matsuda M and Takai Y (2007)
Rap1-PDZ-GEF1 interacts with a neurotrophin receptor at late endosomes,
leading to sustained activation of Rap1 and ERK and neurite outgrowth. *J Cell*
Biol **178**(5):843-860.

Kamakura S, Moriguchi T and Nishida E (1999) Activation of the protein kinase
ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of
a signaling pathway to the nucleus. *J Biol Chem* **274**(37):26563-26571.

Kato Y, Tapping RI, Huang S, Watson MH, Ulevitch RJ and Lee JD (1998) Bmk1/Erk5
is required for cell proliferation induced by epidermal growth factor. *Nature*
395(6703):713-716.

Li G, Lucas JJ and Gelfand EW (2005) Protein kinase C alpha, betaI, and betaII isozymes regulate cytokine production in mast cells through MEKK2/ERK5-dependent and -independent pathways. *Cell Immunol* **238**(1):10-18.

Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z and Xia Z (2003) ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. *Proc Natl Acad Sci U S A* **100**(14):8532-8537.

Liu L CP, Abel G, Wang Y, Faigle R, Sakagami H, Xu M and Xia Z (2006) Extracellular signal-regulated kinase (ERK) 5 is necessary and sufficient to specify cortical neuronal fate. *Proc Natl Acad Sci U S A* **103**(25):9697-9702.

MacInnis BL and Campenot RB (2002) Retrograde support of neuronal survival without retrograde transport of nerve growth factor. *Science* **295**(5559):1536-1539.

Markus A, Patel TD and Snider WD (2002) Neurotrophic factors and axonal growth. *Curr Opin Neurobiol* **12**(5):523-531.

Morimoto H, Kondoh K, Nishimoto S, Terasawa K and Nishida E (2007) Activation of

a C-terminal transcriptional activation domain of ERK5 by autophosphorylation.

J Biol Chem **282**(49):35449-35456.

Nishimoto S, Kusakabe M and Nishida E (2005) Requirement of the MEK5-ERK5

pathway for neural differentiation in *Xenopus* embryonic development. *EMBO*

Rep **6**(11):1064-1069.

Nishimoto S and Nishida E (2006) MAPK signalling: ERK5 versus ERK1/2. *EMBO*

Rep **7**(8):782-786.

Nishimoto S and Nishida E (2007) Fibroblast growth factor 13 is essential for neural

differentiation in *Xenopus* early embryonic development. *J Biol Chem*

282(33):24255-24261.

Obara Y, Horgan AM and Stork PJ (2007) The requirement of Ras and Rap1 for the

activation of ERKs by cAMP, PACAP, and KCl in cerebellar granule cells. *J*

Neurochem **101**(2):470-482.

Obara Y, Labudda K, Dillon TJ and Stork PJ (2004) PKA phosphorylation of Src

mediates Rap1 activation in NGF and cAMP signaling in PC12 cells. *J Cell Sci*

117(Pt 25):6085-6094.

Obara Y, Okano Y, Ono S, Yamauchi A, Hoshino T, Kurose H and Nakahata N (2008)

Betagamma subunits of G(i/o) suppress EGF-induced ERK5 phosphorylation, whereas ERK1/2 phosphorylation is enhanced. *Cell Signal* **20**(7):1275-1283.

Obara Y, Yamauchi A, Takehara S, Nemoto W, Takahashi M, Stork PJ and Nakahata N

(2009) ERK5 activity is required for NGF-induced neurite outgrowth and stabilization of tyrosine hydroxylase in PC12 cells. *J Biol Chem*.

Pazyra-Murphy MF, Hans A, Courchesne SL, Karch C, Cosker KE, Heerssen HM,

Watson FL, Kim T, Greenberg ME and Segal RA (2009) A retrograde neuronal survival response: target-derived neurotrophins regulate MEF2D and bcl-w. *J Neurosci* **29**(20):6700-6709.

Pearson GW, Earnest S and Cobb MH (2006) Cyclic AMP selectively uncouples

mitogen-activated protein kinase cascades from activating signals. *Mol Cell Biol* **26**(8):3039-3047.

