SCYL1 arginine methylation by PRMT1 is essential for neurite outgrowth via Golgi morphogenesis

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ABSTRACT **Arginine methylation is a common posttranslational modification that modulates protein function. SCY1-like pseudokinase 1 (SCYL1) is crucial for neuronal functions and interacts with γ_2-COP to form coat protein complex I (COPI) vesicles that regulate Golgi morphology. However, the molecular mechanism by which SCYL1 is regulated remains unclear. Here, we report that the γ_2-COP-binding site of SCYL1 is arginine-methylated by protein arginine methyltransferase 1 (PRMT1) and that SCYL1 arginine methylation is important for the interaction of SCYL1 with γ_2-COP. PRMT1 was colocalized with SCYL1 in the Golgi fraction. Inhibition of PRMT1 suppressed axon outgrowth and dendrite complexity via abnormal Golgi morphology. Knockdown of SCYL1 by small interfering RNA (siRNA) inhibited axon outgrowth, and the inhibitory effect was rescued by siRNA-resistant SCYL1, but not SCYL1 mutant, in which the arginine methylation site was replaced. Thus, PRMT1 regulates Golgi morphogenesis via SCYL1 arginine methylation. We propose that SCYL1 arginine methylation by PRMT1 contributes to axon and dendrite morphogenesis in neurons.**

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INTRODUCTION

The Golgi apparatus plays a central role in the posttranslational modification and trafficking of proteins and lipids. In neurons, the Golgi apparatus is involved in axon and dendrite outgrowth via vesicle trafficking (Rosso *et al.*, 2004; Ori-McKenney *et al.*, 2012; Villarroel-Campos *et al.*, 2014) and regulates the development of apical and basal dendrites in embryonic pyramidal neurons (Horton *et al.*, 2005; Matsuki *et al.*, 2010). Golgi apparatus destruction causes Purkinje cell neuron loss and ataxia (Liu *et al.*, 2017). Therefore, Golgi organization is vital for neuronal development. Golgi organization is regulated by coat protein complex I (COPI; Papanikou *et al.*, 2015; Ishii *et al.*, 2016). COPI functions not only in protein trafficking but also in transport between Golgi cisternae for Golgi organization (Cosson *et al.*, 2002; Emr *et al.*, 2009). COPI-mediated transport is important for brain growth (Xu *et al.*, 2010; Izumi *et al.*, 2016). Thus, COPI performs a critical role in Golgi organization and brain development.

SCY1-like pseudokinase 1 (SCYL1) is a member of the SCY1-like family of catalytically inactive protein kinases that regulates Golgi morphology (Burman *et al.*, 2010; Chafe and Mangroo, 2010). SCYL1 modulates COPI-mediated retrograde transport by interacting with γ_2-COP, a COPI subunit (Burman *et al.*, 2008; Hamlin *et al.*, 2014; Pelletier, 2016). Therefore, SCYL1 is involved in Golgi apparatus organization via COPI. SCYL1 is prominently expressed in central nervous system (CNS) neurons (Schmidt *et al.*, 2007). SCYL1 variants

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Abbreviations used: AdOx, oxidized adenosine; AMI-1, arginine methyltransferase inhibitor 1; ARF4, ADP ribosylation factor 4; BDNF, brain-derived neurotrophic factor; CALFAN, low γ-glutamyl-transferase cholestasis, acute liver failure and neurodegeneration; CNS, central nervous system; COPI, coat protein complex I; DIV, days in vitro; ER, endoplasmic reticulum; ERGIC53, 53-kDa ER-Golgi intermediate compartment; GM130, 130-kDa *cis*-Golgi matrix protein; NGF, nerve growth factor; NT-3, neurotrophin-3; PRMT1, protein arginine methyltransferase 1; SCYL1, SCY1-like pseudokinase 1; siRNA, small interfering RNA.

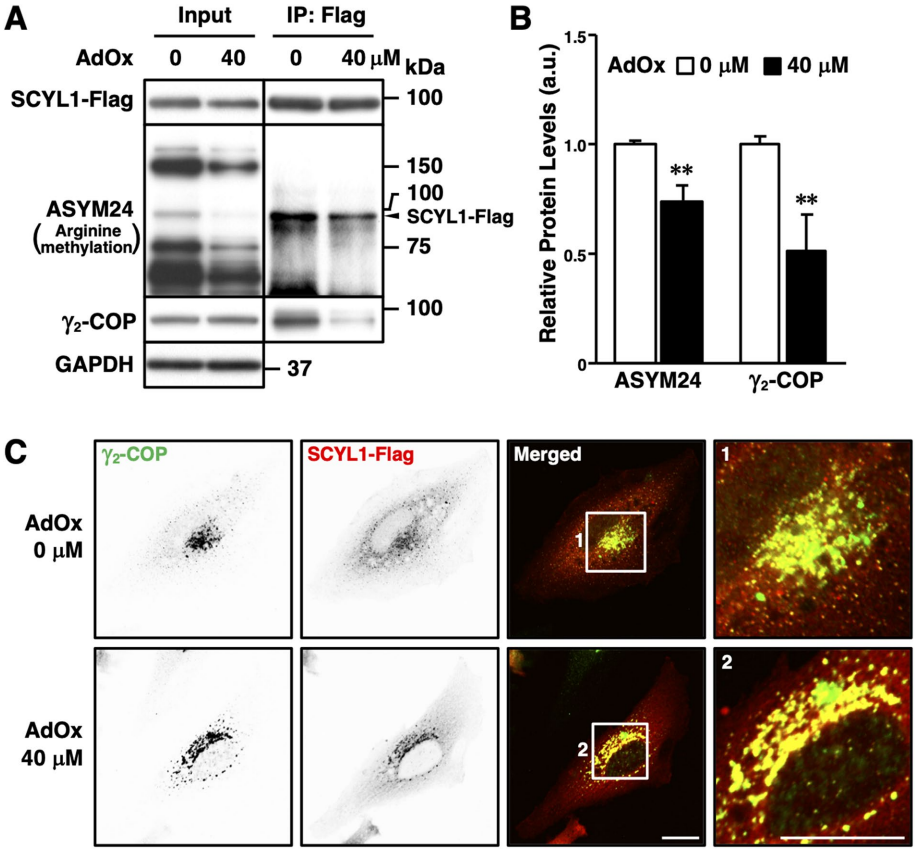

FIGURE 1: Arginine methylation of SCYL1 is important for the interaction between SCYL1 and γ_2-COP. (A) HeLa cells transfected with SCYL1-Flag were treated or not treated with 40 μM AdOx (a methylation inhibitor) for 24 h before immunoprecipitation with an anti-Flag antibody. The immunoprecipitates were subjected to SDS–PAGE and immunoblot analysis with ASYM24 (asymmetrical dimethylated arginine) and anti-Flag and anti-γ_2-COP antibodies. (B) Quantification of immunoblots from immunoprecipitation assays with anti-Flag antibody. Values were normalized to controls. Unpaired, two-tailed Student's *t* test; $^{**}p < 0.01$. Error bars indicate the mean ± SEM ($n = 4$). (C) HeLa cells expressing SCYL1-Flag were treated or not treated with 40 μM AdOx for 24 h before fixation. The cells were immunostained with anti-γ_2-COP (green) and anti-Flag (red) antibodies. The boxed areas (1, 2) are enlarged in the right panels. Results are representative of more than three experiments. Scale bar: 20 μm.

cause brain development delay (Lenz *et al.*, 2018); thus, SCYL1 is important for brain development via COPI regulation. However, little is known of the molecular mechanism by which SCYL1 regulates Golgi organization via γ_2-COP.

Protein arginine methylation is a major posttranslational modification. Protein arginine methyltransferase 1 (PRMT1) performs monomethylation and asymmetric dimethylation of proteins. PRMT1 modulates most of the arginine methylation in mammalian cells, and regulates transcription, cell death, DNA damage responses, and signal transduction (Tang *et al.*, 2000; Bedford and Clarke, 2009; Lorton and Shechter, 2019). PRMT1 is required for CNS development at embryonic and perinatal stages (Hashimoto *et al.*, 2016). Protein arginine methylation by PRMT1 is vital for brain development. However, the association between Golgi organization and PRMT1 in neuronal development remains unclear.

