Cellular/Molecular

GSK3β/Axin-1/β-Catenin Complex Is Involved in Semaphorin3A Signaling

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Semaphorin3A (Sema3A) exerts a wide variety of biological functions by regulating reorganization of actin and tubulin cytoskeletal proteins through signaling pathways including sequential phosphorylation of collapsin response mediator protein 1 (CRMP1) and CRMP2 by cyclin-dependent kinase-5 and glycogen synthase kinase-3β (GSK3β). To delineate how GSK3β mediates Sema3A signaling, we here determined the substrates of GSK3β involved. Introduction of either GSK3β mutants, GSK3β-R96A, L128A, or K85M into chick dorsal root ganglion (DRG) neurons suppressed Sema3A-induced growth cone collapse, thereby suggesting that unprimed as well as primed substrates are involved in Sema3A signaling. Axin-1, a key player in Wnt signaling, is an unprimed substrate of GSK3β. The phosphorylation of Axin-1 by GSK3β accelerates the association of Axin-1 with β-catenin. Immunocytochemical studies revealed that Sema3A induced an increase in the intensity levels of β-catenin in the DRG growth cones. Axin-1 siRNA knockdown suppressed Sema3A-induced growth cone collapse. The reintroduction of RNAi-resistant Axin-1 (rAxin-1)-wt rescued the responsiveness to Sema3A, while that of nonphosphorylated mutants, rAxin S322A/S326A/S330A and T485A/S490A/S497A, did not. Sema3A also enhanced the colocalization of GSK3β, Axin-1, and β-catenin in the growth cones. The increase of β-catenin in the growth cones was suppressed by the siRNA knockdown of Axin-1. Furthermore, either Axin-1 or β-catenin RNAi knockdown suppressed the internalization of Sema3A. These results suggest that Sema3A induces the formation of GSK3β/Axin-1/β-catenin complex, which regulates signaling cascade of Sema3A via an endocytotic mechanism. This finding should provide clue for understanding of mechanisms of a wide variety of biological functions of Sema3A.

Introduction

During development, the neuronal growth cone senses and responds to a variety of guidance cues that are expressed in the developing nervous system, and functions to guide the formation of axonal connections (Tessier-Lavigne and Goodman, 1996). Semaphorin3A (Sema3A), a secreted type of semaphorin, has originally been identified as a repulsive axon guidance molecule and induces growth cone collapse in dorsal root ganglion (DRG) neurons through microtubule and actin cytoskeleton reorganization (Fan and Raper, 1995; Goshima et al., 2002; Tran et al., 2007; Zhou et al., 2008). Sema3A plays roles in regulating dendritic patterning, synapse maturation and neural cell polarity in addition to axon guidance (Sasaki et al., 2002; Yamashita et al., 2007; Nakamura et al., 2009; Pasterkamp and Giger, 2009; Tran et al., 2009).

The semaphorin receptors that mediate Sema3A signals have been identified as a complex of neuropilin-1 (NRP-1) and plexin-As. NRPs and plexin-As are ligand-binding and signal-transducing subunits of class 3 semaphorin receptor complexes, respectively (Takahashi et al., 1999). Intracellular signaling of this prototype of semaphorin has been most extensively studies. Collapsin response mediator proteins (CRMPs) have been identified as one of the intracellular mediators of Sema3A signaling, and the sequential phosphorylation of CRMP2 by Cdk5 and glycogen synthase kinase-3β (GSK3β) is necessary for the growth cone collapse through microtubule reorganization (Goshima et al., 1995; Uchida et al., 2005).

GSK3β is a multifunctional serine/threonine kinase contributing to a wide range of cellular processes including cell polarity, transcription, endocytosis, and cytoskeletal regulation (Hur and Zhou, 2010). Numerous GSK3β substrates have been identified and classified into two categories: primed substrates and unprimed substrates (Sutherland, 2011). Primed substrates, such as CRMP2 and β-catenin, require the priming phosphorylation by another kinase at a serine/threonine residue located four residues C-terminal to the side of GSK3β phosphorylation sites (Hagen and Vidal-Puig, 2002; Uchida et al., 2005). Unprimed substrates, including Axin-1, low-density lipoprotein receptor-related 6 (LRP6), and MAP1B, are also GSK3β substrates but do

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not require the priming phosphorylation (Ikeda et al., 1998; Zeng et al., 2005; Scales et al., 2009).

In this study, we identified Axin-1 as an unprimed substrate of GSK3β participated in intracellular signaling of Sema3A. We find that Axin-1 plays an essential role in the increase of intensity levels of β-catenin in the growth cones in response to Sema3A. We further showed that Axin-1, β-catenin, and GSK3β are involved in the internalization of Sema3A. We here propose that Sema3A induces the formation of GSK3β/Axin-1/β-catenin complex in the growth cones, which plays an important role in the mechanisms of Sema3A signaling through regulation of endocytosis.

Materials and Methods

Plasmid construction. To generate EGFP-tagged GSK3β, human GSK3β cDNA encoding GSK3β was inserted into pEGFP C-1 expression vector. EGFP-GSK3β R96A, K85M, and L128A mutants were constructed by site-directed mutagenesis. The primers directing peptide sequence of FRATtide (Thomas et al., 1999) were purchased from Invitrogen, and the fragment was inserted into pEGFP C-1 expression vector. DNA fragment encoding rat Axin-1 was amplified by PCR from rat cDNA and inserted into pcDNA4 expression vector. DNA fragment encoding Flag-tagged rat Axin-1 was amplified by PCR from rat Axin-1 and inserted into pcDNA3.1 expression vector. Rat Axin-1 S322A/S326A/S330A and T485A/S490A/S497A mutants were constructed by site-directed mutagenesis. To examine the function of Axin-1 in Axin-1 knockdown neurons, the Flag-tagged Axin-1 rescue constructs (RNAi-resistant rat Axin-1) were prepared with PCR-based techniques by introducing silent substitutions in the target site of short interfering RNA (siRNA). The coding region of above-mentioned constructs was subcloned into pHSVPrPUC expression vector for transient expression in chick or mouse DRG explant using herpes simplex virus (HSV) (Uchida et al., 2005).

