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著者	Yamazaki Masakazu, Yamauchi Yohei, Goshima Yoshio, Kanaho Yasunori
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**Phosphatidylinositol 4-phosphate 5-kinase  $\beta$  regulates growth cone morphology and Semaphorin 3A-triggered growth cone collapse in mouse dorsal root ganglion neurons**

Masakazu Yamazaki<sup>a,1,2</sup>, Yohei Yamauchi<sup>b,1</sup>, Yoshio Goshima<sup>c</sup>, Yasunori Kanaho<sup>b,\*</sup>

<sup>a</sup>*Research Center for Biosignal, Akita University, 1-1-1 Hondo, Akita 010-8543, Japan*

<sup>b</sup>*Department of Physiological Chemistry, Faculty of Medicine & Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan*

<sup>c</sup>*Department of Molecular Pharmacology and Neurobiology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan*

\*Correspondence to: Yasunori Kanaho

Department of Physiological Chemistry

Faculty of Medicine & Graduate School of Comprehensive Human Sciences

University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan

Tel: (Japan: +81) 29-853-3282

Fax: (Japan: +81) 29-853-3271

E-mail: ykanaho@md.tsukuba.ac.jp

<sup>1</sup>These authors equally contributed to this work.

<sup>2</sup>Present address: Department of Cell Biology and Morphology, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan

**Abstract**

Growth cone motility and morphology, which are critical for axon guidance, are controlled through intracellular events such as actin cytoskeletal reorganization and vesicular trafficking. The membrane phospholipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P<sub>2</sub>] has been implicated in regulation of these cellular processes in a diverse range of cell types. The main kinases involved in the production of PI(4,5)P<sub>2</sub> are the type I phosphatidylinositol 4-phosphate 5-kinase (PIP5K) family, which consist of three isozymes,  $\alpha$ ,  $\beta$  and  $\gamma$ . Here, we demonstrate the involvement of PIP5K $\beta$  in growth cone dynamics. Overexpression of a lipid kinase-deficient mutant of PIP5K $\beta$  (PIP5K $\beta$ -KD) in mouse dorsal root ganglion (DRG) neurons stimulated axon elongation and increased growth cone size, whereas wild-type PIP5K $\beta$  tended to show opposite effects. Furthermore, PIP5K $\beta$ -KD inhibited growth cone collapse of DRG neurons induced by semaphorin 3A (Sema3A). These results provide evidence that PIP5K $\beta$  negatively regulates axon elongation and growth cone size and is involved in the cellular signaling pathway for Sema3A-triggered repulsion in DRG neurons.

*Keywords:* phosphatidylinositol 4-phosphate 5-kinase; semaphorin 3A; dorsal root ganglion neurons; growth cone dynamics

*Abbreviations:* PIP5K, phosphatidylinositol 4-phosphate 5-kinase; DRG, dorsal root ganglion; Sema3A, semaphorin 3A; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BHK cells, baby hamster kidney cells

## 1. Introduction

A highly ordered neuronal network is established by the precise regulation of axon guidance, which is controlled by extracellular guidance cues that attract or repel neurites during embryogenesis [23,34]. A specialized structure at the axon tip, the growth cone, detects and translates guidance information into changes in morphology to direct the motility necessary for navigation to an appropriate target [24,33]. Several lines of evidence indicate that growth cone morphology and directed motility involve cellular signaling pathways that regulate cytoskeletal dynamics and membrane trafficking [4-6,18,25,29,30]. Several intracellular molecules, including actin-binding proteins, Rho family small GTPases and protein kinases, regulate actin cytoskeleton reorganization and are implicated in the signaling pathways that regulate growth cone morphology and motility [17,29]. However, the molecular mechanisms through which extracellular cues regulate these growth cone dynamics remain unclear.