Rosario M, Franke R, Bednarski C and Birchmeier W (2007) The neurite outgrowth

multiadaptor RhoGAP, NOMA-GAP, regulates neurite extension through SHP2 and Cdc42. *J Cell Biol* **178**(3):503-516.

Stork PJ and Schmitt JM (2002) Crosstalk between cAMP and MAP kinase signaling in

the regulation of cell proliferation. *Trends Cell Biol* **12**(6):258-266.

Sun W, Wei X, Kesavan K, Garrington TP, Fan R, Mei J, Anderson SM, Gelfand EW

and Johnson GL (2003) MEK kinase 2 and the adaptor protein Lad regulate

extracellular signal-regulated kinase 5 activation by epidermal growth factor via

Src. *Mol Cell Biol* **23**(7):2298-2308.

Tatake RJ, O'Neill MM, Kennedy CA, Wayne AL, Jakes S, Wu D, Kugler SZ, Jr.,

Kashem MA, Kaplita P and Snow RJ (2008) Identification of pharmacological

inhibitors of the MEK5/ERK5 pathway. *Biochem Biophys Res Commun*

377(1):120-125.

Wang X and Tournier C (2006) Regulation of cellular functions by the ERK5 signalling

pathway. *Cell Signal* **18**(6):753-760.

Wang Y, Su B and Xia Z (2006) Brain-derived neurotrophic factor activates ERK5 in

cortical neurons via a Rap1-MEKK2 signaling cascade. *J Biol Chem*

281(47):35965-35974.

Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ and Segal RA (2001)

Neurotrophins use the Erk5 pathway to mediate a retrograde survival response.

Nat Neurosci **4**(10):981-988.

Xiao C, Zhang L, Cheng QP and Zhang LC (2008) The activation of extracellular signal-regulated protein kinase 5 in spinal cord and dorsal root ganglia contributes to inflammatory pain. *Brain Res* **1215**:76-86.

York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW and Stork PJ (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature* **392**(6676):622-626.

Zhou G, Bao ZQ and Dixon JE (1995) Components of a new human protein kinase signal transduction pathway. *J Biol Chem* **270**(21):12665-12669.

Zweifel LS, Kuruvilla R and Ginty DD (2005) Functions and mechanisms of retrograde neurotrophin signalling. *Nat Rev Neurosci* **6**(8):615-625.

Figure legends

Figure 1. MAPK cascade and ERK5 structure. (a) Whereas ERK1/2 is activated through Raf isoforms (MAPKKK) and MEK1/2 (MAPKK), ERK5 is activated through MEKK2/3 and MEK5. (b) ERK5 protein contains Thr and Tyr residues in its kinase domain that are phosphorylated by MEK5, two prolin-rich domains (PR1 and 2), nuclear localization signal (NLS) and autophosphorylation sites.

Figure 2. The signaling pathway that leads to ERK5 activation. (a) GPCR agonists activate or inhibit ERK5 activity through their corresponding receptors and heterotrimeric G-proteins. (b) Growth factors and neurotrophic factors activate ERK5 in small G-protein-dependent or independent manners. (c) Other signaling pathways.

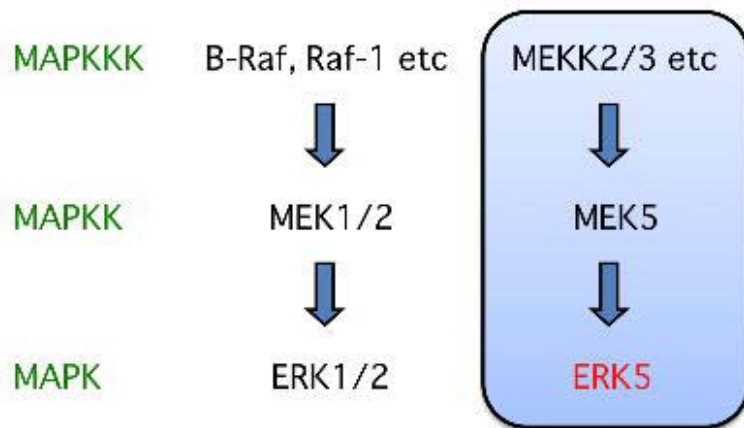
Figure 3. ERK5 activity is required for NGF-induced neurite outgrowth in PC12 cells. (a) PC12 cells were incubated with NGF (100 ng/ml) for a day in the presence of BIX02188 or BIX02189 (30 μ M) for a day. (b) After PC12 cells were pretreated with or without BIX02188 and BIX02189 (3-30 μ M) for 30 min, the cells were stimulated with