Explant DRG culture. DRG explants were dissected from chick E7 or mouse E12.5 embryos (male or female) in PBS. The DRG explants were plated on eight-well glass chamber slides (Nunc) previously coated with 100 μg/ml poly-L-lysine (Wako Pure Chemicals) followed by 8 μg/ml laminin (BD Biosciences). Chick or mouse DRG explants were then incubated in the medium containing 10% fetal bovine serum (FBS), Ham's F-12, and 10 ng/ml 2.5S NGF (Wako) or in the medium containing Neurobasal medium, 2% B-27 supplement (Invitrogen), 1 mM L-glutamine (Nacalai Tesque), and 25 ng/ml 2.5S NGF, respectively. Cultured mouse DRG explants were stimulated with 0.1 or 0.5 nM Sema3A for 5 min. In some experiments, 10 μM *N*-(4-methoxybenzyl)-*N*′-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418) (Sigma-Aldrich), which is a specific GSK3 inhibitor (Bhat et al., 2003), or DMSO was added to the explants 30 min before Sema3A stimulation.

Recombinant HSV preparations and infections. Recombinant HSV preparations and infections of cultured chick E7 or mouse E12.5 DRG explants were performed as described previously (Sasaki et al., 2002). In brief, recombinant viruses possessing EGFP, EGFP-GSK3β mutants, EGFP-FRATtide, Flag-Axin-1 mutants, or Flag-RNAi-resistant Axin-1 mutants genes were added to the explants cultures for 24 h before growth cone collapse assay. The percentage of axons expressing EGFP- or Flag-tagged proteins ranged from 60 to 80% in infected cultures.

siRNA transfection. Nontargeting scramble and Axin-1 siRNA were purchased from Invitrogen, and β-catenin, and neuropilin-1 siRNA were purchased from Santa Cruz Biotechnology. β-Catenin or neuropilin-1 siRNA was a mixture of three or four different sequences against each molecule. The RNA interference of these molecules in cultured mouse DRG explants or Neuro2A cells was performed using DharmaFECT-3 (Thermo Fisher Scientific) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, respectively. Neuro2A cells were transfected with each siRNA (final concentration, 20 nM) 24 h after the start of cultures. Cultured mouse DRG explants were transfected with each siRNA (final concentration, 50 nM) 10–12 h after the start of cultures. The medium was replaced with preequilibrated and prewarmed fresh culture medium 4–6 h after transfection. These cells were incubated for 24 h before a series of experiments.

Growth cone collapse assay. Growth cone collapse assay using chick E7 and mouse E12.5 DRG neurons were performed using purified recombinant chick Sema3A as previously described (Goshima et al., 1995). Cultured chick or mouse DRG explants were stimulated with Sema3A or lysophosphatidic acid (LPA) for 30 min and were fixed with 3.7% formalin and 10% sucrose in PBS at room temperature for 20 min, permeabilized for 10 min using 0.1% Triton X-100/PBS, and then incubated with Alexa 488-phalloidin (Invitrogen) at 37°C for 1 h (Sasaki et al., 2002). Individual growth cone morphology was analyzed using Olympus Optical IX71 microscopy and scored as previously described (Uchida et al., 2005).

Immunocytochemistry and microscopy observation. The DRG explants were fixed with fresh 2 or 4% PFA including 4% sucrose at room temperature for 20 min, washed twice with PBS, permeabilized for 10 min using 0.1% Triton X-100/PBS, and then blocked for 1 h in blocking buffer (5% goat serum/PBS). The DRG explants were incubated with anti-Axin-1 antibody (1:200; Cell Signaling Technology), anti-β-catenin antibody (1:200; BD Biosciences Transduction Laboratories), anti-GSK3β antibody (1:400; Cell Signaling Technology), or anti-GSK3β antibody (1:400; BD Biosciences Transduction Laboratories) diluted in Dako REAL Antibody Diluent (Dako) at 4°C overnight, and then incubated at room temperature for 1 h with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Invitrogen), and with 5-(4, 6-dichlorotriazin-2-yl)aminofluorescein (DTAF) (Invitrogen) for measurement of growth cone morphology and volume. The fluorescence microscopic images were visualized using a laser confocal microscope (LSM510) with a water-immersed object at 63 (CApochromat) equipped with Axioplan 2 imaging microscope (Zeiss).

Venus-Sema3A internalization assay. To visualize internalization of Sema3A, we constructed his-venus-tagged human Sema3A (venus-Sema3A). Recombinant venus-Sema3A was purified in a similar way to the chick Sema3A as previously described (Goshima et al., 1995). Mouse E12.5 DRG explants were plated on Thermanox plastic coverslips (Nunc) previously coated with 100 μg/ml poly-L-lysine (Sigma-Aldrich) followed by 8 μg/ml laminin (BD Biosciences), and then incubated in the medium containing Neurobasal medium, 2% B-27 supplement, 1 mM L-glutamine, and 10 ng/ml 2.5S NGF. The DRG explants were incubated in the medium containing 10% FBS for 2 h before venus-Sema3A application. The DRG explants were stimulated with 0.5 nM venus-Sema3A for 3 min. To detect binding of venus-Sema3A to neuropilin-1, the DRG explants were fixed with 3.7% formalin and 10% sucrose in PBS at room temperature for 20 min, washed twice with PBS, and then blocked for 1 h in blocking buffer (5% goat serum/PBS). To detect internalization of venus-Sema3A, the DRG explants washed once with cold PBS and then acid stripped with acidic buffer (0.2 M acetic acid and 0.5 M NaCl) for 2 min on ice. The DRG explants were washed three times with cold PBS and then fixed with 3.7% formalin and 10% sucrose in PBS at room temperature for 20 min, permeabilized for 10 min using 0.1% Triton X-100/PBS, and then blocked for 1 h in blocking buffer (5% goat serum/PBS). The DRG explants were incubated at 4°C overnight with anti-GFP antibody (1:2500; MBL), and then incubated at room temperature for 1 h with Alexa Fluor 594-conjugated secondary antibodies and DTAF. The fluorescence microscopic images were visualized using the laser confocal microscope (LSM510) as described above.

