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# Knockdown of pre-mRNA cleavage factor Im 25 kDa promotes neurite outgrowth

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ABSTRACT

Mammalian precursor mRNA (pre-mRNA) cleavage factor I (CFIm) plays important roles in the selection of poly(A) sites in a 3′-untranslated region (3′-UTR), producing mRNAs with variable 3′ ends. Because 3′-UTRs often contain cis elements that impact stability or localization of mRNA or translation, alternative polyadenylation diversifies utilization of primary transcripts in mammalian cells. However, the physiological role of CFIm remains unclear. CFIm acts as a heterodimer comprising a 25 kDa subunit (CFIm25) and one of the three large subunits—CFIm59, CFIm68, or CFIm72. CFIm25 binds directly to RNA and introduces and anchors the larger subunit. To examine the physiological roles of CFIm, we knocked down the CFIm25 gene in neuronal cells using RNA interference. Knockdown of CFIm25 increased the number of primary dendrites of developing hippocampal neurons and promoted nerve growth factor (NGF)-induced neurite extension from rat pheochromocytoma PC12 cells without affecting the morphology of proliferating PC12 cells. On the other hand, CFIm25 knockdown did not influence constitutively active or dominantly negative RhoA suppression or promotion of NGF-induced neurite extension from PC12 cells, respectively. Taken together, our results indicate that endogenous CFIm may promote neuritogenesis in developing neurons by coordinating events upstream of NGF-induced RhoA inactivation.



## 1. Introduction

The 3′-end of precursor mRNA (pre-mRNA) is processed by endonucleolytic cleavage of the primary transcript followed by addition of a poly(A)-tail to the upstream cleavage product [1,2]. The 3′-end processing is an essential step in gene expression that is closely associated with transcription termination and translation initiation [3,4]. However, it also promotes diversity of RNA molecules with distinct lengths and structures of 3′-untranslated regions (3′-UTR) by alternative poly(A) site selection in the last or 3′-most exon [4,5]. In the nervous system, in particular, a number of neuron-specific RNA-binding proteins and micro RNAs (miRNAs) regulate stability, localization, and translational efficiency of their target RNA molecules by binding cis-elements within the 3′-UTR [6,7]. Thus, basic machineries for 3′-end processing control selective elimination of cis-elements and are very likely to play roles in precipitating subcellular proteomics, thereby strongly impacting differentiation and/or functional maturation of neurons.

Six factors are necessary and sufficient to reconstitute cleavage and polyadenylation *in vitro*—cleavage and polyadenylation stimulating factor (CPSF), cleavage stimulating factor (CstF), mammalian cleavage factor I and II (CFIm and CFIIm), poly(A) polymerase (PAP), and poly(A) binding protein II (PABII) [1,2]. CPSF and CstF recognize their own target sequences on pre-mRNAs, AAUAAA hexamer and U/GU-rich sequences upstream or downstream of the cleavage site, respectively. CFIm is an essential pre-mRNA 3′-end processing factor unique to metazoans, and it was first purified from HeLa cell nuclear extracts as four polypeptides of 25, 59, 68, and 72 kDa (CFIm25, CFIm59, CFIm68, and CFIm72, respectively [8]). CFIm59, CFIm68, and CFIm72 are structurally related and CFIm can be reconstituted with CFIm68 and CFIm25 subunits, suggesting that CFIm is a heterodimer composed of the smallest CFIm25 subunit and any one of the three large subunits [9]. In addition to the fact that the smallest subunit is one of the basic components for 3′-end processing, the following observation prompted us to investigate the function of CFIm25 in the differentiation of neurons: (1) CFIm preferentially binds to pre-mRNA through CFIm25 at an early step of 3′-end processing [10] and facilitates assembly of the other factors on pre-mRNA [8] and (2) knockdown of CFIm25 gene expression in HeLa cells alters the selected polyadenylation site in the 3′-UTR of several genes [11].