NGF (100 ng/ml) for 5 min. Then, phospho-ERK5, total ERK5, phospho-ERK1/2 and total ERK1/2 were examined by Western blotting. (c) PC12 cells were co-transfected with GFP and empty vector or ERK5KD. The cells were incubated with NGF (100 ng/ml) or dbcAMP (0.5 mM) for one day. The nuclei were stained with Hoechst-33258 (blue) and morphological changes of GFP-positive cells were observed under fluorescence microscope. *Fig. 3 is reproduced from *J Biol Chem* (Obara et al. 2009)

Table 1: Representative reports of ERK5-dependent effects on neuronal cells.

Neuronal cell types	ERK5-dependent effects	References
•Cortical neural progenitor cells	Cell fate determination (neuronal differentiation)	Liu et al. (2006)
•DRG neurons	Retrograde transport-dependent cell survival	Watson et al. (2001) Pazyra-Murphy et al. (2009)
•Cortical neurons	ERK5/MEF2-depedent survival	Wang et al. (2006) Liu et al. (2003)
•MN9D cells (dopaminergic cells)	ERK5/MEF2-dependent survival	Cavanaugh et al. (2006)
•Superior cervical ganglion neurons	ERK5/CREB, Akt-dependent survival	Finegan et al. (2009)
•DRG and spinal dorsal horn	Inflammatory pain	Xiao et al. (2008)
•PC12 cells	Neurite outgrowth TH stabilization	Obara et al. (2009)
<i>In vivo</i>		
• <i>Xenopus laevis</i>	Neuronal differentiation	Nishimoto et al. (2005; 2007)
•conditional KO in neurons	Normal neuronal development	Hayashi et al. (2004a)