A candidate for a signaling mediator involved in the regulation of growth cone dynamics is type I phosphatidylinositol 4-phosphate 5-kinase (PIP5K), which catalyzes phosphorylation of phosphatidylinositol 4-phosphate [PI(4)P] at the D-5 position of the inositol ring to generate the versatile signaling lipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P<sub>2</sub>] [2,15,19,20,26]. The mammalian PIP5K family consists of three gene products,  $\alpha$ ,  $\beta$  and  $\gamma$ , including three  $\gamma$  spliced variants,  $\gamma$ 635,  $\gamma$ 661 and  $\gamma$ 687 [10,19,20,26]. The potential relevance of this candidate is supported by the following reports: (1) the PIP5K product, PI(4,5)P<sub>2</sub>, reorganizes the actin cytoskeleton through direct interaction with and regulation of actin-associated proteins [15,35], and (2) we [39] and van Horck et al [37] have demonstrated that mouse PIP5K $\alpha$  regulates lysophosphatidic acid (LPA)- and semaphorin 3A (Sema 3A)-induced neurite retraction of mouse neuroblastoma N1E-115 cells, respectively. In addition, PIP5K $\gamma$ 687 and PIP5K $\gamma$ 661 have been implicated in axon elongation and Sema3A-mediated repulsion of outgrowing axons, respectively (10,36). However, the role of PIP5K $\beta$  in axon guidance is poorly understood. In this study, therefore, we investigated whether PIP5K $\beta$  is involved in the regulation of growth cone morphology, axon elongation and

chemorepulsion of mouse dorsal root ganglion (DRG) neurons using wild-type PIP5K $\beta$  and a lipid kinase-deficient mutant of PIP5K $\beta$  (PIP5K $\beta$ -WT and PIP5K $\beta$ -KD, respectively). The results indicate that mouse PIP5K $\beta$  negatively regulates axon elongation and growth cone size and is involved in the signal transduction pathway for semaphorin 3A (Sema3A)-induced growth cone collapse in DRG neurons.

## 2. Materials and methods

### 2.1. Generation of Sindbis pseudovirions

Mouse PIP5K $\beta$  cDNA was amplified from mouse brain total RNA using RT-PCR, and the cDNA sequence, verified. cDNA for PIP5K $\beta$ -KD was constructed using a PCR-based strategy. To coexpress EGFP and PIP5Ks, cDNAs for PIP5K $\beta$  and its mutant were inserted into the *XbaI/StuI* site in pSinRep5-IRES-EGFP [1], a generous gift from Dr Sehara. The plasmids were linearized and transcribed *in vitro* as previously reported [38]. These transcripts and the transcript of DH (26S) helper DNA (Invitrogen, Carlsbad, CA, USA) were cotransfected into BHK cells by electroporation. After 48 h, Sindbis pseudovirions were harvested and stored at -80°C until use.

### 2.2. Preparation and culture of DRG neurons, and PIP5K expression

Dorsal root ganglia were dissected from embryonic day 12.5 mice and plated on glass-bottom culture dishes (MatTek Corporation, Ashland, MA, USA) precoated sequentially with poly-D-lysine (PDL) and laminin (Biomedical Technologies, Stoughton, MA, USA). DRG explants were then cultured for 6 h in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 20 ng/ml of nerve growth factor (NGF) (Alomone Labs, Jerusalem, Israel) and infected with Sindbis pseudovirions for 2 h to express PIP5Ks. To analyze PIP5K localization, axon elongation and growth cone morphology, the infected DRG explants were further cultured in the growth medium for 10 h. For analysis of growth

cone collapse, the infected DRG explants were cultured in DMEM/F12 medium supplemented with 0.5% FCS and 20 ng/ml of NGF for 6 h and then incubated with recombinant chick Sema3A, which was purified as previously described [12].

### *2.3. Immunofluorescence microscopy*

The DRG explants treated as described above were fixed, permeabilized and blocked according to the method reported previously by Fournier et al [9]. DRG neurons were then immunostained for EGFP using a polyclonal rabbit EGFP antibody (MBL, Nagoya, Japan; 1:500 dilution) and for PIP5K $\beta$  using a polyclonal rabbit anti-PIP5K $\beta$  antibody (Upstate Biotechnology, Lake Placid, NY, USA; 1:1000 and 1:100 dilutions for overexpressed and endogenous PIP5K $\beta$  staining, respectively). EGFP and PIP5K $\beta$  were visualized with Alexa488- and Cy3-conjugated secondary antibodies, respectively (Molecular Probes, Eugene, OR, USA and Jackson ImmunoResearch Laboratories, West Grove, PA, USA, respectively). Images of the immunofluorescently stained DRG neurons were obtained by means of an immunofluorescence microscope (Axiovert S100; Zeiss, Gottingen, Germany).