Quantitative analysis. Quantitation of the intensity of Axin-1 and β-catenin immunofluorescence was performed using MetaMorph (Molecular Devices). Growth cone area and volume were measured by DTAF fluorescence, and the intensity within the area of control or Sema3A-treated growth cone was calculated. The intensity of background fluorescence in an adjacent area was similarly calculated and subtracted from the growth cone value to give final intensity measurement. To normalize Axin-1 or β-catenin intensity in growth cones, the ratios between Axin-1 or β-catenin and DTAF intensity were calculated in each growth cones. The relative intensity value was calculated by dividing the value for each sample by the average value of control. Quantitation of colocalization was performed using the plug-in RG2B colocalization to ImageJ (written by Christopher Philip Mauer, Northwestern University, Chicago, IL).

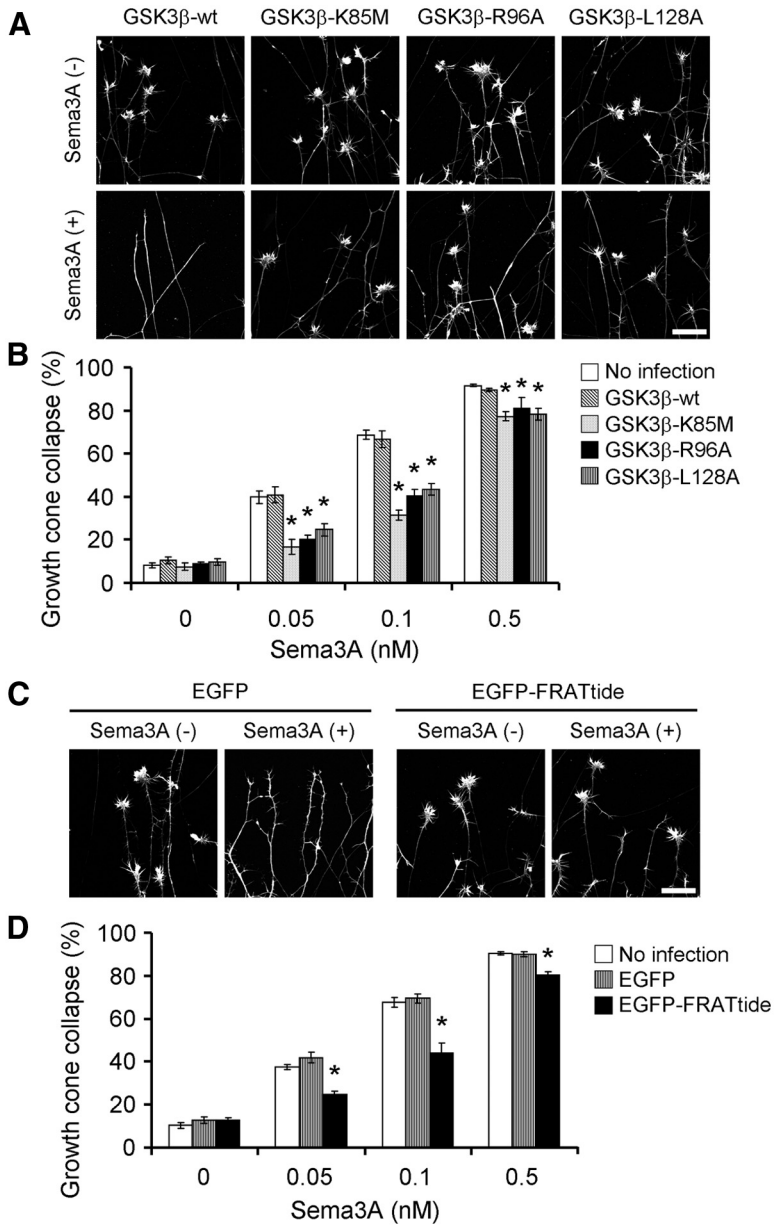

Figure 1. Suppression of Sema3A-induced response by introduction of GSK3β mutants. ***A***, Cultured chick E7 DRG neurons were infected with recombinant HSV preparations directing the expression of EGFP, EGFP-GSK3β-wt (GSK3β-wt), EGFP-GSK3β-K85M (GSK3β-K85M), EGFP-GSK3β-R96A (GSK3β-R96A), or EGFP-GSK3β-L128A (GSK3β-L128A). Cultured DRG neurons were treated with Sema3A for 30 min, fixed, and stained with Alexa 488-phalloidin. Overexpression of GSK3β-K85M, GSK3β-R96A, and GSK3β-L128A suppressed Sema3A-induced growth cone collapse, while that of EGFP or GSK3β-wt did not interfere with the Sema3A response. Scale bar, 50 μm. ***B***, Quantitative analysis of growth cone collapse response in the neurons expressing GSK3β mutant. Data are shown as the mean $\pm$ SEM ($n = 6$). *$p < 0.01$, by one-way ANOVA, compared with no infection neurons. ***C***, Cultured DRG neurons were infected with recombinant HSV preparations directing the expression of EGFP and EGFP-FRATtide. Overexpression of EGFP-FRATtide suppressed Sema3A-induced growth cone response, while that of EGFP did not interfere with the Sema3A response. Scale bar, 50 μm. ***D***, Quantitative analysis of growth cone collapse response in the expressing EGFP-FRATtide. Data are shown as the mean $\pm$ SEM ($n = 6$). *$p < 0.01$, by one-way ANOVA, compared with EGFP-expression neurons.