a) MAPK Cascade



b) ERK5 Structure

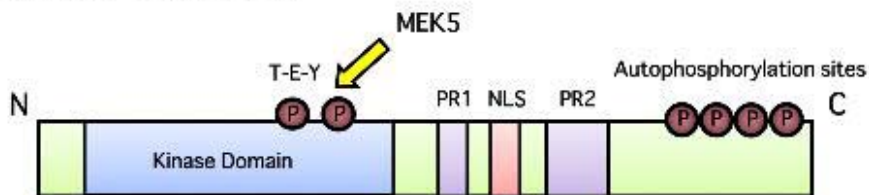


Fig. 1

a) GPCRs/Heterotrimeric G-proteins

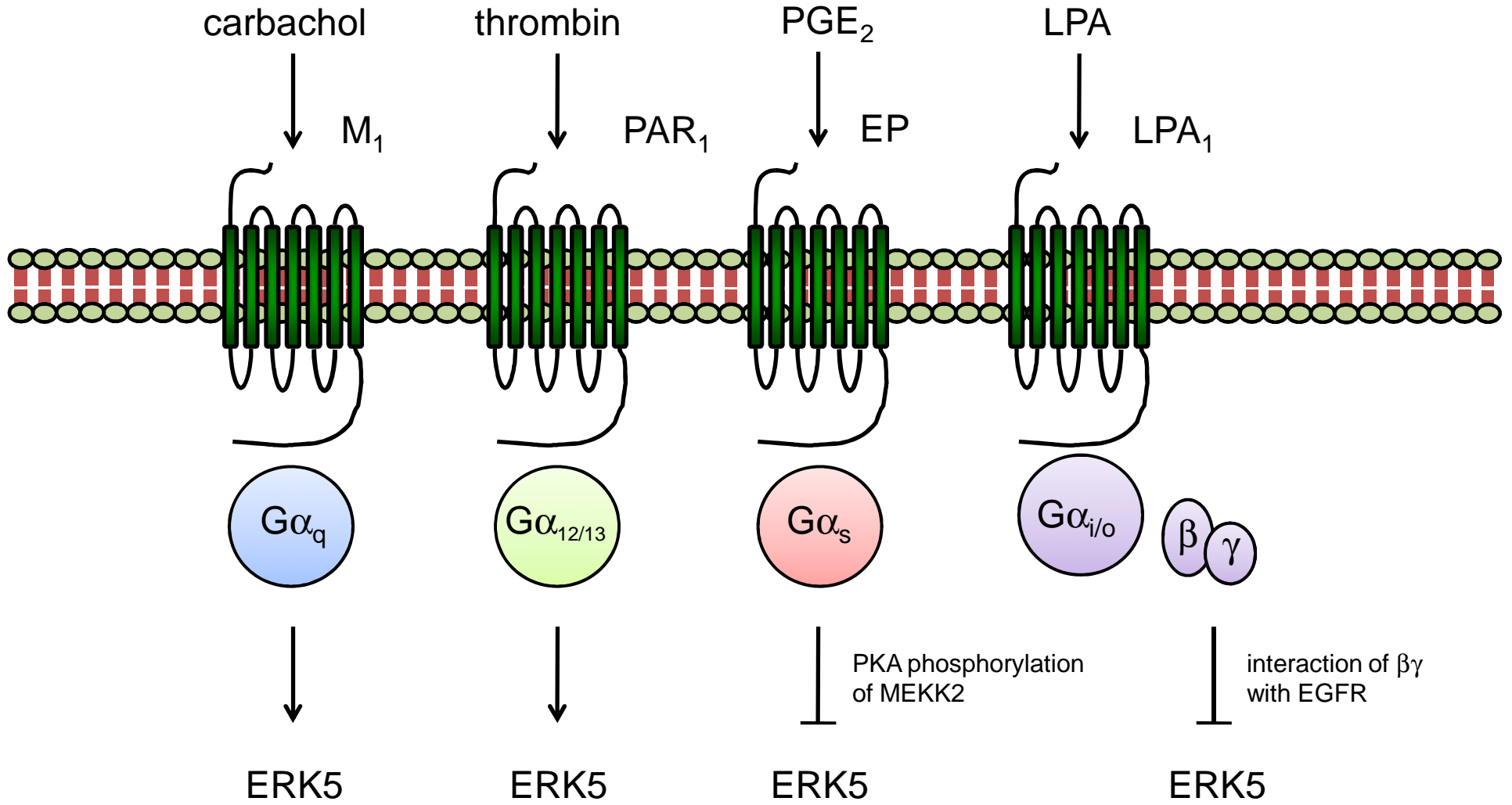
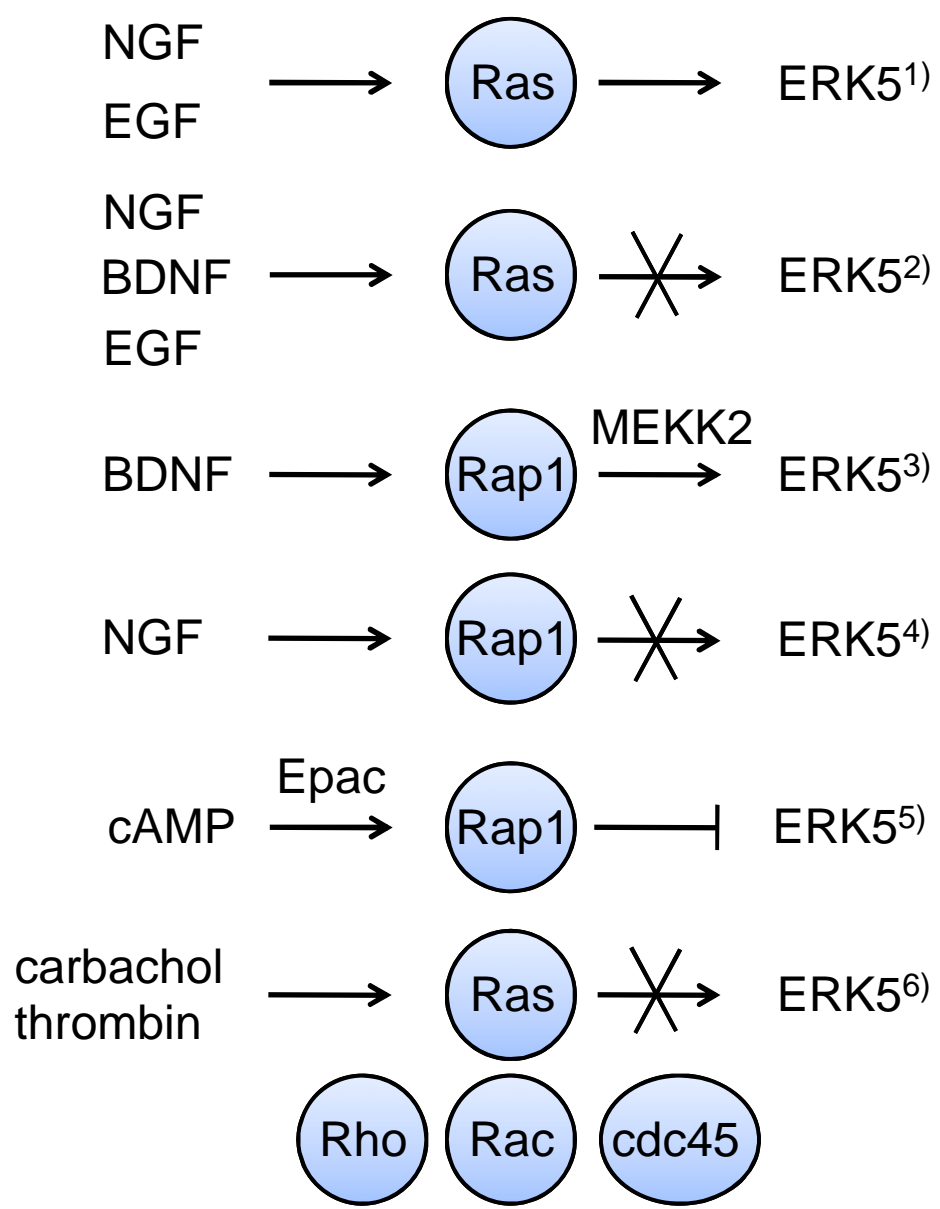
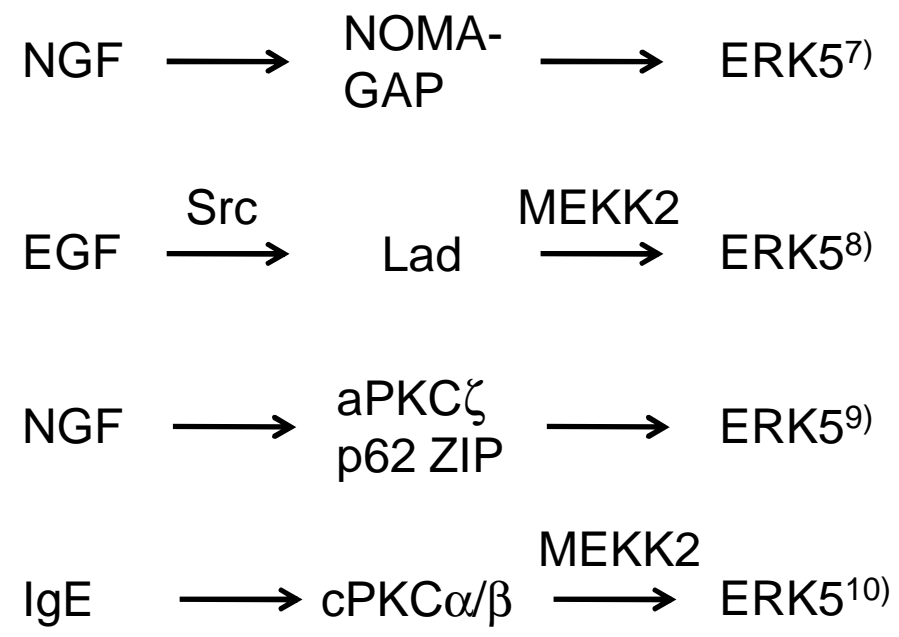