### *2.4. Measurement of axon length and growth cone area*

Morphological analyses of DRG neurons expressing EGFP, PIP5K $\beta$ -WT or PIP5K $\beta$ -KD were performed using IPLab image analysis software (Scanalytics, Fairfax, VA, USA). The length of an axon was defined as the length of trace from the contour limit of the DRG explant to the tip of the process. To quantify axon lengths and growth cone areas, more than 100 neurons from 3-4 explants were analyzed in an individual experiment. Statistical significance was evaluated using the Tukey-Kramer multiple comparison test.

### *2.5. PIP5K activity assay*

cDNAs for Myc-PIP5K $\beta$ -WT and Myc-PIP5K $\beta$ -KD were subcloned into a pcDNA3-myc vector and transfected into COS7 cells using Lipofectamine Plus (Invitrogen). Proteins were

overexpressed by incubation of the cells for 24 h. After cells were lysed in lysis buffer and centrifuged as previously described [16], expressed proteins were immunoprecipitated with the anti-Myc antibody affinity resin 9E10 (Santa Cruz Biotechnology). The immunoprecipitates, which contained PIP5K $\beta$ -WT and PIP5K $\beta$ -KD, were incubated at 37°C for 30 min in the presence of 50  $\mu$ M of phosphatidic acid (PA), 50  $\mu$ M of PI(4)P (Sigma, St Louis, MO, USA), and 50  $\mu$ M of [ $\gamma$ -<sup>32</sup>P]ATP (1  $\mu$ Ci/assay). [<sup>32</sup>P]PI(4,5)P<sub>2</sub> produced by the incorporation of <sup>32</sup>Pi from [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Biosciences, Arlington Heights, IL, USA) into PI(4)P was detected by thin-layer chromatography as previously reported [16].

## 2.6. Immunoblot analysis

Immunoblot analysis was performed as previously reported [40]. In brief, proteins contained in the samples were separated by SDS-PAGE on 10% gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated with anti-PIP5K $\beta$  antibody (1:400 dilution) or anti-Myc antibody (Santa Cruz Biotechnologies; 1:500 dilution) and then with peroxidase-conjugated rabbit anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive proteins were detected with an ECL immunoblotting detection reagent (Amersham).

## 2.7. Assay for *Sema3A*-induced growth cone collapse

Growth cone collapse of DRG explants was assayed using purified recombinant chick *Sema3A* as previously described [11]. The individual growth cone area of each explant was scored as previously described [12].

# 3. Results

## 3.1. PIP5K $\beta$ is enriched in the axon and growth cone

As shown in Figure 1A and B, endogenous PIP5K $\beta$  was detected in DRG neurons by Western blot analysis and found to be localized in the punctate structures of the growth cone and in the axon shaft as analyzed by immunostaining. Overexpressed PIP5K $\beta$  in DRG explants was also localized in the growth cone as well as in the axonal shaft and soma (Fig. 1C and D). These results raised the possibility that PIP5K $\beta$  is implicated in the regulation of growth cone morphology and axon guidance of DRG neurons.

### *3.2. PIP5K $\beta$ negatively regulates axon elongation and growth cone size*

To investigate whether PIP5K $\beta$  is implicated in the regulation of axon elongation and growth cone morphology, PIP5K $\beta$ -WT and PIP5K $\beta$ -KD, in the latter of which Ala was substituted for Asp<sup>307</sup>, which is a critical amino acid residue for the putative ATP and PI(4)P binding site [31], were overexpressed in DRG neurons, and their effects, examined. This PIP5K $\beta$ -KD mutant was shown to be almost completely deficient of kinase activity (Fig. 2A). As shown in Figure 2B and C, overexpression of PIP5K $\beta$ -WT slightly inhibited axon elongation when compared with EGFP-expressing control neurons. In contrast, overexpression of PIP5K $\beta$ -KD significantly stimulated axon elongation (Fig. 2B and C), leading us to conclude that this PIP5K $\beta$ -KD functions as a dominant negative mutant of PIP5K $\beta$ . Taken together, these results suggest that PIP5K $\beta$  negatively regulates axon elongation of DRG neurons.