The colocalization measurements were taken using a minimum threshold pixel intensity adjusted between 100 and 200 and set equivalently for both channels to correspond to the image intensity. The minimum ratio for pixel intensity between the two channels was set to 0.5. Colocalization pixels in growth cones were displays as an image with maximum pixel intensity. Each image was quantitated by measuring the total pixels in growth cones above the threshold intensity used in colocalization determination (see above). Results are displayed as percentage colocalization as determined by dividing the number of each colocalization pixels by the number of total Axin-1 pixels in the case of colocalization between Axin-1 and GSK3β, or Axin-1 and β-catenin, and by the number of total GSK3β pixels in the case of GSK3β and β-catenin. Quantitative analysis of venus-Sema3A was performed using MetaMorph and ImageJ. Growth cone area and volume was measured by DTAF fluorescence, and the number of particles within the area of the growth cone was counted. Binding of venus-Sema3A was calculated by dividing the number by each growth cone area. Internalization of venus-Sema3A was calculated by dividing the number by DTAF intensity of each growth cone area. The relative intensity value was calculated by dividing the value for each sample by the average value of control siRNA knockdown neurons.

Dissociated mouse DRG neuron culture. DRGs were removed from mouse E12.5 embryos (male or female) and were treated with 0.25% trypsin (Invitrogen) in Ca^{2+}- and Mg^{2+}-free PBS at 37°C for 5 min, and stopped by adding DMEM (Wako) containing 10% FBS. The DRGs were washed with Neurobasal medium containing 2% B-27 supplement, 1 mM L-glutamine, and 25 ng/ml 2.5S NGF. The cells were plated onto previously 100 μg/ml poly-L-lysine and 8 μg/ml laminin-coated culture dish, and cultured at 37°C for 24 h before Sema3A stimulation.

Western blot analysis. Neuro2A cells transfected with each siRNA and dissociated mouse DRG neurons were washed two times with ice-cold PBS, and lysed in lysis buffer [25 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 10 μg/ml aprotinin, and 50 μM ρ-APMSF (ρ-amidinophenylmethanesulfonyl fluoride)]. These lysates were centrifuged, and protein extracts were separated on 8% SDS-polyacrylamide gels and transferred to Immobilon transfer membranes (PVDF; Millipore). The membranes were washed, and then incubated in TBST containing 5% skim milk for 1 h. The membranes were treated with the anti-Axin-1 (1:2000; Cell Signaling Technology), anti-β-catenin (1:2000; BD Biosciences Transduction Laboratories), or anti-β-actin antibody (1:10,000; Sigma-Aldrich) for overnight at 4°C. HRP-conjugated secondary antibody was used at 1:5000 (GE Healthcare).

Statistical significance. Data are shown as mean $\pm$ SEM. The statistical significance of the results was analyzed using one-way ANOVA or Student's *t* test.

Results

GSK3β mutants suppress Sema3A-induced growth cone collapse

We have previously reported that CRMP2 is endogenously phosphorylated by Cdk5 in the embryonic brain, and the sequential phosphorylation of CRMP2 by Cdk5/GSK3β mediates growth cone collapse response to Sema3A (Uchida et al., 2005). To further elucidate how GSK3β is involved in intracellular signaling of Sema3A, we used three GSK3β mutants, GSK3β K85M, GSK3β R96A, and GSK3β L128A mutant. Mutation of lysine 85 to methionine on GSK3β (GSK3β K85M mu-

tant) is a catalytically inactive form, in which the ATP binding site is mutated. Arginine 96 on GSK3β is one of the crucial basic residues of the positive pocket within the catalytic groove together with arginine 180 and lysine 205, and the positive pocket is essential to the interaction with phosphate group on primed substrates followed by phosphorylation of primed substrates. Mutation of arginine 96 to alanine (GSK3β R96A mutant) disrupts the positive pocket and results in inconvenient binding with primed substrates (Frame et al., 2001). Therefore, GSK3β R96A mutant structurally lacks the ability to phosphorylate primed substrates. It has been reported that overexpression of GSK3β R96A mutant suppresses the phosphorylation of endogenous CRMP2 in DRG neurons in a dominant-negative manner (Kim et al., 2006). Using HSV gene transfer, EGFP-tagged wild-type GSK3β (EGFP-GSK3β-wt), EGFP-GSK3β-K85M or EGFP-GSK3β-R96A mutants were overexpressed in cultured chick DRG neurons, which did not affect the growth cone morphology. Both EGFP-GSK3β-K85M and R96A mutants suppressed Sema3A-induced collapse response (Fig. 1*A*,*B*), while the overexpression of EGFP or EGFP-GSK3β-wt did not affect the response. This result supports that GSK3β mediates Sema3A signaling through the phosphorylation of primed substrates including CRMP2 (Uchida et al., 2005). To further clarify the involvement of unprimed substrates in Sema3A signaling, we overexpressed EGFP-GSK3β-L128A, another GSK3β mutant, in the DRG neurons. Leucine 128 on GSK3β is equivalent to leucine 155 on PDK1 (3-phosphoinositide-dependent protein kinase-1), which is involved in the activation of the enzyme and its specific interaction with several substrates. Mutation of leucine 128 to alanine (GSK3β L128A mutant) lacks the ability to phosphorylate Axin-1, an unprimed substrate, but retains the ability to phosphorylate primed substrates in *in vitro* kinase assay (Frame et al., 2001). In DRG neurons expressing EGFP-GSK3β-L128A mutant, Sema3A-induced growth cone response was suppressed when compared with neurons expressing EGFP. This result suggests that, in addition to a primed substrate CRMP2, unprimed substrates are also involved in Sema3A signaling (Fig. 1*A*,*B*).

To further determine the involvement of GSK3β-unprimed substrates, we examined the effect of overexpression of a partial fragment of frequently rearranged in advanced T-cell lymphomas 1 (FRATtide) on the Sema3A response. FRATtide has been shown to interfere the interaction between GSK3β and Axin-1, followed by the inhibition of the phosphorylation of Axin-1 (Thomas et al., 1999). Again, overexpression of EGFP-FRATtide in the DRG neurons suppressed the response to Sema3A at a concentration of 0.05, 0.1, and 0.5 nM (Fig. 1*C*,*D*). Together, these results suggest that phosphorylation of Axin, an unprimed substrate of GSK3β probably participates in Sema3A signaling to induce growth cone collapse.