In this study, we aimed to unravel the molecular mechanism behind the relationship between Golgi morphogenesis and arginine methylation by PRMT1. We report that PRMT1 regulates the interaction between SCYL1 and γ_2-COP for Golgi organization via SCYL1 arginine methylation. Arginine methylation by PRMT1 is involved in axon outgrowth and dendrite complexity. We propose that PRMT1 control of Golgi morphogenesis is important for axon outgrowth and dendrite complexity in neurons.

RESULTS

Methylation of the C-terminal arginine of SCYL1 modulates its interaction with γ_2-COP

SCYL1 is known to interact with γ_2-COP (Hamlin *et al.*, 2014). Arginine methylation can be involved in protein–protein interactions (Hofweber *et al.*, 2018); therefore, we speculated that the methylation of SCYL1 arginine enables SCYL1 interaction with γ_2-COP. We investigated whether SCYL1 arginine is methylated (Figure 1A). Oxidized adenosine (AdOx) is a methylation inhibitor, and 40 μM AdOx is effective in cultured cells (Bartel and Borchardt, 1984; Hoffman and McConnell, 1987; Le Romancer *et al.*, 2008; Toriyama *et al.*, 2017). HeLa cells were transfected with SCYL1-Flag and cultured in the presence of 40 μM AdOx. SCYL1-Flag was then immunoprecipitated with an anti-Flag antibody. The immunoprecipitates were analyzed by immunoblotting using anti-asymmetrical dimethylated arginine antibody (ASYM24) to detect SCYL1 arginine methylation (Figure 1A). SCYL1-Flag arginine methylation was detected, and the methylation levels were decreased by AdOx treatment (Figure 1, A and B). Under these conditions, AdOx treatment did not affect the expression levels of SCYL1-Flag (Figure 1A). These results indicate that SCYL1 is methylated at the arginine residues. We then examined whether the arginine methylation of SCYL1 affects its binding to γ_2-COP. γ_2-COP was coimmunoprecipitated with SCYL1-Flag after the cells were incubated with and without AdOx. The amount of coimmunoprecipitated γ_2-COP was reduced in the presence of AdOx (Figure 1, A and B). AdOx treatment did not change γ_2-COP expression levels (Figure 1A). These results indicate that SCYL1 arginine methylation is important for the interaction of SCYL1 with γ_2-COP. To investigate the effect of SCYL1 and γ_2-COP distribution on the inhibition of arginine methylation, HeLa cells expressing SCYL1-Flag in the presence of AdOx were fixed and then immunostained with anti-γ_2-COP (green) and anti-Flag (SCYL1, red) antibodies (Figure 1C). The inhibition of arginine methylation did not affect the distribution of SCYL1 and γ_2-COP in HeLa cells (Figure 1C). These results indicate that SCYL1 arginine methylation is not crucial for the distribution of γ_2-COP.

SCYL1 has two isoforms, and the binding site for γ_2-COP in both isoforms is the C-terminus Arg–Lys–Leu–Asp amino acid sequence (Figure 2A; Hamlin *et al.*, 2014). To investigate whether the C-terminal arginine of SCYL1 is methylated, we used Flag-tagged SCYL1 isoform B wild-type (RKLD) and mutant (AKLD; C-terminal Arg was replaced by Ala). HeLa cells were transfected with SCYL1-Flag RKLD or SCYL1-Flag AKLD mutant, and then the cell lysates were immunoprecipitated with an anti-Flag antibody. Immunoblotting

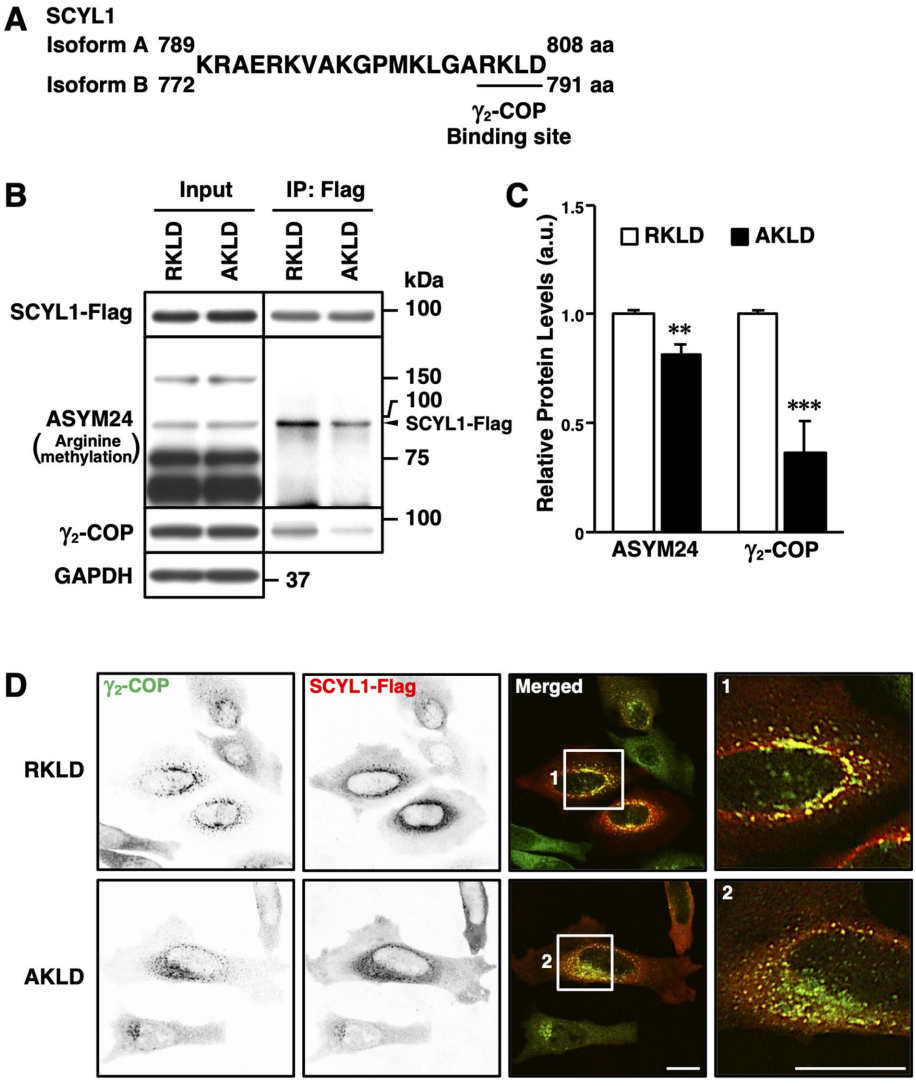

FIGURE 2: C-terminal arginine methylation of SCYL1 is crucial for the interaction of SCYL1 with γ_2-COP. (A) Alignment of the human SCYL1 amino acid sequence. SCYL1 has two isoforms. Both isoforms have an Arg–Lys–Leu–Asp sequence at the C-terminus. aa: amino acid. (B) HeLa cells were transfected with wild-type (RKLD) or mutant (AKLD) SCYL1-Flag for 24 h. Immunoprecipitation assays were performed with an anti-Flag antibody. The immunoprecipitates were subjected to SDS–PAGE and immunoblot analysis with ASYM24 (asymmetrical dimethylated arginine) and with anti-Flag and anti-γ_2-COP antibodies. (C) Quantification of immunoblots from immunoprecipitation assays with the anti-Flag antibody. Values were normalized to controls. Unpaired, two-tailed Student's *t* test; $^{**}p < 0.01$, $^{***}p<0.001$. The data are shown as the mean ± SEM ($n = 7$). (D) HeLa cells were transfected with wild-type (RKLD) or mutant (AKLD) SCYL1-Flag for 24 h. The cells were fixed and immunostained with anti-γ_2-COP (green) and anti-Flag (red) antibodies. The boxed areas (1, 2) are enlarged in the right panels. Results are representative of more than three experiments. Scale bar: 20 μm

analysis showed that the levels of arginine methylation in the SCYL1-Flag AKLD mutant were decreased compared with those in SCYL1-Flag RKLD (Figure 2, B and C). We then examined whether the C-terminus arginine methylation of SCYL1 affects its binding to γ_2-COP. The amount of γ_2-COP coimmunoprecipitated with the SCYL1-Flag AKLD mutant was decreased compared with that of SCYL1-Flag RKLD (Figure 2, B and C). These results indicate that C-terminal arginine methylation of SCYL1 plays a key role in the interaction between SCYL1 and γ_2-COP. SCYL1 and γ_2-COP distribution were then investigated by inhibiting C-terminal arginine methylation of SCYL1 in HeLa cells expressing SCYL1-Flag RKLD or SCYL1-Flag AKLD mutant. These cells were fixed and then immunostained with anti-γ_2-COP (green) and anti-Flag (SCYL1, red) antibodies (Figure 2D). Inhibition of arginine methylation did not affect the distribution of SCYL1 and γ_2-COP (Figure 2D). These results indicate that SCYL1 C-terminal arginine methylation is not essential for the distribution of γ_2-COP.