Several reports have indicated that molecules such as small GTPase RhoA and LIM-kinase, which possess the ability to reduce the elongation rate of neurites, cause growth cone collapse [1,3,8,28]. Since PIP5K $\beta$  phenocopied these molecules in its effect on axon elongation, we examined the effects of PIP5K $\beta$ -WT and PIP5K $\beta$ -KD on growth cone morphology. When PIP5K $\beta$ -WT was overexpressed in DRG neurons, growth cone size tended to be reduced in comparison with EGFP-expressing control cells (Fig. 2D and E), although the difference was not statistically significant (Fig. 2E). In contrast, overexpression of PIP5K $\beta$ -KD markedly increased the growth cone area (Fig. 2D and E). Thus, PIP5K $\beta$  also negatively regulates growth cone morphology.



### *3.3. Implication of PIP5K $\beta$ in the signal transduction pathway of the Sema3A-induced repulsive response*

To investigate whether PIP5K $\beta$  is involved in the repulsive response induced by Sema3A, we employed an assay of growth cone collapse of DRG neurons, which is most useful and a characteristic response upon Sema3A stimulation (Fig. 3A and B). Overexpression of PIP5K $\beta$ -WT and PIP5K $\beta$ -KD tended to decrease growth cone collapse in the unstimulated control cells, but the difference was not statistically significant. Again, overexpression of PIP5K $\beta$ -WT had little, if any, effect on Sema3A-induced growth cone collapse when compared with control EGFP overexpression. On the other hand, PIP5K $\beta$ -KD significantly suppressed Sema3A-induced growth cone collapse, suggesting that PIP5K $\beta$  participates in the signaling pathway for Sema3A-induced growth cone collapse in DRG neurons. Although we currently cannot clearly explain why overexpression of PIP5K $\beta$ -WT had no effect on the number of collapsed growth cones in unstimulated and Sema3A-stimulated cells, it is conceivable that endogenous PIP5K $\beta$  is sufficient to transduce the signaling for growth cone collapse.

## **4. Discussion**

In the present study, we demonstrated that PIP5K $\beta$  negatively regulates the axon elongation and growth cone size of mouse DRG neurons. Hernandez-Deviez et al [13] have recently reported that overexpressed PIP5K $\alpha$  in hippocampal neurons localizes in the axon and suppresses axon elongation and branching induced by the dominant negative form of Arf6. We and van Horck et al have demonstrated that PIP5K $\alpha$  regulates LPA- and Sema3A-induced neurite retraction of neuroblastoma N1E-115 cells [37,39]. These results support the notion that PI(4,5)P<sub>2</sub> produced by PIP5K functions as a negative regulator of axon elongation, although the PIP5K isozymes used for the experiments of these two studies were different. In conflict with these findings, the

neuron-specific splice variant of PIP5K $\gamma$ , PIP5K $\gamma$ 687, has been reported to positively regulate axon elongation and branching [10], suggesting that PI(4,5)P<sub>2</sub> produced by PIP5K functions as a positive regulator of axon elongation. Although we currently cannot clearly explain why the effect of PIP5K $\gamma$ 687 differs from that of PIP5K $\alpha$  and  $\beta$ , it is plausible that the PI(4,5)P<sub>2</sub> pool produced by PIP5K $\gamma$ 687 differs from those produced by PIP5K $\alpha$  and  $\beta$ . It would be interesting to investigate the expression and localization of PIP5K $\gamma$ 687 in DRG neurons in comparison with those of other PIP5K isozymes.