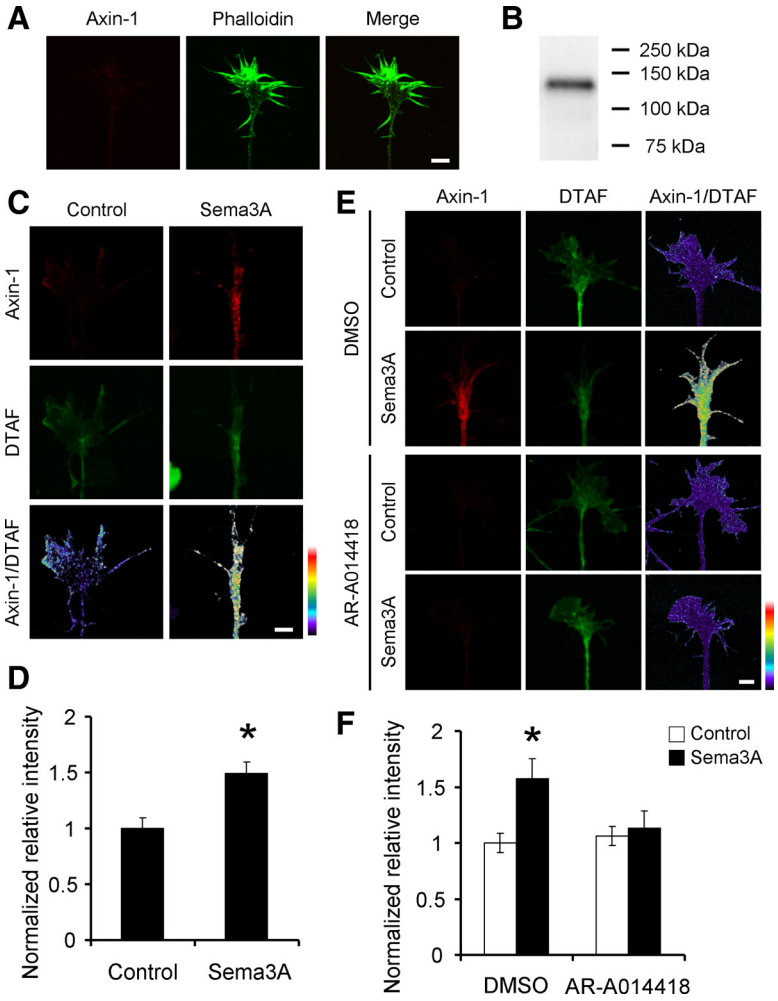

Figure 2. Sema3A induces an increase in the intensity levels of Axin-1 in the growth cones. ***A***, Cultured mouse E12.5 DRG neurons stained with anti-Axin-1 antibody (red) and Alexa 488-phalloidin (green). Scale bar, 5 μm. ***B***, Dissociated DRG neurons lysate was separated by SDS-PAGE and immunoblotted with anti-Axin-1 antibody. ***C***, Axin-1 immunofluorescence signal in growth cones before and after Sema3A stimulation. Cultured DRG neurons were treated with 0.1 nM Sema3A for 5 min. The cultures were stained with anti-Axin-1 antibody (red) and DTAF (green). Scale bar, 5 μm. ***D***, Normalized relative intensity of Axin-1 immunofluorescence signal at a single growth cone after control or Sema3A application. Data are shown as the mean ± SEM ($n = 40$). *$p < 0.01$, by Student's *t* test, compared with control neurons. ***E***, Cultured DRG neurons were treated with DMSO or AR-A014418 at 10 μM for 30 min, and then treated with 0.5 nM Sema3A for 5 min. The cultures were stained with anti-Axin-1 antibody (red) and DTAF (green). Scale bar, 5 μm. ***F***, Normalized relative intensity of Axin-1 immunofluorescence signal at a single growth cone with or without Sema3A after treatment with DMSO or AR-A014418. Data are shown as the mean ± SEM ($n = 25$). *$p < 0.01$, by one-way ANOVA, compared with control neurons.

Axin-1 is involved in Sema3A-induced growth cone collapse

To further confirm the involvement of Axin-1, we examined whether Axin-1 is expressed in cultured mouse DRG neurons. Axin-1 was expressed in the axonal shaft and growth cone of the DRG neurons (Fig. 2*A*). The expression was also confirmed in the DRG neurons by Western blot analysis (Fig. 2*B*). We next examined localization change of Axin-1 following stimulation with Sema3A in the DRG neurons. The distribution of Axin-1 in the growth cones and axons showed a high fluorescence dot-like staining pattern after Sema3A stimulation (Fig. 2*C*). To correct change in the growth cone volume, we labeled total protein using DTAF. To normalize the fluorescent intensity of Axin-1, we divided it by the DTAF intensity of the same growth cone area. Quantification of normalized fluorescent intensity showed ~1.5-fold increase over the basal level of unstimulated control after Sema3A application (Fig. 2*C*,*D*).