During differentiation, neurons drastically alter their morphology. Rearrangement of the cytoskeleton induced by extracellular molecules has been implicated in the regulation of neuron morphology. Typically, neurons extend long processes called neurites from their cell bodies, and actin is one of the main components of the neurite cytoskeleton. The Rho family of small GTPases, including Rho, Rac, and Cdc42, regulates various aspects of the actin cytoskeleton [12,13]; therefore, these GTPases are good candidates for signal transducers linking extracellular molecules to the cytoskeleton. Moreover, several recent studies have suggested a

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functional association between Rho-GTPases and 3′-end processing that operates as follows: (1) transcripts for RhoA are localized to developing neurites, and their intra-neurite translation influences the morphology of the neurite. Targeting of RhoA mRNA to the neurite is mediated by unidentified elements located in the RhoA 3′-UTR [14]; and (2) miRNA-185 binds to its mRNA 3′-UTR, thus inhibiting the proliferation potential of HeLa cells by translationally repressing RhoA and Cdc42 [15]. The bipotential cell line PC12, derived from rat pheochromocytoma, is an ideal *in vitro* model that has been extensively used to study neuronal differentiation and signaling [16,17]. Therefore, in this study, we knocked down CFIm25 in neuronal cells and evaluated the effects on the development of neuronal morphology using cultured PC12 cells and hippocampal neurons.

## 2. Materials and methods

### 2.1. Plasmids and antibodies

The plasmid vectors used in this study were generated as described in the Supplementary Materials and Methods and included the vector encoding enhanced green fluorescent protein (EGFP), V5-tagged constitutive active ($RhoA^{G14V}$) and dominant negative ($RhoA^{T19N}$) form of RhoA, FLAG-tagged full-length CFIm25, and small interfering RNA (siRNA) targeting CFIm25.

### 2.2. Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin.

Neurons were prepared from the hippocampi of embryonic day 18 rats and cultured as described previously [18] (culturing methods are briefly described in the Supplementary Materials and Methods).

### 2.3. Gene transfection

For silencing experiments, PC12 cells ($4 \times 10^5$ cells/well in 6-well plates) were transfected with siRNA duplexes (125 pmol/well) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and harvested at indicated time points after transfection to obtain whole-cell extract. For the morphological assay, PC12 cells ($4 \times 10^4$ cells/well in 24-well plates) and hippocampal neurons ($2 \times 10^4$ cells/well in 24-well plates) were co-transfected with pCAG-EGFP (for PC12 cells, 0.25 μg/well; for neurons, 0.0625 μg/well) and siRNA duplexes (for PC12 cells, 25 pmols/well) and/or each indicated expression vector (for PC12 cells, 0.5 μg/well; for neurons, 0.125 μg/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For differentiation of PC12 cells, the medium was replaced with DMEM containing 50 ng/ml of nerve growth factor (NGF) and 1% FBS 6 h after transfection. Three siRNAs targeting rat CFIm25, 5′-AAUUAAACCUGCUCAGC AGCUGAGG-3′ (siRNA-1; target sequence nos. 274–298 of the rat CFIm25 gene, GenBank ID: NM_001039004), 5′-AUCCCAAUCUUAUC AAAUUCCUCCC-3′ (siRNA-2; nos. 284–308), and 5′-UAUCAAAUUCC UCCCGCAUGCGCUG-3′ (siRNA-3; nos. 727–751), were synthesized by Invitrogen (Stealth™ RNAi). Transfection efficiency of siRNA was controlled by BLOCK-iT™ Alexa Fluor® Red Fluorescent Oligo (a red fluorescent-labeled double-strand RNA, Invitrogen). The red-labeled standard dsRNA (Rs siRNA) or Stealth™ RNAi negative control duplex [Medium GC Duplex #2 (negative siRNA), Invitrogen] was used as control siRNA. These control siRNAs are not homologous to any known gene and are double-stranded RNA with the same length, charge, and configuration as those of standard siRNA.

### 2.4. Western blotting and immunocytochemistry

Western blotting and immunocytochemistry were performed as described previously [19,20] (these methods are briefly described in the Supplementary Materials and Methods).

### 2.5. Reverse-transcription (RT)-PCR

Total RNA was isolated from cultured PC12 cells using Trizol® Reagent (Invitrogen). One microgram of total RNA was used for the RT reaction with the PrimeScript® RT Reagent Kit (Takara, Ohtsu, Japan), according to the manufacturer's recommendation. Quantitative PCR (qPCR) was performed with SYBR®Premix Ex-Taq™ (Takara) and analyzed with the Thermal Cycler Dice™ Real-time System (Takara). Primer sequences used for qPCR are shown in Supplementary Table 1.