Fig. 2

b) Small G-proteins



c) Others



References

- 1) Kamakura et al. (1999)
- 2) Obara et al. (2009); Wang et al. (2006); Kato et al. (1998)
- 3) Wang et al. (2006)
- 4) Obara et al. (2009)
- 5) Dodge-Kafka et al. (2005)
- 6) Fukuhara et al. (2000)
- 7) Rosario et al. (2007)
- 8) Sun et al. (2003)
- 9) Geetha and Wooten (2003)
- 10) Li et al. (2005)

Fig. 2

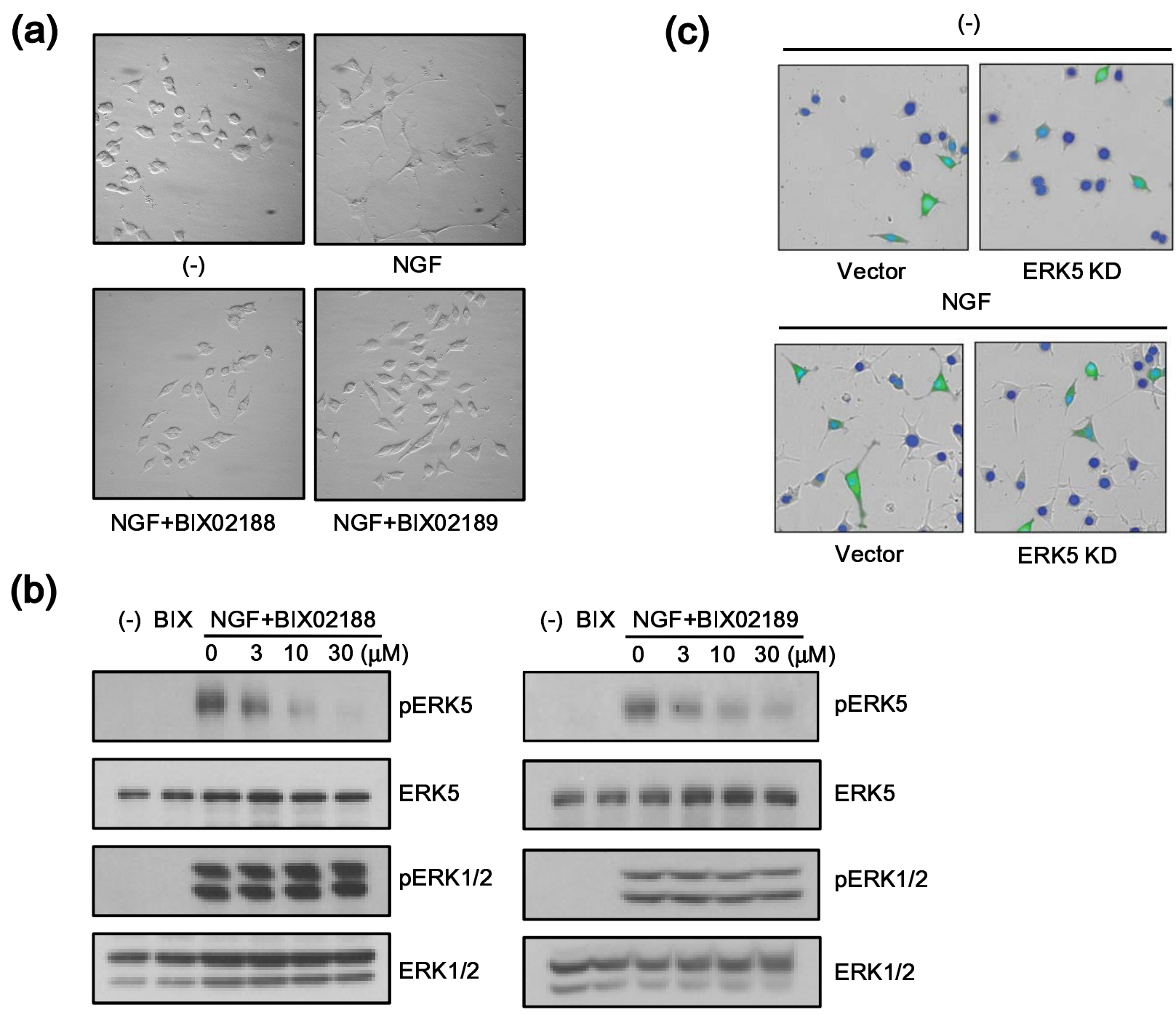


Fig. 3