To further investigate whether the GSK3β was involved in the Sema3A-induced increase in the intensity of Axin-1, we exam-

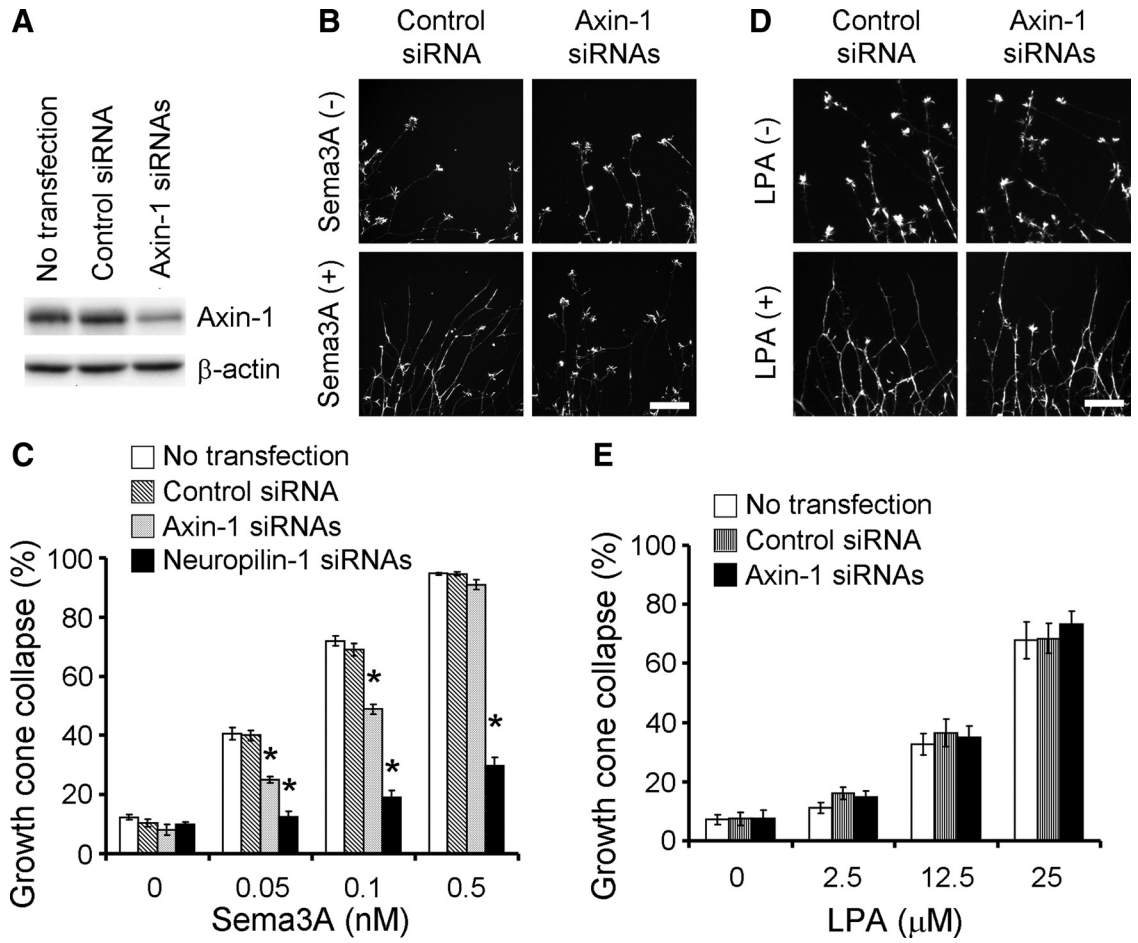

Figure 3. Axin-1 knockdown suppresses Sema3A-induced growth cone collapse. ***A***, Neuro2A cells were transfected with control or Axin-1 siRNA. The cell lysates were separated by SDS-PAGE and immunoblotted with anti-Axin-1 and anti-β-actin antibody. ***B***, Cultured mouse E12.5 DRG neurons were transfected with the indicated siRNA. Transfection of Axin-1 and neuropilin-1 siRNAs suppressed Sema3A-induced growth cone collapse. Scale bar, 50 μm. ***C***, Quantitative analysis of growth cone collapse of nontransfected, control, and Axin-1 and neuropilin-1 siRNAs-transfected DRG neurons. *$p < 0.01$, by one-way ANOVA, compared with control siRNA-treated neurons. ***D***, Cultured mouse E12.5 DRG neurons were transfected with the indicated siRNA. Transfection of Axin-1 siRNAs has no effect on LPA-induced growth cone collapse. ***E***, Growth cone collapse response to LPA in nontransfected, control, and Axin-1 siRNA-transfected DRG neurons. Scale bar, 50 μm. Data are shown as the mean ± SEM ($n = 6$).

ined the effect of AR-A014418, a specific GSK3 inhibitor (Bhat et al., 2003), on the increase of Axin-1. In control DRG neurons treated with DMSO, Sema3A increased the normalized fluorescent intensity levels of Axin-1 by 1.6-fold in the growth cones over unstimulated control. In the neurons treated with AR-A014418 at 10 μM, the Sema3A-induced increase in the intensity levels of Axin-1 in the growth cones was markedly suppressed (Fig. 2*E*,*F*). These results suggest that GSK3β activation is involved in the Sema3A-induced increase in the intensity levels of Axin-1 in the growth cones.

Next, we examined the effect of RNAi-mediated knockdown of Axin-1 on the Sema3A response by using siRNA targeting Axin-1 in cultured mouse DRG neurons. We first examined the knockdown efficiency of three different Axin-1 siRNAs. Transfection of any of the three different Axin-1 siRNAs into Neuro2A cells effectively reduced the expression of Axin-1 when compared with that in the cells transfected with nontargeting scramble siRNA (control siRNA) (data not shown). To further improve the efficiency of Axin-1 siRNA knockdown, we used a mixture of the three Axin-1 siRNAs. Transfection of these Axin-1 siRNAs into Neuro2A cells significantly reduced the expression of Axin-1 when compared with that in the cells transfected with control siRNA (Fig. 3*A*). As a positive control, we performed siRNA knockdown of neuropilin-1, the binding receptor of Sema3A. Knockdown of neuropilin-1 almost completely suppressed Sema3A-induced growth cone collapse, while control siRNA produced no effect on the Sema3A response in the DRG neurons (Fig. 3*C*). In Axin-1 siRNA knockdown DRG neurons, growth cone collapse induced by the lower concentration of Sema3A (0.05 and 0.1 nM) was significantly suppressed, but the response induced by a higher concentration of 0.5 nM was not affected (Fig. 3*C*). LPA is reported to induce growth cone collapse through phosphorylation of CRMP2 by Rho/ROCK kinase (Arimura et al., 2000). LPA induced growth cones collapse to a similar extent in both control and Axin-1 siRNA knockdown neurons (Fig. 3*D*,*E*), indicating that Axin-1 is not involved in LPA signaling to induce growth cone collapse. This result suggests that Axin-1 regulates responsiveness to the repulsive signals of Sema3A, but not of LPA.