The chemorepellant Sema3A restricts axonal elongation and causes growth cone collapse through its specific receptor, the plexin-neuropilin complex, located on the surface of growth cones [17,29]. Sema3A-induced growth cone collapse is believed to be triggered by actin cytoskeletal reorganization [17,29]. Accumulating evidence suggests that growth cone morphology is regulated by actin cytoskeleton reorganization [5,6,24,33]. Since the PIP5K product, PI(4,5)P<sub>2</sub>, regulates actin dynamics through direct binding to and regulation of actin-associated proteins such as alpha-actinin, gelsolin and cofilin [15,35], the effects of PIP5K $\beta$  shown in this study may be mediated by reorganization of actin filaments. However, we could not detect any significant change in actin filaments in DRG neuron growth cones overexpressing PIP5K $\beta$ -WT and PIP5K $\beta$ -KD as assessed by phalloidin staining (data not shown). Although we cannot totally rule out the possibility that overexpression of PIP5K $\beta$  causes subtle changes in actin filaments that cannot be detected by phalloidin staining, it is plausible that PIP5K $\beta$  regulates growth cone morphology by regulating membrane dynamics such as endocytosis. This notion is supported by reports that PI(4,5)P<sub>2</sub> regulates both clathrin-dependent and clathrin-independent endocytosis [21,27] and that Sema3A-induced growth cone collapse is accompanied by macropinocytosis, which is categorized as clathrin-independent endocytosis [9]. Therefore, it could be speculated that PIP5K $\beta$  regulates Sema3A-induced collapse response of growth cones through clathrin-independent membrane invagination regulated by PI(4,5)P<sub>2</sub>. On the other hand, it has been reported that Sema3A induces a repulsive response through clathrin-dependent endocytosis [9] and that the GSK3 $\beta$ /axin-1/ $\beta$ -catenin complex plays an important role in Sema3A-induced axon repulsion via

an endocytic mechanism [14]. Taken together, the findings of these reports and of the present study suggest that PIP5K $\beta$  and the GSK3 $\beta$ /axin-1/ $\beta$ -catenin complex coordinately regulate Semaphorin 3A-induced axon repulsion through clathrin-dependent and -independent endocytosis, respectively. In addition, it has been reported that several molecules such as CRMP, Rac1, LIM-kinase, Fyn and Cdk5 are involved in the signaling pathway for Semaphorin 3A-induced responses [1,7,12,22,32]. PIP5K $\beta$  may also coordinate with these molecules in Semaphorin 3A-induced axon repulsion.

Recently, it has been reported that PIP5K $\gamma$ 661, a crucial mediator of neuronal adhesion, is inactivated by sequestration from talin through interaction with FARP2 in response to Semaphorin 3A in DRG neurons [36]. The authors demonstrated that this decrease in PIP5K $\gamma$ 661 activity leads to suppression of neuronal adhesion and is required for Semaphorin 3A-mediated repulsion of outgrowing axons [36]. Thus, PIP5K $\gamma$ 661 negatively regulates Semaphorin 3A-mediated repulsion of outgrowing axons, whilst PIP5K $\beta$  seems to function as a positive regulator, as was demonstrated by the present study. These observations led us to speculate that each PIP5K isozyme produces a distinct PI(4,5)P<sub>2</sub> pool in spatially and temporally distinct manners, thereby playing a role in different cellular events. Further studies on spatial and temporal analyses of PI(4,5)P<sub>2</sub> localization will provide insight into the molecular mechanisms by which PI(4,5)P<sub>2</sub> regulates the chemorepulsive response of axonal growth cones.

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## Figure legends

**Fig. 1.** Localization of endogenous and overexpressed PIP5K $\beta$  in isolated DRG explants.

(A) Western blot analysis of endogenous PIP5K $\beta$  expression in mouse DRG neurons. DRG neuron homogenates prepared from embryonic day 12.5 mice were immunoblotted with anti-PIP5K $\beta$  antibody. (B) Immunofluorescent staining of endogenous PIP5K $\beta$  in DRG neurons prepared from embryonic day 12.5 mice. Scale bar: 10  $\mu$ m. (C and D) PIP5K $\beta$ -WT was overexpressed in mouse DRG explants and then immunostained. (D) Magnified image of the growth cone shown in (C). Scale bars: 200  $\mu$ m and 10  $\mu$ m in C and D, respectively.