PRMT1 regulates the SCYL1 arginine methylation that is necessary for the interaction between SCYL1 and γ_2-COP

PRMT1 is the primary arginine methyltransferase and is localized in both the nucleus and the cytoplasm. (McBride and Silver, 2001; Herrmann *et al.*, 2005; Suchánková *et al.*, 2014). To examine whether PRMT1 is localized with SCYL1 in the microsomes, 12 endoplasmic reticulum (ER)–Golgi fractions were prepared by iodixanol density gradient centrifugation and subjected to immunoblot analysis with several antibodies (Figure 3A). GM130, a *cis*-Golgi marker, was contained in the low-density fractions (fractions 1–3); ERGIC53, an ER-Golgi intermediate compartment marker, was present in the intermediate- and high-density fractions (fractions 4–12); and Calnexin, an ER-associated protein, was detected in the high-density fractions (fraction 10 and 11; Figure 3A). Under these conditions, we found that PRMT1 was colocalized with ADP-ribosylation factor 4 (ARF4), γ_2-COP, and SCYL1 in Golgi fractions, indicating colocalization with COPI vesicles (Figure 3A). We investigated whether SCYL1-Flag RKLD or SCYL1-Flag AKLD mutant are localized in Golgi fractions. Both SCYL1-Flag RKLD and SCYL1-Flag AKLD mutant were colocalized with γ_2-COP and PRMT1 in Golgi fractions (Supplemental Figure S1). The result indicates that SCYL1 C-terminal arginine methylation is not important for the localization of SCYL1 in the Golgi apparatus. Next, to investigate whether SCYL1 is arginine-methylated by PRMT1, knockdown experiments were performed using small interfering RNA (siRNA) of PRMT1. HeLa cells expressing SCYL1-Flag were transfected with PRMT1 siRNA. The expression levels of PRMT1 were dramatically decreased in the PRMT1 knockdown cells (Figure 3B). We found that the levels of arginine methylation in SCYL1-Flag were decreased by PRMT1 knockdown (Figure 3, B and C). The amount of γ_2-COP coimmunoprecipitated with SCYL1-Flag in PRMT1 knockdown cells was decreased in comparison with that in control cells (Figure 3, B and C). PRMT1 knockdown did not affect the expression levels of SCYL1-Flag and γ_2-COP. These results indicate that PRMT1 regulates the interaction between SCYL1 and γ_2-COP in Golgi fractions via SCYL1 arginine methylation.

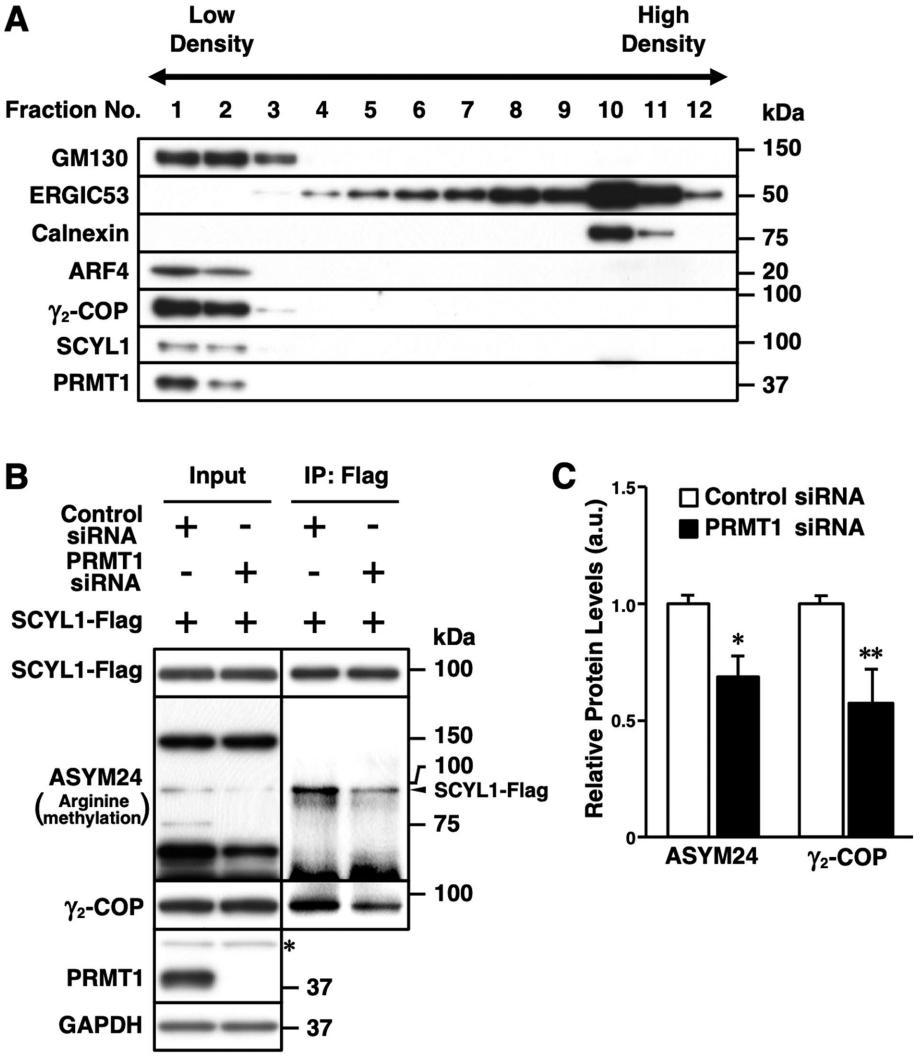

FIGURE 3: PRMT1 is colocalized with SCYL1 in microsomal fractions and regulates SCYL1 arginine methylation for the interaction between SCYL1 and γ_2-COP. (A) Microsomal fractions extracted from HeLa cells were ultracentrifuged using iodixanol concentration gradients. After ultracentrifugation, 12 fractions were collected from the upper layer and immunoblot analysis was performed with anti-GM130 (a *cis*-Golgi marker), anti-ERGIC53 (an ER-Golgi intermediate compartment marker), anti-Calnexin (an ER-associated protein), anti-ARF4 (a COPI vesicle-associated small GTPase), anti-γ_2-COP, anti-SCYL1, and anti-PRMT1 antibodies. (B) HeLa cells were treated with control or PRMT1 siRNA for 24 h before being transfected with SCYL1-Flag. Immunoprecipitation assays were performed with an anti-Flag antibody. The immunoprecipitates were subjected to SDS–PAGE and immunoblot analysis with ASYM24 (asymmetrical dimethylated arginine) and with anti-Flag and anti-γ_2-COP antibodies. The asterisk indicates nonspecific bands. (C) Quantification of immunoblots of immunoprecipitates. Values were normalized to controls. Unpaired, two-tailed Student's *t* test; $*p < 0.05$, $**p < 0.01$. Error bars indicate the mean ± SEM ($n = 5$).