Phosphorylation of Axin-1 regulates Sema3A signaling to induce growth cone collapse response

Because GSK3β mediated the Sema3A-induced increase in the intensity levels of Axin-1 in the growth cones, we determined whether phosphorylation of Axin-1 is involved in Sema3A-induced growth cone collapse response. For this purpose, we

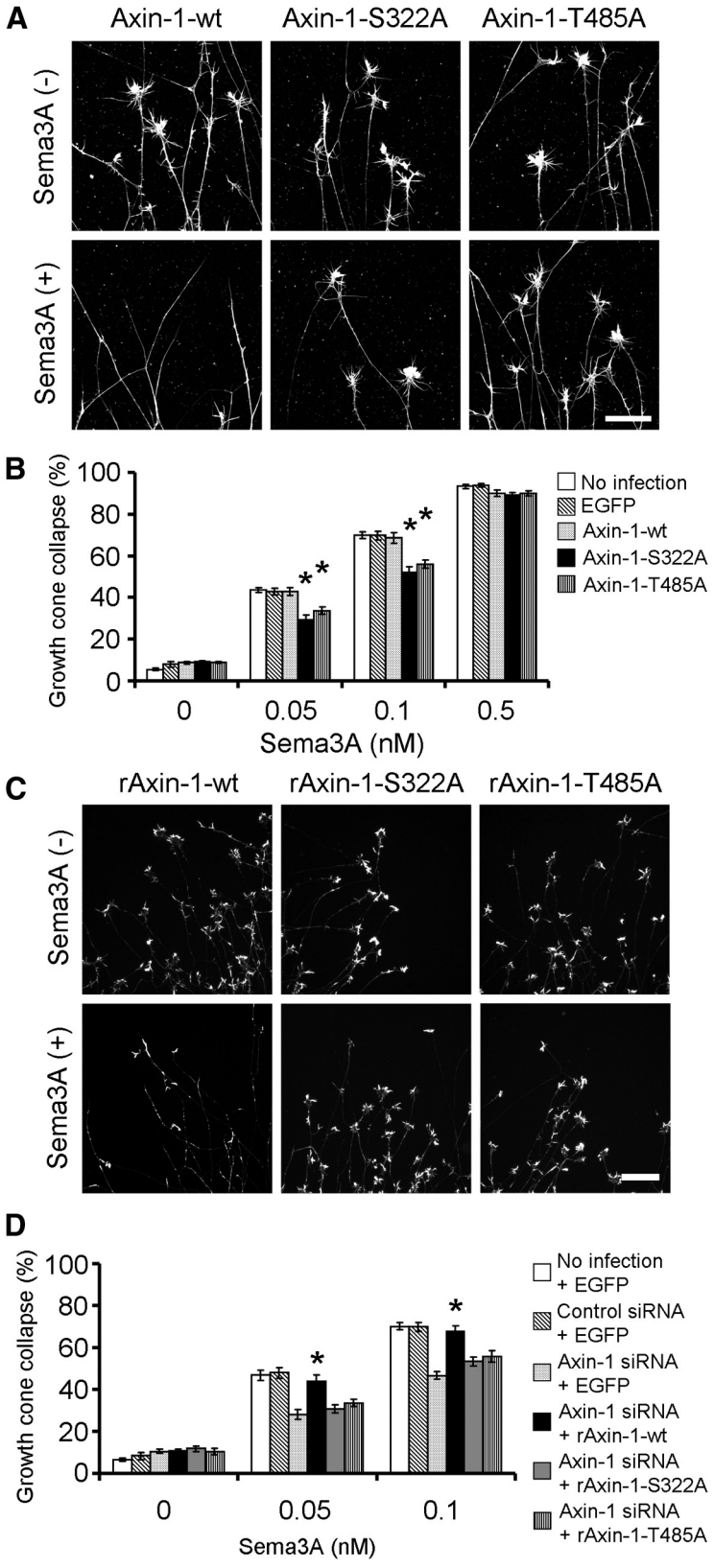

Figure 4. Phosphorylation of Axin-1 is involved in Sema3A-induced growth cone collapse. ***A***, Cultured chick E7 DRG neurons were infected with recombinant HSV preparations directing the expression of EGFP, Flag-Axin-1-wt (Axin-1-wt), Flag-Axin-1-S322A/S326A/S330A (Axin-1-S322A), and Flag-Axin-1-T485A/S490A/S497A (Axin-1-T485A). Overexpression of EGFP and Axin-1-wt did not interfere with Sema3A-induced growth cone collapse, whereas that of Axin-1-S322A and Axin-1-T485A suppressed the Sema3A response. Scale bar, 50 μm. ***B***, Quantitative analysis of growth cone collapse response in DRG neurons expressing Axin-1 mutants. Data are shown as the mean ± SEM ($n = 6$). *$p < 0.01$, by one-way ANOVA, compared with control neurons expressing EGFP alone. ***C***, Sema3A-induced growth cone collapse response in the Axin-1 knockdown DRG neurons reintroduced by Axin-1 siRNA resistance Flag-rAxin-1-wt (rAxin-1-wt), Flag-rAxin-1-S322A/S326A/S330A (rAxin-1-S322A), and Flag-rAxin-1-T485A/S490A/S497A (rAxin-1-T485A). After transfection with control or Axin-1 siRNA, the DRG neurons were infected with recombinant HSV preparations directing the expression of EGFP, rAxin-1-wt, and rAxin-1 mutants, rAxin-1-S322A and rAxin-1-T485A. Overexpression of rAxin-1-wt in Axin-1 knockdown DRG neurons rescued the responsiveness to Sema3A, whereas that of rAxin-1-S322A and rAxin-1-T485A mutants did not. Scale bar, 50 μm. ***D***, Quantitative analysis of growth cone collapse response in DRG neurons transfected with each siRNA and rAxin-1 mutants. Data are shown as the mean ± SEM ($n = 6$). *$p < 0.01$, by one-way ANOVA, compared with Axin-1 siRNA plus EGFP, rAxin-1-S322A, or rAxin-1-T485A-expressing neurons.