### 2.6. Morphological analysis

Cells were fixed at the indicated times and the immunofluorescence images were acquired with a confocal microscope (LSM 510; Carl Zeiss, Oberkochen, Germany). For PC12 cells, GFP-positive processes were recorded and measured with Scion Image software (Scion, Frederick, MD, USA). Neurite length was measured by tracing the longest neurite starting at the cell body. More than 100 cells were evaluated in each of the six independent culture replicates for each gene transfection group. GFP-positive fluorescent images were digitally converted into monochrome using Photoshop (Adobe Systems, San Jose, CA, USA). For hippocampal neurons, the cells were fixed and reacted with anti-microtubule-associated protein 2 (MAP2) antibodies. All non-axonal protrusions from the GFP-/MAP2-positive cell soma longer than 10 μm were defined as primary dendrites. More than 40 cells were collected per construct from three independent experiments.

### 2.7. RhoA activation assay

Quantification of RhoA activation was performed using G-LISA RhoA Activation Assay Biochem Kit (Cytoskeleton, Denver, CO, USA) according to the manufacturer's instructions. Methods are briefly described in the Supplementary Materials and Methods.

### 2.8. Statistical analysis

Data are presented as mean ± SE. The statistical significance of the differences among multiple groups was assessed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* tests.

## 3. Results

### 3.1. Knockdown of CFIm25 in PC12 cells

To evaluate the effects of CFIm on neuronal cells, we first examined the expression of each component of CFIm in PC12 cells. As observed in HeLa and COS7 cells, CFIm25, CFIm59, and CFIm68 were much more prominent than CFIm72 in PC12 cells, irrespective of NGF treatment (Fig. 1A, data not shown).

Because CFIm25 is essential for CFIm binding to RNA, we reasoned that depleting cells of CFIm25 by RNAi would block CFIm function. Thus, three siRNAs targeting CFIm25 were designed (siRNA-1, siRNA-2, and siRNA-3) and were independently transferred to PC12 cells with the negative siRNA as a control. Twenty-four hours after transfection, the cells were harvested and the whole-cell extracts were subjected to Western blotting. The gene-transfected cells appeared healthy and grew normally.

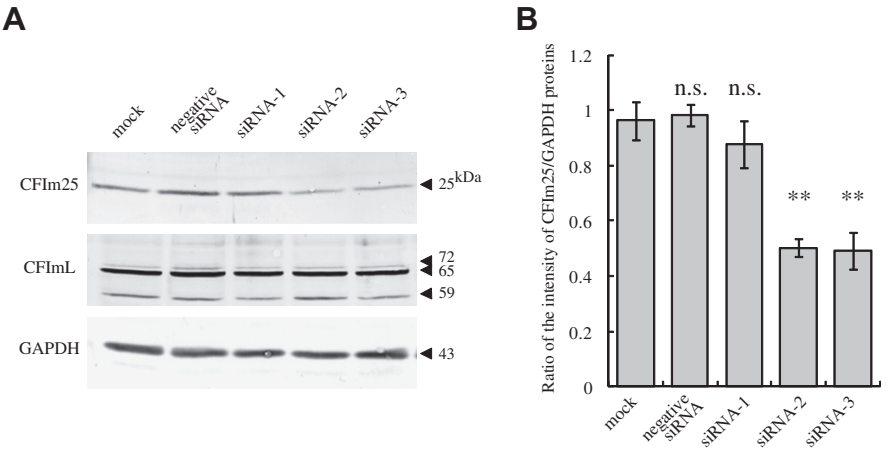


**Fig. 1.** Knockdown efficiency of siRNAs as measured by CFIm25 protein levels in PC12 cells. (A) Three siRNAs (siRNA-1, siRNA-2, and siRNA-3) were designed to target CFIm25. Whole-cell lysates were subjected to polyacrylamide gel electrophoresis (PAGE) (20-μg protein/lane) and blotted onto membranes for Western blotting with anti-CFIm25, anti-CFIm68, and anti-GAPDH antibodies. (B) The intensity of the target bands for CFIm25 was densitometrically quantified, and the ratio of the intensity of CFIm25/GAPDH proteins was expressed as onefold increase in the value of the corresponding non-siRNA-treated cells (mock). The values are expressed as mean ± SE ($n = 6$). Significant differences from the value of the non-siRNA treated cells (mock) were determined by one-way ANOVA followed by Tukey's test. $^{**}p < 0.01$, n.s. not significant.