**Fig. 2.** Effects of overexpressed PIP5K $\beta$ -WT and PIP5K $\beta$ -KD on axon elongation and growth cone size of mouse DRG neurons.

PIP5K $\beta$ -WT and PIP5K $\beta$ -KD were overexpressed in mouse DRG neuron explants. EGFP was also expressed as a control. (A) Lipid kinase activities of recombinant PIP5K $\beta$ -WT and PIP5K $\beta$ -KD expressed in COS7 cells were analyzed (upper panel). The lower panel shows the expression levels of PIP5K $\beta$ -WT and PIP5K $\beta$ -KD as assessed by Western blot analysis. (B and C) DRG explants expressing EGFP, PIP5K $\beta$ -WT or PIP5K $\beta$ -KD were immunostained for EGFP and PIP5K $\beta$  (B). Scale bar: 200  $\mu$ m. (C) The axon lengths were determined, and the results presented as the means  $\pm$  SEMs of 4 independent experiments. \* $p$ <0.01. (D and E) Growth cone morphology of DRG neurons expressing EGFP, PIP5K $\beta$ -WT or PIP5K $\beta$ -KD. Proteins overexpressed in DRG neurons were immunostained (D). Scale bar: 10  $\mu$ m. (E) The growth cone areas of DRG neurons were determined, and the results presented as the means  $\pm$  SEMs of 3 independent experiments. N.S., not significant. \* $p$ <0.01.

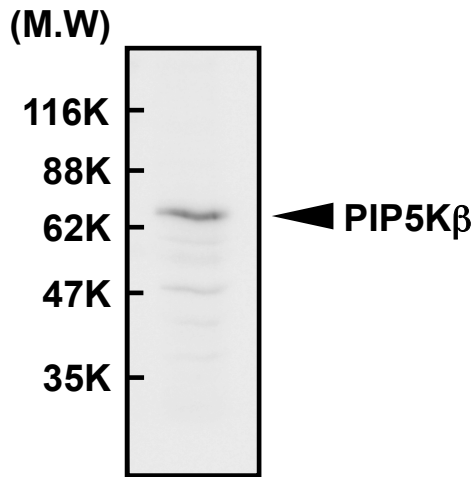
**Fig. 3.** Inhibition of Sema3A-induced growth cone collapse by PIP5K $\beta$ -KD.

DRG explants overexpressing EGFP, PIP5K $\beta$ -WT and PIP5K $\beta$ -KD were incubated with or

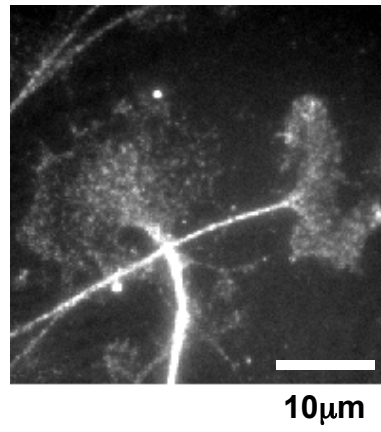


withou 4 U/ml of Sema3A for 30 min. (A) DRG neurons were then immunostained for EGFP, PIP5K $\beta$ -WT and PIP5K $\beta$ -KD. Scale bar: 10  $\mu$ m. (B) Collapsed growth cones were scored, and the results presented as the means  $\pm$  SEMs of 3 independent experiments. N.S., not significant. \*p<0.05.