PRMT1 is essential for Golgi morphology

To examine whether PRMT1 modulates Golgi organization, HeLa cells were transfected with PRMT1 siRNA and then the cell lysates were subjected to immunoblot analysis with Golgi apparatus, ER, ER–Golgi intermediate compartment, and COPI vesicle markers (Figure 4, A and B). The expression levels of PRMT1 were dramatically decreased by PRMT1 knockdown (Figure 4, A and B). Under these conditions, the expression levels of GM130 were increased about 2.2-fold by PRMT1 knockdown (Figure 4, A and B), while there was no effect on the expression levels of other organelle and COPI vesicle markers as compared with those of the controls (Figure 4, A and B). SCYL1 knockdown affects Golgi morphogenesis (Burman *et al.*, 2010). We examined whether SCYL1-Flag AKLD mutant affects the expression levels of GM130. The expression levels of GM130 were not affected by SCYL1-Flag AKLD mutant compared with SCYL1-Flag RKLD (Supplemental Figure S2, A and B). Next, we investigated whether PRMT1 regulates Golgi morphology. PRMT1 knockdown cells were fixed and then immunostained with anti-PRMT1 (green) and anti-GM130 (red) antibodies (Figure 4C). The Golgi apparatus was enlarged and fragmented in PRMT1 knockdown cells (Figure 4C). These results indicate that PRMT1 is essential for Golgi morphology.

SCYL1 is arginine-methylated in neurons

We examined whether endogenous SCYL1 is arginine-methylated by PRMT1. HEK293T cells were transfected with PRMT1 siRNA. Endogenous SCYL1 was then immunoprecipitated with an anti-SCYL1 antibody and subjected to immunoblot analysis with ASYM24 and anti-SCYL1 antibodies (Figure 5A). SCYL1 arginine methylation was detected, and the methylation levels were decreased by PRMT1 knockdown. These results indicate that endogenous SCYL1 is arginine methylated by PRMT1 in HEK293T cells.

SCYL1 is essential for brain development (Schmidt *et al.*, 2007; Lenz *et al.*, 2018). To determine whether SCYL1 is arginine-methylated in the brain, we immunoprecipitated SCYL1 from mouse embryonic brain lysates. Immunoblot analysis showed that SCYL1 was arginine-methylated in the mouse brain (Figure 5B). Next, we investigated whether SCYL1 is arginine-methylated in cultured neurons. We immunoprecipitated SCYL1 from cultured rat hippocampal neuron lysates. The immunoprecipitates were analyzed by immunoblotting using anti-asymmetrical dimethylated arginine (ADMA) antibody to detect SCYL1 arginine methylation (Figure 5C). SCYL1 arginine methylation was detected. The result indicates that endogenous SCYL1 is arginine-methylated in cultured neurons.

Golgi abnormality caused by PRMT1 inhibition affects axon outgrowth and dendrite complexity

To examine the effect of PRMT1 inhibition on Golgi morphology in neurons, hippocampal neurons were cultured in the presence of the PRMT1 inhibitor, arginine methyltransferase inhibitor 1 (AMI-1). Immunoblot analysis with an anti-GM130 antibody revealed that the expression levels of GM130 were increased about 2-fold by AMI-1 treatment (Figure 6, A and B). These results are consistent with the effect of PRMT1 knockdown in HeLa cells.

Next, we investigated whether PRMT1 is involved in axon outgrowth. Hippocampal neurons were cultured with AMI-1 or AdOx

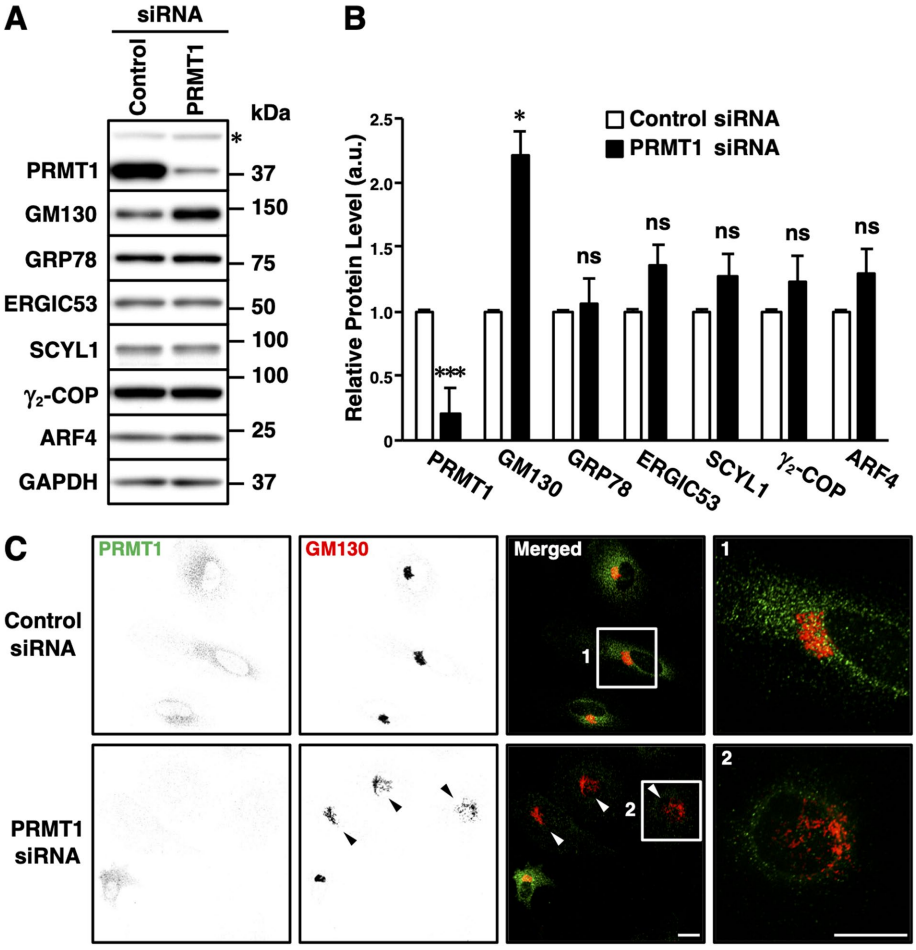

FIGURE 4: PRMT1 regulates Golgi organization. (A) HeLa cells were transfected with control or PRMT1 siRNA for 24 h. The samples were subjected to SDS–PAGE and immunoblot analysis with anti-PRMT1, anti-GM130 (a *cis*-Golgi marker), anti-GRP78 (an ER chaperone protein), anti-ERGIC53 (an ER-Golgi intermediate compartment marker), anti-SCYL1, anti-γ_2-COP, anti-ARF4 (a COPI vesicle-associated small GTPase), and anti-GAPDH antibodies. The asterisks indicate nonspecific bands. (B) Expression levels of indicated proteins were measured by quantitative immunoblotting. Values were normalized to controls. Unpaired, two-tailed Student's *t* test; $^*p < 0.05$, $^{***}p < 0.001$. Error bars indicate the mean ± SEM ($n = 6$). ns: not significant. (C) HeLa cells were transfected with control siRNA or PRMT1 siRNA. The cells were immunostained with anti-PRMT1 (green) and anti-GM130 (red) antibodies. The boxed areas (1, 2) are enlarged in the right panels. The arrowheads indicate PRMT1 knockdown cells. Representative results from three independent experiments are shown. Scale bars: 20 μm.

for 48 h and fixed at 3 d in vitro (DIV). These neurons were immunostained with Tau-1 antibody, an axonal marker. We measured the length of the longest Tau-1–positive neurite as an axon. Both AMI-1 and AdOx decreased axon outgrowth (Figure 6, C–E). To examine whether inhibition of arginine methylation affects dendrite complexity, hippocampal neurons were treated with AMI-1 and AdOx for 72 h and fixed at 14 DIV (Figure 7A). Dendrite complexity was assessed by Sholl analysis (Figure 7B). Both AMI-1 and AdOx decreased dendrite complexity (Figure 7C). These results indicate that PRMT1 regulates axon outgrowth and dendrite complexity by modulating Golgi morphology.

Methylation of the C-terminal arginine of SCYL1 plays an essential role in axon outgrowth

Rat-1 cells were transfected with SCYL1 siRNA, and then the cell lysates were subjected to immunoblot analysis with anti-SCYL1 and anti-GAPDH antibodies. The expression of SCYL1 was dramatically decreased by the siRNA treatment (Figure 8A), indicating that the siRNA for SCYL1 is effective in rat cells. It is reported that SCYL1 knockdown causes Golgi fragmentation (Burman *et al.*, 2010). We confirmed that SCYL1 knockdown affects Golgi morphology. Rat-1 cells were transfected with SCYL1 siRNA. The Golgi apparatus was fragmented in SCYL1 knockdown cells (Supplemental Figure S3A). To investigate whether the abnormal Golgi morphology caused by SCYL1 knockdown is rescued by expressing SCYL1-Flag RKLD, rescue experiments were performed using siRNA-resistant SCYL1-Flag RKLD or AKLD mutant in SCYL1 knockdown cells (Supplemental Figure S3B). The fragmented Golgi morphology was rescued by the expression of siRNA-resistant SCYL1-Flag RKLD but not AKLD mutant. These results indicate that C-terminal arginine methylation of SCYL1 is essential for Golgi morphogenesis.