As shown in Fig. 1A and B, the protein level was reduced by 50% in siRNA-2 or siRNA-3-transfected cells. However, the protein level in siRNA-1 or negative siRNA transfected cells was comparable to that of native PC12 cells. The amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein, analyzed as a loading control, was not altered by CFIm25 knockdown, indicating that each siRNA specifically knocked down CFIm25. Moreover, the expression levels of the larger subunits of CFIm were not changed by CFIm25 knockdown (Fig. 1A). Therefore, siRNA-2 and siRNA-3 were used for further analyses. Preliminary time-course experiments using qPCR revealed that an approximately 50% reduction in expression of the CFIm25 mRNA was detected 24 h after transfection of either siRNA-2 or siRNA-3, and the expression levels recovered to approximately 77% and 60% at 48 h and to approximately 99% and 65% 72 h after transfection of siRNA-2 and siRNA-3, respectively. The transfection efficiency of siRNA in PC12 cells was estimated to be 63.0 ± 6.3% ($n = 6$) of the total as determined by the signals detected 24 h after transfection of Rs siRNA. Therefore, the expected rate of reduced CFIm25 expression per gene-transfected cell would be a maximum of approximately 80% at 24 h and would recover within several days of transfection with any siRNA.

### *3.2. CFIm25 knockdown enhances NGF-induced neurite extension in PC12 cells and dendrite outgrowth of hippocampal neurons*

To study the functional significance of CFIm in NGF-induced neuron-like differentiation of PC12 cells, we knocked down CFIm25 with the siRNAs and assessed neurite outgrowth. The cells transfected with CFIm25 siRNAs were identified by co-transfection with pCAG-GFP, and their morphologies were compared with that of cells co-transfected with pCAG-GFP and negative siRNA. The transfection efficiency of siRNA was estimated to be 93.8 ± 1.4% of GFP-positive cells as determined from the detected red fluorescent signals 24 h after transfection of Rs siRNA ($n = 4$, 176 GFP-positive cells). Compared with the relative control, the neurite lengths were significantly increased in siRNA-2 or siRNA-3-transfected PC12 cells treated with NGF for 48 h (Fig. 2A and B). To rule out off-target effects of siRNAs, we performed a genetic rescue experiment by CFIm25 expression recovery and found that neuritogenesis promotion of PC12 cells induced by siRNA-3 transfection subsided to the relative control level when the RNA duplex was co-transfected with the CFIm25 expression vector (Supplementary Fig. 1). In addition, each of the gene-transfected cells, which were cultured in serum-containing medium without NGF stimulation, grew normally and did not extend neurites (data not shown). Therefore, CFIm did not directly affect neuronal differentiation but modified the mechanism underlying NGF-induced neuronal differentiation.

In cultured rat hippocampal neurons, the neurites (with the exception of one axon per neuron) grew at a slower rate to form dendrites after 4 days of culturing (4 days *in vitro*, 4DIV). To evaluate CFIm25 functions in morphological maturation of neuronal dendrites, pCAG-CFIm25 or pU6-CFIm25 siRNA, with a longer expected gene-silencing effect than RNA oligomer duplex, was generated and independently transferred to hippocampal neurons. Prior to gene transfection, we confirmed the effects of pU6-CFIm25 siRNA using COS7 cells and the CFIm25 protein level was approximately 50% decreased in COS7 cells co-transfected with pCAG-CFIm25 and pU6-CFIm25 siRNA compared with cells co-transfected with pCAG-CFIm25 and pU6 empty vector (data not shown). Neurons were cultured for 96 h after transfection at 4DIV, fixed, and then immunostained with anti-MAP2 antibodies. As observed by MAP2 immunostaining and GFP fluorescence, CFIm25 knockdown increased the number of dendritic outgrowths from cell bodies, although CFIm25 overexpression did not alter the number of dendrites (Fig. 3A and B). These results indicate that CFIm25 depletion rather than overproduction has a greater impact on neuron morphology.