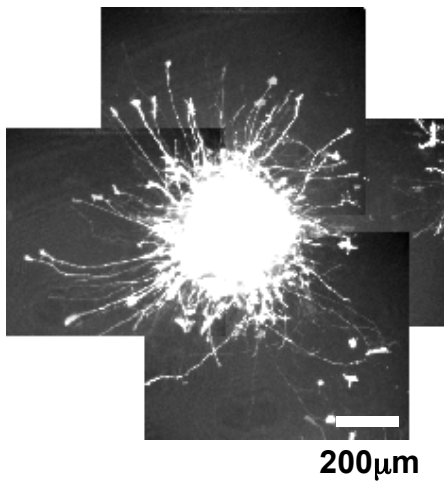
**A**



**B**



**C**



**D**

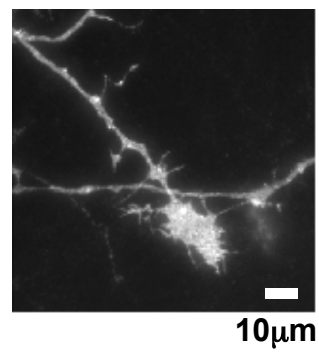


Fig.1. Yamazaki et al.



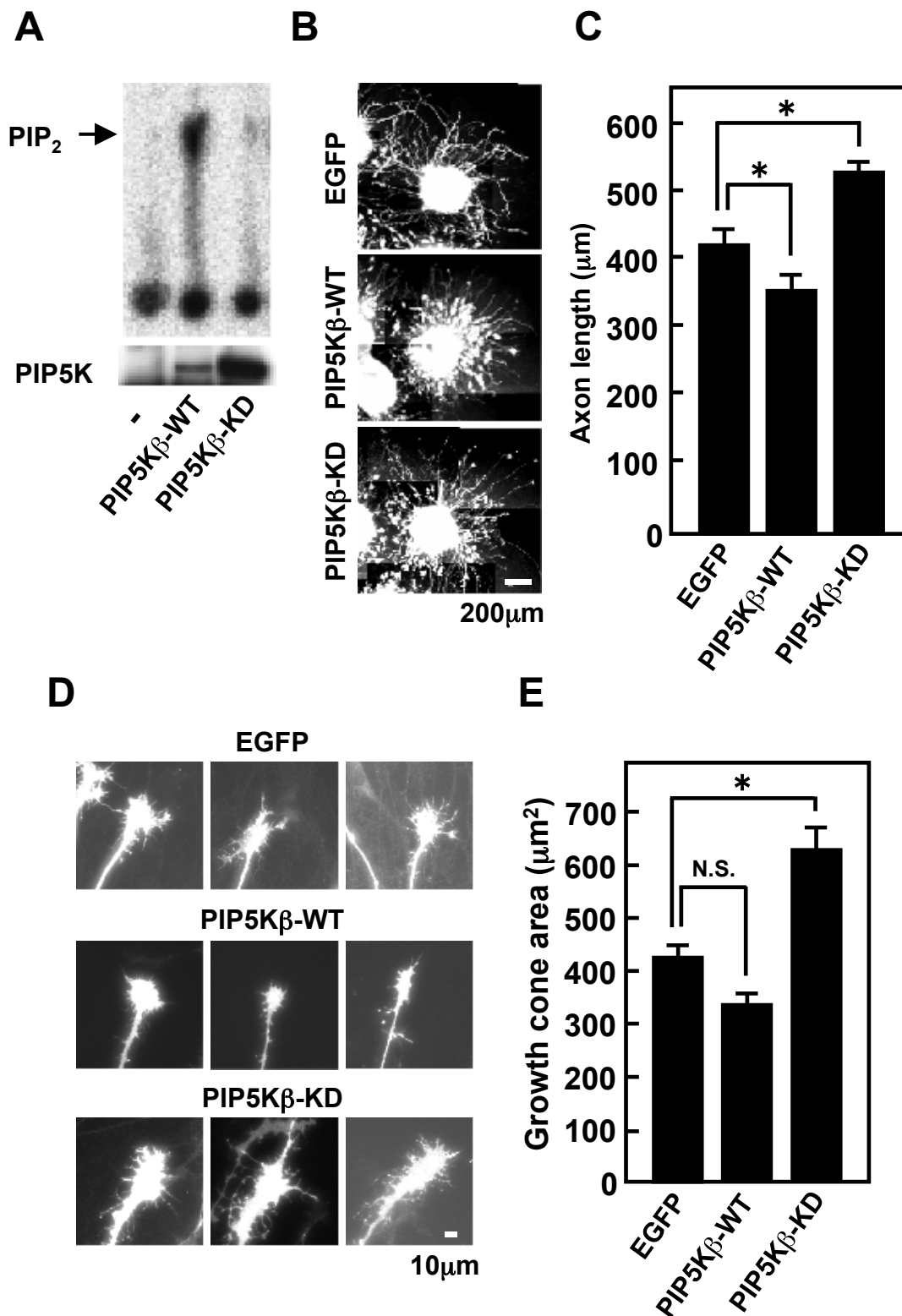


Fig.2. Yamazaki et al.