To investigate whether SCYL1 knockdown affects axon outgrowth, cultured rat hippocampal neurons were cotransfected with SCYL1 siRNA and GST using electroporation. These neurons were fixed at 3 DIV and immunostained with anti-Flag (green) and anti-GST (red) antibodies. Neuronal morphology was visualized using GST immunostaining, and the length of the longest neurite was measured as an axon. Knockdown of SCYL1 by siRNA inhibited axon outgrowth (Figure 8, B and C). Next, rescue experiments were performed using siRNA-resistant SCYL1-Flag RKLD or AKLD mutant. The inhibition of axon outgrowth by SCYL1 knockdown was fully rescued by the expression of siRNA-resistant SCYL1-Flag RKLD but not AKLD mutant (Figure 8, B and C). These results indicate that PRMT1 regulates axon outgrowth via SCYL1 arginine methylation.

DISCUSSION

In the present study, we have shown that the C-terminal arginine of SCYL1 is asymmetrically dimethylated by PRMT1. The interaction between SCYL1 and γ_2-COP was decreased by the inhibition of SCYL1 arginine methylation. Therefore, SCYL1 arginine methylation via PRMT1 is necessary for SCYL1 to interact with γ_2-COP. In PRMT1 knockdown cells, the expression of the *cis*-Golgi marker, GM130, was increased and the Golgi apparatus was enlarged and fragmented. The Golgi apparatus was shown to be similarly fragmented, with an increased volume in SCYL1 knockdown cells (Burman *et al.*, 2010). COPI knockdown was also shown to increase the size of the Golgi complex (Guo *et al.*, 2008). These observations are similar to the Golgi abnormalities caused by PRMT1 knockdown. Together, the results of this study demonstrate that PRMT1 regulates Golgi morphology via SCYL1 arginine methylation and the formation of COPI vesicles (Figure 9).

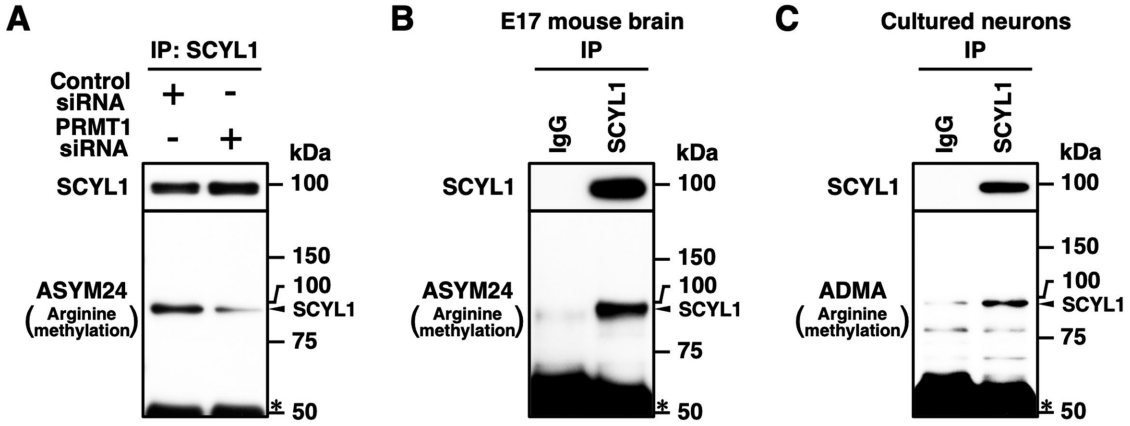

FIGURE 5: Endogenous SCYL1 is arginine- methylated. (A) HEK293T cells were treated with control or PRMT1 siRNA for 48 h. Endogenous SCYL1 was immunoprecipitated with an anti-SCYL1 antibody. The immunoprecipitates were subjected to SDS–PAGE and immunoblot analysis with ASYM24 and anti-SCYL1 antibodies. The asterisk indicates an immunoglobulin heavy chain. (B) Immunoprecipitation of endogenous SCYL1 from mouse brain lysate. Extract of developmental mouse brain was incubated with rabbit IgG or anti-SCYL1 antibody. The immunoprecipitates were subjected to SDS–PAGE and immunoblot analysis with ASYM24 and anti-SCYL1 antibodies. The asterisk indicates an immunoglobulin heavy chain. (C) Immunoprecipitation of endogenous SCYL1 from cultured neuron lysate. After rat hippocampal neurons were cultured for 3 d, immunoprecipitation assays were performed with rabbit IgG or anti-SCYL1 antibody. The immunoprecipitates were analyzed by immunoblotting with ADMA (asymmetrical dimethylated arginine) and anti-SCYL1 antibodies. The asterisk indicates an immunoglobulin heavy chain.

We showed that the expression levels of GM130, a *cis*-Golgi marker, were similarly increased in HeLa cells by AMI-1 treatment and PRMT1 knockdown. The treatment with arginine methylation inhibitors led to the suppression in the axon outgrowth and dendrite complexity via abnormal Golgi morphology. Moreover, the knockdown of SCYL1 by siRNA inhibited axon outgrowth. The inhibition of axon outgrowth by SCYL1 siRNA was fully rescued by siRNA-resistant SCYL1-Flag RKLD, but not AKLD mutant. Therefore, SCYL1 arginine methylation by PRMT1 is essential for axon outgrowth via altering Golgi morphology. Rab family small GTPase proteins affect neurite outgrowth by regulating vesicle trafficking, and are colocalized with the Golgi apparatus (Nakazawa *et al.*, 2012; Villarroel-Campos *et al.*, 2014). Both downregulation and overexpression of them in mouse primary hippocampal neurons affect neuronal branching (Mori *et al.*, 2012; Vanmarsenille *et al.*, 2014). These studies show that Golgi morphology is important for neuronal maturation via Rab family protein–controlled vesicle trafficking. The inhibition of axon outgrowth by SCYL1 siRNA suggests that the abnormal Golgi morphology via SCYL1 knockdown is involved in the dysfunction of Rab family proteins. Therefore, our findings support a model where SCYL1 arginine methylation by PRMT1 participates in Rab family vesicle trafficking via Golgi morphology alteration and modulates axon outgrowth and dendrite complexity through the axon and dendrite trafficking (Figure 9).