### *3.3. Effects of CFIm25 knockdown on neurite extension are canceled by RhoA activity*

To address how CFIm influences NGF-induced neurite extension, we first examined the effects of CFIm25 knockdown on the activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. Sustained protein phosphorylation induced by NGF treatment permits its translocation to the nucleus, which is necessary for activation of new gene expression, leading to neurite outgrowth from PC12 cells [21]. Quantitative analysis revealed that NGF treatment the number of cells with pERK1/2 positive nuclei and the time-dependent alteration in this number was comparable in CFIm25 siRNA-3- and negative siRNA-transfected cells (data not shown), suggesting that NGF-induced ERK1/2 activation was not modified in CFIm25-depleted cells.

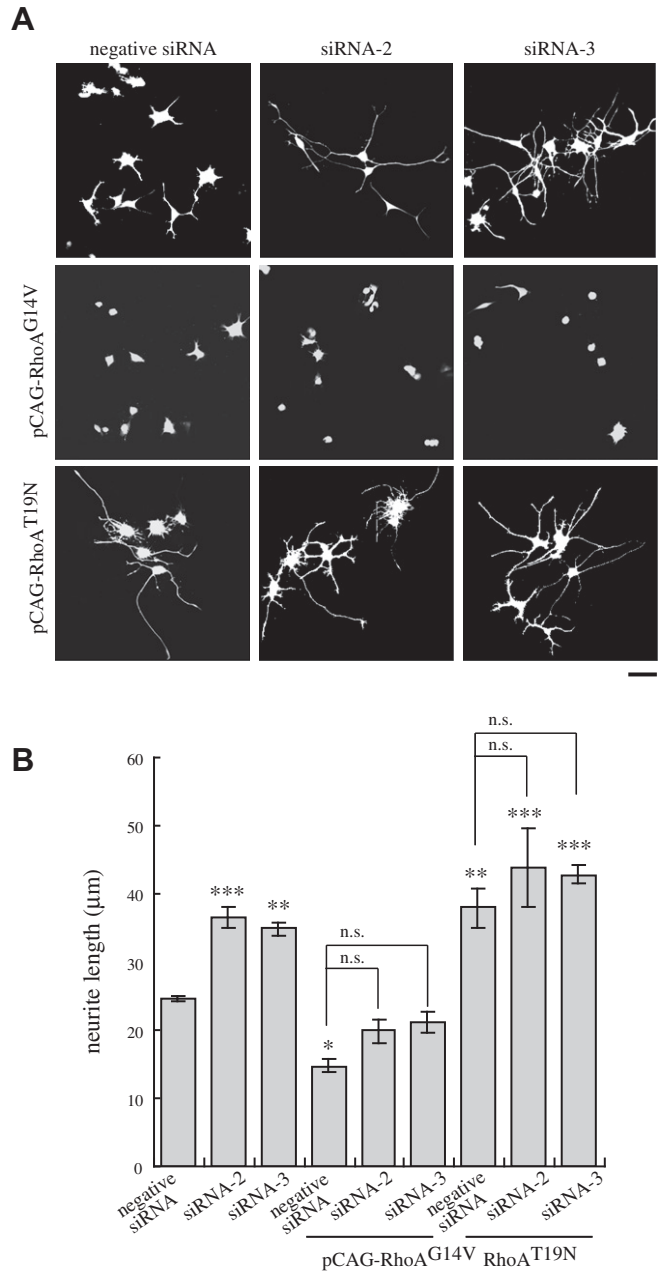


**Fig. 2.** Effects of CFIm25 siRNAs and RhoA mutants on NGF-induced neurite extension in PC12 cells. (A) PC12 cells were transiently transfected with pCAG-GFP and one siRNA (negative siRNA or siRNA-2, siRNA-3, for targeting CFIm25) and/or one expression vector encoding RhoA mutants (pCAG-RhoA$^{G14V}$, pCAG-RhoA$^{T19N}$). At 48 h after NGF treatment, GFP-positive fluorescence images were acquired with a confocal microscope. Scale bar, 20 μm. (B) Neurite length of GFP-positive PC12 cells was measured as described in Section 2. The values are expressed as mean ± SE ($n$ = 6). Significant differences from the value of the negative siRNA-treated cells were determined by one-way ANOVA followed by Tukey's test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, n.s. not significant.