↑ Top

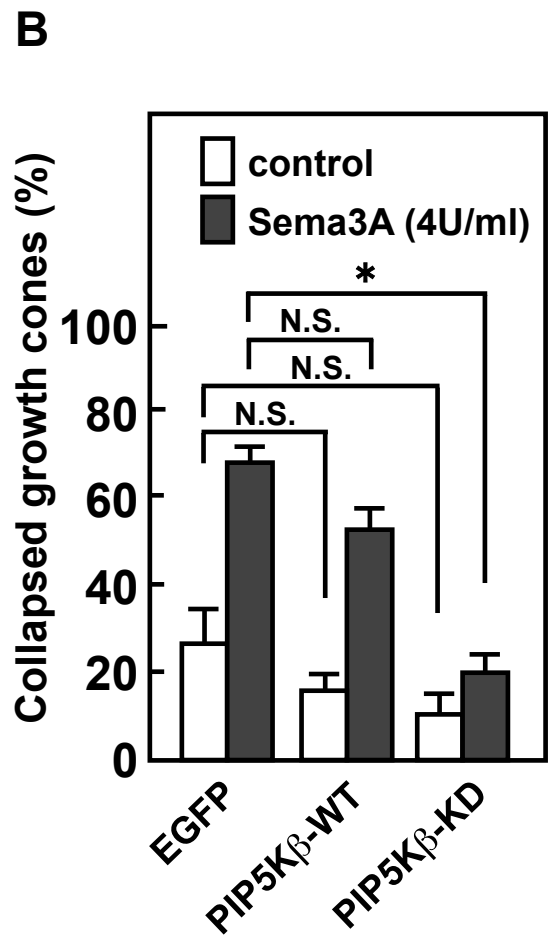
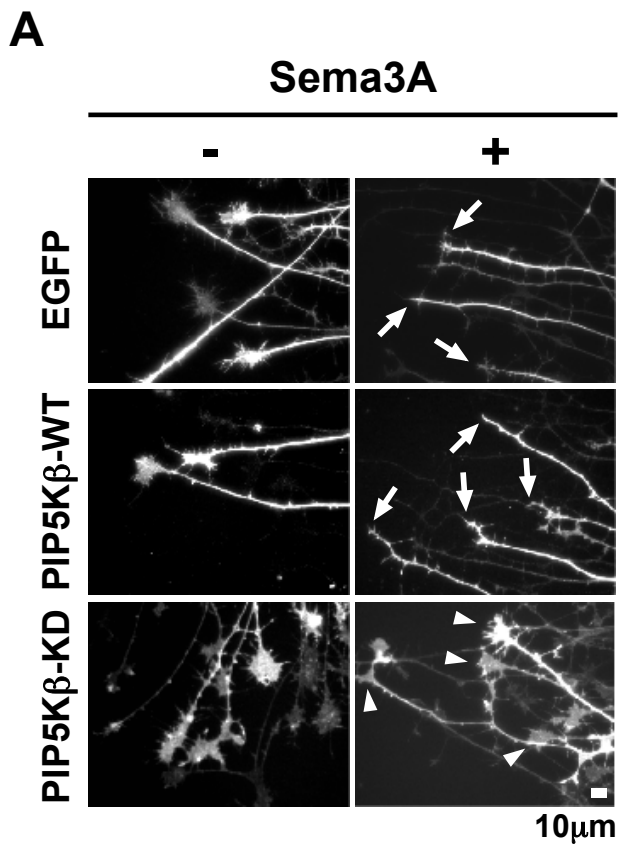


Fig.3. Yamazaki et al.

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