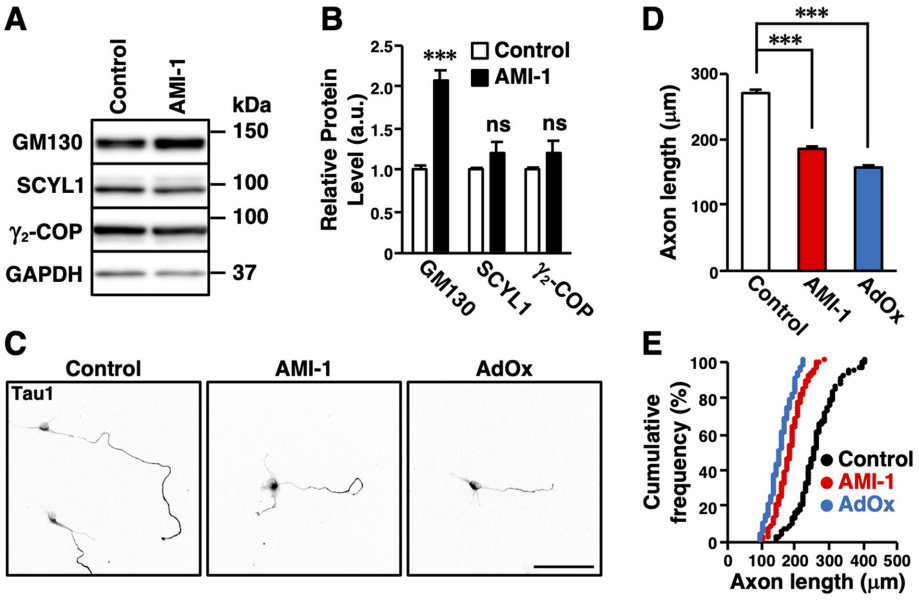

FIGURE 6: Arginine methylation is important for axon outgrowth. (A) Hippocampal neurons were treated for 48 h with 10 μM AMI-1 (a PRMT1 inhibitor). The samples were subjected to SDS–PAGE and immunoblot analysis with the indicated antibodies. (B) Expression levels of indicated proteins were measured by quantitative immunoblotting. Values were normalized to controls. Unpaired, two-tailed Student's *t* test; ***$p < 0.001$. Error bars indicate the mean ± SEM ($n = 3$). ns: not significant. (C) Hippocampal neurons were cultured in the presence of AMI-1 or AdOx for 48 h before fixation. Neurons were fixed at 3 DIV and then immunostained with Tau-1 antibody, an axonal marker. Scale bar: 100 μm. (D) Axon length was measured at 3 DIV in neurons treated with the indicated inhibitors. Unpaired, two-tailed Student's *t* test; ***$p < 0.001$. The data are shown as the mean ± SEM of three independent experiments (120 axons from each group). (E) Cumulative frequency plots showing the distribution of axon length from individual neurons that were analyzed in *D*.

PRMT1 is activated by nerve growth factor (NGF) in PC12 cells (Cimato *et al.*, 1997). Inhibition of arginine methylation decreases NGF-induced neurite outgrowth in PC12 cells (Cimato *et al.*, 2002). Brain-derived

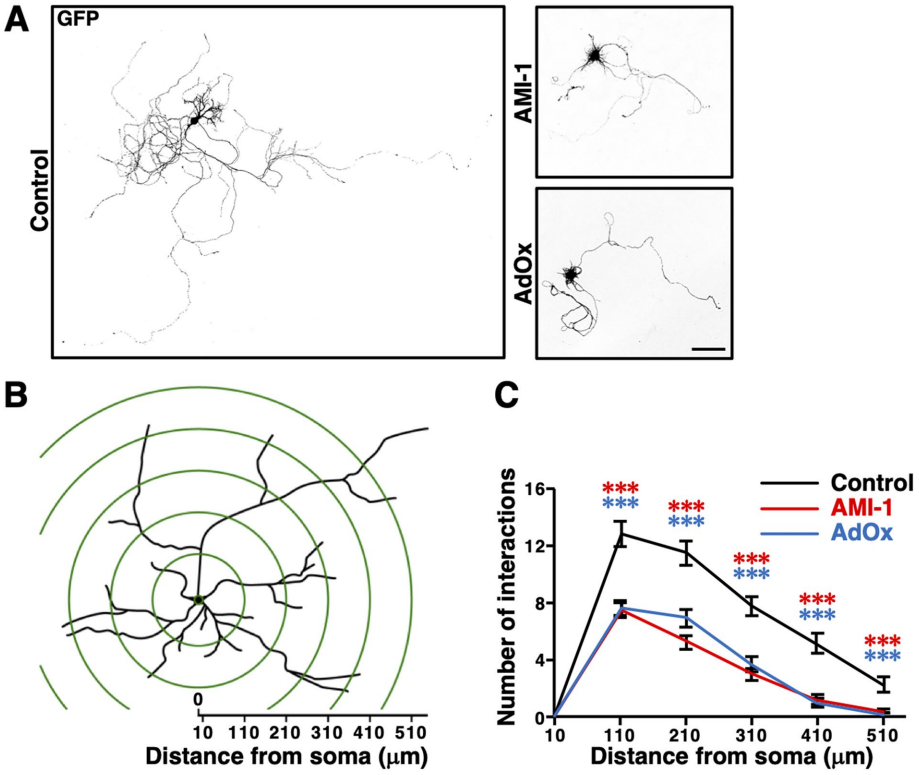

FIGURE 7: Arginine methylation is essential for dendrite complexity. (A) Hippocampal neurons were transfected with GFP at 10 DIV. Neurons were fixed at 14 DIV after treatment with indicated inhibitors for 72 h and then immunostained with an anti-GFP antibody. Scale bar: 100 μm. (B) Illustration of Sholl analysis used to quantify neurite complexity. The number of interactions made by the extending neurites with each circle was counted and used as a measure of neurite complexity. (C) Sholl analysis of neurite complexity in neurons treated as in (A). The mean numbers of intersections are plotted. Unpaired, two-tailed Student's *t* test; ***$p < 0.001$. Error bars indicate the mean ± SEM of four independent experiments (40 hippocampal neurons from each group).

neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), but not NGF, enhance neurite outgrowth and branching in hippocampal neurons (Ip *et al.*, 1993; Morfini *et al.*, 1994; Labelle and Leclerc, 2000). BDNF and NT-3 may lead to the activation of PRMT1 in hippocampal neurons. It is possible that BDNF and NT-3 enhance axon outgrowth and dendrite complexity through SCYL1 arginine methylation by PRMT1.

PRMT1 plays a vital role in the CNS development (Hashimoto *et al.*, 2016). In the mouse brain, the expression levels of PRMT1 are remarkably up-regulated during early embryonic to early fetal stages (Ikenaka *et al.*, 2006). CNS-specific PRMT1 knockout mice exhibit morphological abnormalities of the brain and die within 17 d after birth (Hashimoto *et al.*, 2016). It is possible that the early lethality of CNS-specific PRMT1 knockout mice is caused by the failure of COPI vesicle formation via inhibition of SCYL1 arginine methylation. A SCYL1 variant causes a delay of speech development and mild mental retardation (Lenz *et al.*, 2018). SCYL1 is expressed in the corpus callosum, cortical neurons, and brain-stem neurons and is subjected to axonal transport (Schmidt *et al.*, 2007). These studies indicate that SCYL1 is essential for neuronal development. We propose that SCYL1, methylated by PRMT1, is important for brain development.

A syndrome with low γ-glutamyl-transferase cholestasis, acute liver failure and neurodegeneration (CALFAN) is caused by SCYL1 variants (Lenz *et al.*, 2018). Recently, an induced pluripotent stem cell line was generated from a patient with CALFAN syndrome (Lenz *et al.*, 2019). The induced pluripotent stem cell lines generated from patients with CALFAN syndrome may be helpful to reveal that abnormal arginine methylation of SCYL1 is involved in CALFAN syndrome. SCYL1 variants induce chronic residual fibrotic liver disease and pronounced hepatomegaly (Schmidt *et al.*, 2015; Li *et al.*, 2019; Shohet *et al.*, 2019). In the hepatomegaly tissue, the Golgi apparatus is fragmented, and COPI vesicles are distributed throughout the cytoplasm (Li *et al.*, 2018). These findings indicate that SCYL1 is essential not only in the brain but also in the liver for Golgi morphology. The deletion of SCYL1 causes myopathological abnormalities and motor movement disfunction (Pelletier *et al.*, 2012). It has recently been shown that SCYL1 variants induce multiple skeletal anomalies, including short stature, lumbar lordosis, and abnormal bone anatomy (Lenz *et al.*, 2018). SCYL1 may, therefore, be involved in the function and morphology of other organs. Our demonstration of SCYL1 methylation by PRMT1 may be important not only for brain development but also for the development and maintenance of other organs.

In conclusion, this study provides evidence that PRMT1 acts via SCYL1 arginine methylation and is critical for Golgi morphology. In addition, PRMT1 is a crucial regulator for axon outgrowth and dendrite complexity. Therefore, this report contributes to the elucidation of brain development via arginine methylation.