Because activation of RhoA or its downstream kinase ROCK inhibits NGF-induced morphological differentiation of PC12 cells [22,23], we examined the possibility that CFIm25 knockdown may influence RhoA activity. In the cells transfected with negative siRNA, although protein levels were not altered by NGF treatment, the amount of the active GTP-bound form was rapidly suppressed to 80% at 1 min and recovered to the original level within 3 min of NGF treatment (Supplementary Fig. 2). CFIm25 depletion did not influence RhoA protein levels independent of NGF treatment. However, RhoA activity in the CFIm25-depleted cells was 10% lower than that in the negative siRNA transfected cells prior to NGF treatment. Concerning the efficiency of red-labeled siRNA transfection (63.0 ± 6.3%), the effect of CFIm25 depletion on RhoA activity was comparable to NGF action; however, the effect was transient because RhoA activity was restored from 3 min after NGF treatment (Supplementary Fig. 2).

The constitutively active and dominant negative properties of the G14 V and T19 N mutations in RhoA, respectively, have been established previously, and these mutants have been used extensively in a variety of cellular and *in vitro* assays [24,25]. To test whether the RhoA/ROCK signaling pathway may be involved in the mechanism of neuritogenesis enhanced by CFIm25 depletion, CFIm25 siRNA was co-transfected with vectors expressing GFP and either RhoA$^{G14V}$ or RhoA$^{T19N}$ mutants of PC12 cells. Then, we examined neurite lengths 48 h after NGF stimulation, and their morphologies were compared with those of cells expressing RhoA mutant and/or GFP only. The co-transfection efficiency of siRNA/RhoA mutant/GFP was estimated to be 97.1 ± 0.9% of GFP-positive cells judging from the detected V5-tag expression and fluorescent signals 24 h after transfection ($n$ = 4, 207 GFP-positive cells). As in previous studies, most cells expressing RhoA$^{G14V}$ did not exhibit neurite outgrowth, whereas those cells expressing RhoA$^{T19N}$ enhanced morphological differentiation in response to NGF. This suggests that NGF-induced neuritogenesis is negatively regulated by RhoA activation. Interestingly, when PC12 cells were co-transfected with either of the two RhoA mutants, CFIm25 depletion did not have any additional influence on neurite extension (Fig. 2). These results suggest that depletion of CFIm25 is likely to influence NGF-induced neuritogenesis through regulation of the RhoA/ROCK signaling pathway.

## 4. Discussion

To the best of our knowledge, we have demonstrated for the first time that CFIm plays a role in the development of neuronal cells because knockdown of the CFIm25 gene expression promoted NGF-induced neurite extension in PC12 cells and formation of the primary dendrite in hippocampal neurons.

Here, we demonstrated that the knockdown efficiency of siRNA-2 against the CFIm25 gene was comparable to that of siRNA-3 at 24 h after transfection, following which it was weak and transient; however, the effects of both the siRNAs on neurite extension was indistinguishable (Fig. 2B). In addition, when the cells were proliferating in serum-containing medium, CFIm25 knockdown did not affect the morphology, cell viability, and proliferative activity of PC12 cells (data not shown). A similar observation was reported in CFIm25 knockdown in HeLa cells [11]. Given the importance of CFIm in gene expression, these results indicate that depletion of CFIm25 would likely alter 3′-end processing of primary transcripts, which is essential for neuron-like differentiation and expression induced by NGF at an early stage of neuritogenesis.