MATERIALS AND METHODS

Materials and chemicals

The following antibodies were used: anti-ARF4 (11673-1-AP, Proteintech, Chicago, IL); anti-asymmetrical dimethylated arginine (ADMA, #13522, Cell Signaling Technology, Beverly, MA; ASYM24, 07-414, Millipore, Billerica, MA); anti-Bip/GRP78 (BD Bioscience, Franklin Lakes, NJ); anti-Calnexin (10427-2-AP, Proteintech); anti-ERGIC53 (sc-398777, Santa Cruz Biotechnology, Santa Cruz, CA); anti-Flag (F1804, Sigma-Aldrich, St. Louis, MO); anti-GAPDH (#5174, Cell Signaling Technology); anti-green fluorescent protein (GFP) (sc-9996, Santa Cruz Biotechnology); anti-GM130 (BD Bioscience); anti-γ_2-COP (sc-14165, Santa Cruz Biotechnology); anti-glutathione-S-transferase (GST) (ab9085, Abcam, Cambridge, UK); nonimmune immunoglobulin (30000-0-AP, Proteintech); anti-PRMT1 (#2449, Cell Signaling Technology); anti-SCYL1 (HPA015015, Atlas, Bromma, Sweden; ab95074, Abcam) and Tau-1 (#MAB3420, Millipore) antibodies. Secondary antibodies HRP-conjugated anti-IgG, Alexa Fluor 488, 568, and 595 were purchased from Abcam, Cell Signaling Technology, Invitrogen, and Proteintech. Arginine methylation inhibitor (oxidized adenosine [AdOx]) and PRMT1 inhibitor (AMI-1) were purchased from Sigma-Aldrich.

Plasmid constructs

To prepare the SCYL1-Flag plasmids, a DNA fragment encoding human SCYL1 isoform B and the Flag octapeptide was inserted into the *Eco*RI site of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The

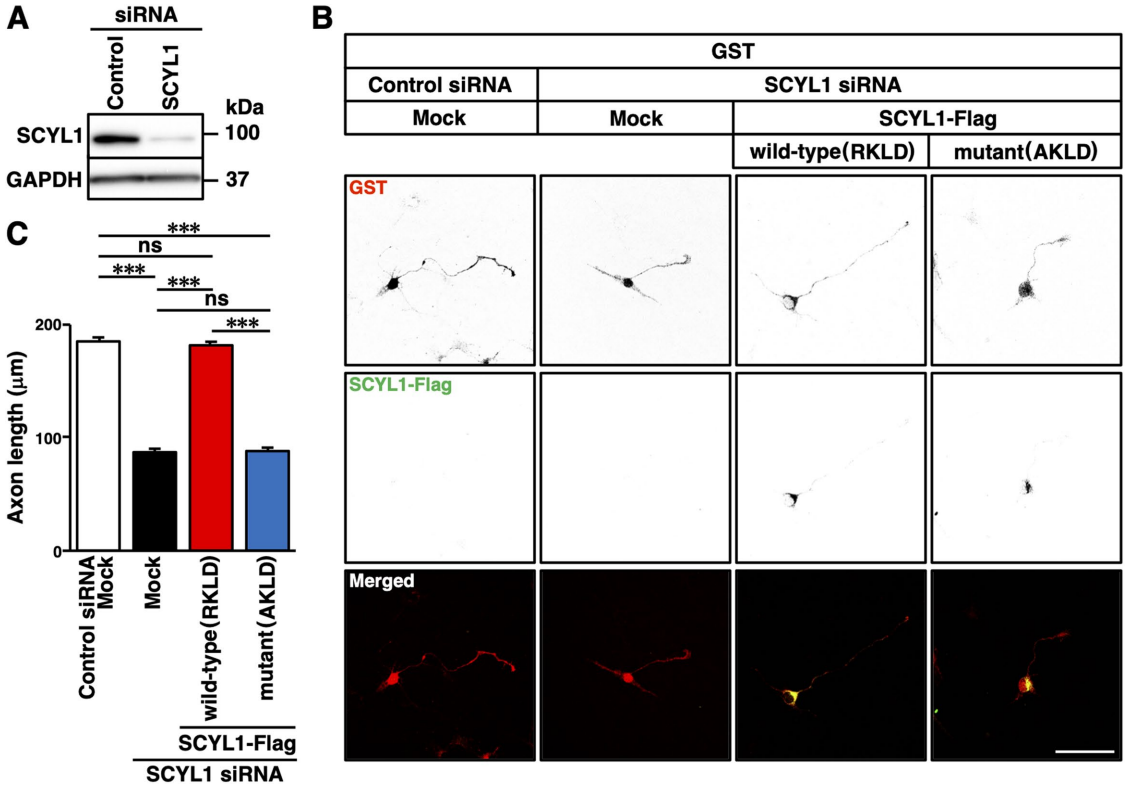

FIGURE 8: C-terminal arginine methylation of SCYL1 is important for axon outgrowth. (A) Rat-1 cells were transfected with control or SCYL1 siRNA. The samples were subjected to SDS–PAGE and immunoblot analysis with anti-SCYL1 and anti-GAPDH antibodies. (B) Cotransfection of GST and SCYL1 siRNA with siRNA-resistant SCYL1-Flag (wild-type or mutant) was performed in cultured hippocampal neurons by electroporation. Neurons were fixed at 3 DIV and then immunostained with anti-Flag (green) and anti-GST (red) antibodies. Scale bar: 100 μm. (C) Axon length was measured at 3 DIV in neurons treated as in B. Unpaired, two-tailed Student's *t* test; ***$p<0.001$. ns: not significant. The data are shown as the mean ± SEM of 120 axons from each group.

SCYL1-Flag AKLD mutant was generated by PCR using a mutation primer in which C-terminal Arg was replaced by Ala and the SCYL1-Flag construct as the template. All fragments were confirmed by DNA sequencing.

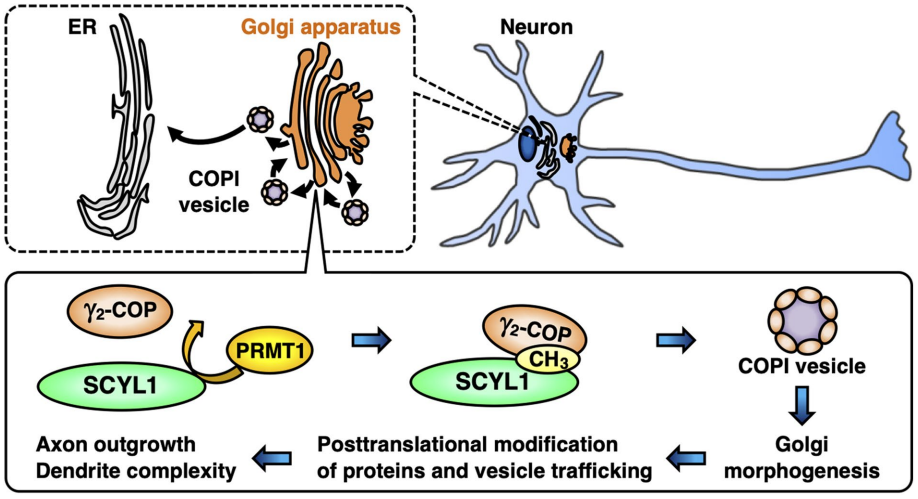

FIGURE 9: Schematic representation illustrating roles of SCYL1 arginine methylation by PRMT1 in neurons. C-terminal arginine of SCYL1 is methylated by PRMT1. SCYL1 methylated by PRMT1 interacts with γ_2-COP. COPI vesicles are formed via the interaction between methylated SCYL1 and γ_2-COP. Golgi organization by COPI vesicles enables posttranslational modification of axonal and dendritic proteins and vesicle trafficking, thereby enhancing axon elongation and dendrite branching.

Animals

All experimental procedures were approved by the Animal Experiment Committee of Osaka University and conducted in accordance with the Guidelines and Regulations on Animal Experimentation at Osaka University. Wistar rats and ICR mice were purchased from Japan SLC (Hamamatsu, Japan).

Preparation of whole-brain lysates

Brains from embryonic day 17 mouse embryos were dissected and homogenized in ice-cold homogenization buffer (1% NP-40 substitute, 1 mM EDTA, 150 mM NaCl, protease-inhibitor cocktail and 20 mM Tris-HCl, pH 8.0). The homogenates were then centrifuged at 100,000 × *g* for 1 h at 4°C. After centrifugation, the supernatants were collected as the mouse brain lysates. The brain lysates were used for immunoprecipitation.

Culture and transfection of HeLa, HEK293T, and Rat-1 cells

HeLa cells were seeded on 10-cm dishes (Iwaki, AGC Techno Glass, Haibara-gun, Japan) or on a Falcon 4-well Culture Slide (Corning, Corning, NY) in DMEM (Nacalai Tesque, Kyoto, Japan) containing

10% (vol/vol) fetal bovine serum (FBS; Life Technologies, Waltham, MA) and 2 mM GlutaMAX (Life Technologies, Waltham, MA), and cultured at 37°C under air/5% CO_2 at constant humidity. Transfections were carried out using Polyethylenimine MAX transfection reagent (Polyscience, Warrington, PA) for immunoblot analysis and immunofluorescence analysis according to the manufacturer's protocols. HEK293T were seeded on 60-mm dishes in DMEM containing 10% (vol/vol) FBS and 2 mM GlutaMAX and cultured at 37°C under air/5% CO_2 at constant humidity. Rat-1 cells were seeded on 60-mm dishes in DMEM (10567014, Life Technologies) containing 10% (vol/vol) FBS and cultured at 37°C under air/5% CO_2 at constant humidity.

Hippocampal neuron culture and transfection

Brains from embryonic day 18 rat embryos were collected into ice-cold Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium (Nacalai Tesque). Embryonic hippocampi were dissected and collected in ice-cold HBSS. Preparation of hippocampal neurons from these tissues was performed using papain as described previously (Yoshimura *et al.*, 2005). These neurons were seeded on 60-mm dishes coated with poly-D-lysine (Sigma-Aldrich) for immunoblot analysis or coverslips coated with poly-D-lysine for immunofluorescence analysis. Neurons were grown in neurobasal medium (21103049, Life Technologies) supplemented with B-27 supplement (A3582801, Life Technologies) and 2 mM GlutaMAX. For immunoblotting, after being cultured for 1 day on 60-mm dishes, neurons were treated with the PRMT1 inhibitor AMI-1 for 48 h. For immunofluorescence, hippocampal neurons were grown for 3 DIV to measure axon length or 14 DIV to measure dendrite complexity. Cells were transfected at 10 DIV with pEGFP-C1 (Clontech, Palo Alto, CA) using ViaFect transfection reagent (E4981, Promega, Madison, WI) according to the manufacturer's protocol. At 4 h after transfection, the medium was replaced with conditioned medium. For electroporation experiments, hippocampal neurons mixed with pEF-BOS-GST, pcDNA3.1 (+)-SCYL1-Flag, and rat-SCYL1 siRNA in Opti-MEM medium (Life Technologies) were transferred to a cuvette (EC-002S, Nepagene, Chiba, Japan). Electroporation was performed with a NEPA type II electroporation system (Nepagene) according to the manufacturer's instructions. After electroporation, these neurons were seeded on coverslips coated with poly-D-lysine and cultured in neurobasal medium supplemented with B-27 supplement and 2 mM GlutaMAX. For immunofluorescence, hippocampal neurons were grown for 3 DIV to measure axon length. Negative control siRNA and rat-SCYL1 siRNA (SIC-001 and SASI_Rn01_00091450) were purchased from Sigma. The target sequence of rat SCYL1 siRNA had no effect on the expression of human SCYL1-Flag.

Immunoblot analysis

HeLa cells or hippocampal neurons were solubilized in phosphate-buffered saline (PBS) containing 1% NP-40 substitute (Wako, Osaka, Japan) and protease inhibitor cocktail (cOmplete EDTA-free; Sigma-Aldrich). After insoluble debris were removed by centrifugation, the supernatant was collected to obtain cell lysates. SDS–PAGE sample buffer was added to the lysates and the samples were boiled for 10 min at 95°C. Proteins were separated on 5–20% SDS–PAGE gels (ATTO, Tokyo, Japan) in SDS running buffer. Proteins were transferred to PVDF membranes (Millipore), which were then blocked with 5% skimmed milk/PBS containing 0.1% Tween 20 (Wako). Membranes were then incubated first with primary antibodies and then with secondary antibodies. The bands were visualized by chemiluminescence using an ECL kit (GE Healthcare, Piscataway, NJ) and detected using x-ray film (Fujifilm, Tokyo, Japan). Immunostaining data were quantified using NIH ImageJ software.

Immunofluorescence analysis

HeLa cells were fixed with 4.0% paraformaldehyde in PBS for 30 min at 4°C and incubated in PBS containing 0.05% Triton X-100 (Sigma-Aldrich) and 10% bovine serum albumin for 1 h at room temperature. Cells were then incubated with primary antibodies, followed by secondary antibodies. Hippocampal neurons were fixed at 3 DIV or 14 DIV with 4.0% paraformaldehyde in PBS for 30 min on ice. Neurons were then treated for 15 min on ice with 0.1% Triton-X for the measurement of axonal length or 0.3% Triton-X for measurement of dendrite branching, followed by incubation in 10% normal goat serum in PBS for 1 h at room temperature. These neurons were then incubated with primary antibodies, followed by secondary antibodies. Immunofluorescence was visualized, and images were collected using a confocal laser microscope (Nikon C2, Nikon, Tokyo, Japan).

Immunoprecipitation

HeLa cells or HEK 293T cells were solubilized with PBS containing 1% NP-40 substitute and protease inhibitor cocktail. After centrifugation, the supernatant was incubated overnight at 4°C with anti-Flag or anti-SCYL1 antibodies conjugated with Dynabeads (10002D and 10004D, Invitrogen). The beads were then washed five times with PBS containing 1% NP-40 substitute and protease inhibitor cocktail, and proteins were extracted by adding SDS–PAGE sample buffer. The samples were boiled at 95°C for 10 min, and Dynabeads were removed.

Hippocampal neurons were solubilized with PBS containing 1% NP-40 substitute and protease inhibitor cocktail. After centrifugation, the supernatants of the cells or brain lysates were incubated with an anti-SCYL1 antibody conjugated with Dynabeads for 2 h at 4°C. The beads were then washed five times with PBS containing 1% NP-40 substitute and protease inhibitor cocktail, and proteins were extracted by adding SDS–PAGE sample buffer. The samples were boiled at 95°C for 10 min, and Dynabeads were removed.

Iodixanol concentration gradient ultracentrifugation

Ten milliliters of each iodixanol solution (1.5%, 3.0%, 4.5%, 6.0%, 7.5%, 10%, 15%, 20%, and 30%) were prepared by mixing 50% (wt/vol) iodixanol solution (Abbott, Chicago, IL) with the homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4). One milliliter of 1.5%, 2 ml of each of 3.0%, 4.5%, and 6.0%, 0.5 ml of 7.5%, 2 ml of 10%, and 0.5 ml of each of 15%, 20%, and 30% solutions were prepared in centrifuge tubes (331372, Beckman Coulter, Brea, CA). HeLa cells were washed twice with PBS to remove the culture medium and then once with homogenization buffer. The cells were then resuspended in 1.0 ml homogenization buffer and disrupted by passing them 12 times through a 26G needle. The homogenate was centrifuged at 1000 × *g* for 10 min at 4°C. The supernatants were collected and centrifuged at 3000 × *g* for 10 min at 4°C. The supernatants were then applied to the iodixanol gradient. After centrifugation at 100,000 × *g* for 2.5 h at 4°C, the 12 upper-layer fractions were collected and analyzed for the presence of proteins.

RNA interference

Negative control siRNA and PRMT1 siRNA (sc-37007 and sc-41069) were purchased from Santa Cruz Biotechnology (Yamaguchi and Kitajo, 2012). HeLa cells, HEK293T cells, and Rat-1 cells seeded on 60-mm dishes were transfected with siRNAs using Lipofectamine RNAiMAX transfection reagent (Invitrogen) for immunoblot analysis according to the manufacturer's instructions. For immunofluorescence analysis, HeLa cells were seeded on a Falcon 4-well culture slide after each siRNA transfection for 24 h. These cells were cultured for 24 h before fixation.

Morphological study of the hippocampal neurons

The length of the longest Tau-1 or GST-positive neurite was measured as a cell axon at 3 DIV using NIH ImageJ software. Dendrite complexity of GFP-positive neurons at 14 DIV without overlap from other GFP-positive cells was evaluated by Sholl analysis. Sholl analysis was used to quantify the number of dendrite intersections in concentric rings centered about the soma, beginning at a radius of 10 μm from the center of the soma and at 100 μm intervals thereafter.

Statistical analysis

Statistical significance was determined by unpaired, two-tailed Student's *t* tests. *p*-values of <0.05 was considered statistically significant. Data were collected and processed randomly and were analyzed using Microsoft Excel.

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