In the NGF-induced signaling response, rapid transient inactivation of RhoA and sustained activation of ERK1/2 are essential for neurite outgrowth from PC12 cells [21,22]. CFIm25 gene depletion did not affect activation of ERK1/2 induced by NGF, but decreased RhoA activity prior to NGF treatment. In addition, both hyper- and hypoactivation of RhoA attenuated the effect of CFIm25 knockdown on neurite extension. Therefore, CFIm25 gene knockdown can modify the signaling pathway that inactivates RhoA activity without affecting ERK1/2 activity in response to NGF. However, preliminary observations with qPCR suggested that RhoA genes do not undergo alternative poly(A) selection (Supplementary Results), leading to a hypothesis that CFIm25 knockdown is likely to affect the 3′-end processing of specific transcripts and not all transcripts. RhoA activity is regulated by several factors and such

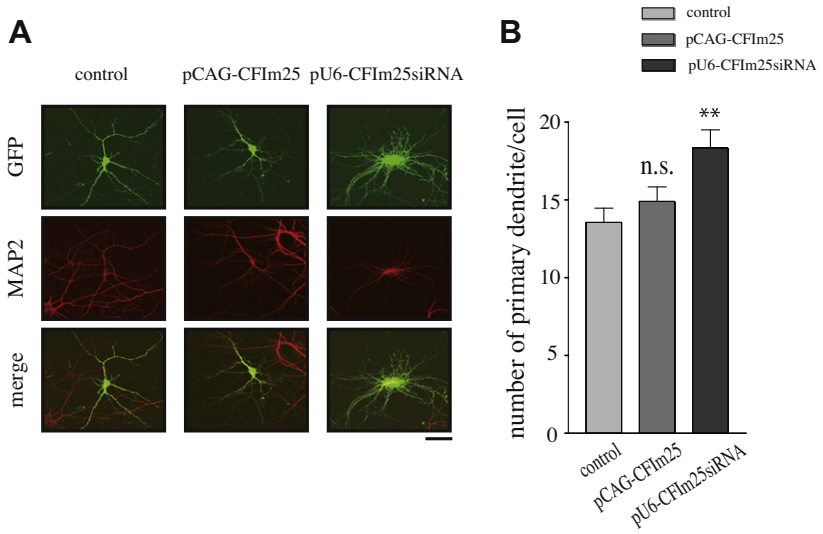


**Fig. 3.** Effects of CFIm25 overexpression and CFIm25 siRNAs on morphology of neurons. (A) Hippocampal neurons were transiently transfected with pCAG-GFP and pU6 empty vector (control) and pCAG-CFIm25 or pU6-CFIm25 siRNA. Neurons were transfected at 4DIV and the immunofluorescence images were acquired with a confocal microscope 96 h after transfection. Scale bar, 50 μm. (B) The number of primary dendrites of GFP/MAP2-positive neurons was measured as described in Materials and Methods. The values are expressed as mean ± SE ($n = 4$). Significant differences from the value of the control cells were determined by one-way ANOVA followed by Tukey's test. $^{**}p < 0.005$, n.s. not significant.

factors are strong candidates for molecules influenced by CFIm25 depletion. Recently, Jeon et al. [26] suggested that inactivation of RhoA induced by NGF treatment is mediated by two GTPase-activating proteins (RhoGAP), p190RhoGAP and Rap-dependent RhoGAP. Alternatively, because CFIm25 depletion was relatively weak and transient in PC12 cells compared with that in in HeLa cells [11], it is possible that 3′-end processing was affected in fewer pre-mRNA species in the present study. For future studies, it will be important to investigate the relationship between the amount of CFIm25 and 3′-end processing in subsets of neural transcripts. In fact, an *in vitro* study demonstrated that the amount of CFIm controls the efficacy of cleavage sites and poly(A) selection of CFIm68 mRNA [10].

Expressions of many neural transcripts need to be regulated in an activity-dependent manner, allowing neurons to develop their morphology and adapt their connectivity in an experience-dependent manner. Currently, very little is known about how 3′-end processing is controlled. However, recent studies suggest the possibility that environmental cues induce phosphorylation or acetylation of at least one subunit of CFIm, including CFIm25, and modify 3′-end processing in HeLa cells [27,28]. Such regulatory molecules of CFIm are also known to play key roles in biochemical processes involved in neuronal development by controlling activity-dependent gene transcription and/or signaling pathways [29,30]. Thus, the function and/or expression of CFIm may be regulated by neuronal activity, which leads to spatio-temporal control of neuronal morphology and function by collaboration of 3′-end processing with RNA-binding proteins and miRNA for post-transcriptional modification.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.164.

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