generated two types of nonphosphorylated Axin-1 mutants, S322A/S326A/S330A and T485A/S490A/S497A. It has been reported that the phosphorylation of S322/S326/S330 residues on rat Axin-1 by GSK3β increases the Axin-1 stability (Yamamoto et al., 1999). Axin-1 residues T485/S490/S497 in rat correspond with its residues S609/S614/S621 in mouse, and phosphorylation at these sites by GSK3β increases the interaction of Axin-1 with β-catenin, a major Axin-1-binding protein (Jho et al., 1999). We overexpressed Flag-tagged Axin-1-wild type (Flag-Axin-1-wt), nonphosphorylated form of Flag-Axin-1 S322A/S326A/S330A or T485A/S490A/S497A mutants in DRG neurons. Both Flag-Axin-1 S322A/S326A/S330A and T485A/S490A/S497A mutants suppressed the growth cone response to Sema3A at a concentration of 0.05 and 0.1 nM (Fig. 4*A*,*B*). This result suggests that phosphorylation of Axin-1 at some of these sites is involved in Sema3A-induced growth cone collapse response.

To further confirm the role of Axin-1 phosphorylation in Sema3A-induced growth cone collapse, we designed rescue experiments using Axin-1 constructs containing resistance to the Axin-1 siRNA [Flag-tagged RNAi-resistant Axin-1 (Flag-rAxin-1)]. We observed that the expression of Flag-rAxin-1 was unaffected against the Axin-1 siRNA, when compared with the RNAi no-resistant Axin-1 using Western blot analysis and immunocytochemical analysis (data not shown). Six hours after transfection of the Axin-1 and control siRNA into cultured mouse DRG neurons, we introduced EGFP, Flag-rAxin-1-wt, S322A/S326A/S330A, or T485A/S490A/S497A mutants in the DRG neurons using HSV gene transfer. The introduction of Flag-rAxin-1-wt rescued the responsiveness to Sema3A and induced growth cone col-

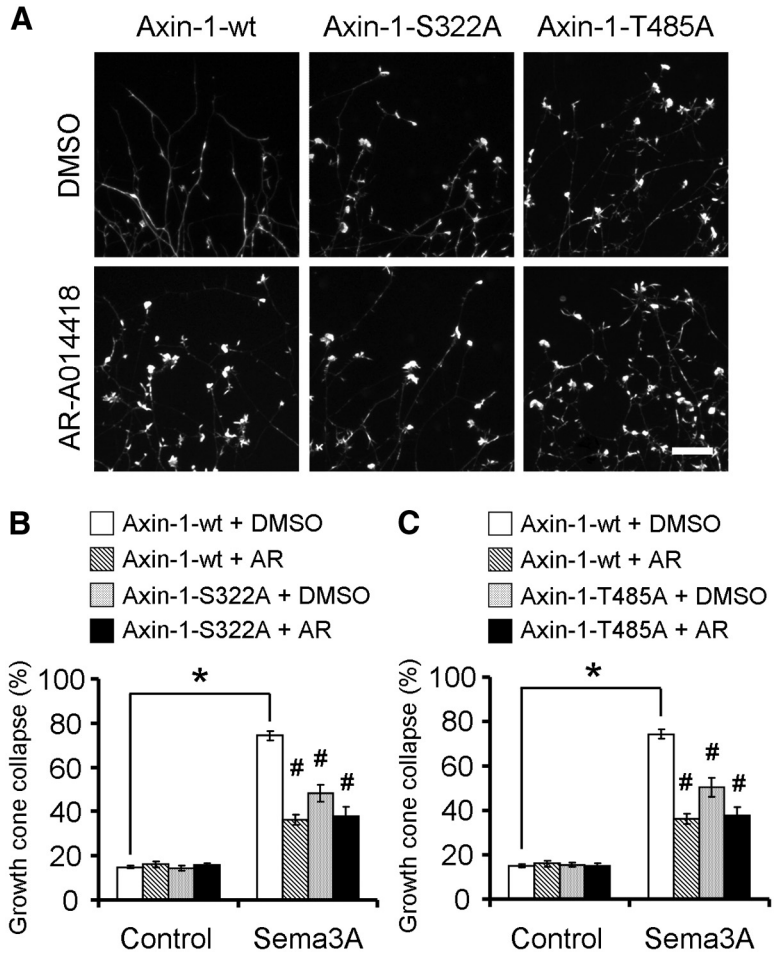

Figure 5. Phosphorylation of Axin-1 by GSK3β is involved in Sema3A-induced growth cone collapse. ***A***, Cultured mouse E12.5 DRG neurons were infected with recombinant HSV preparations directing the expression of Flag-Axin-1-wt (Axin-1-wt), Flag-Axin-1-S322A/S326A/S330A (Axin-1-S322A), and Flag-Axin-1-T485A/S490A/S497A (Axin-1-T485A). Twenty-four hours after infection, the DRG neurons were treated with DMSO or AR-A014418 (AR) at 10 μM for 30 min. Scale bar, 50 μm. ***B***, ***C***, Quantitative analysis of growth cone collapse response in DRG neurons expressing Axin-1-wt or Axin-1 mutants treated with DMSO or AR-A014418 at 10 μM. Introduction of Axin-1-S322A (***B***) or Axin-1-T485A (***C***) suppressed Sema3A-induced growth cone collapse. AR, but not DMSO, suppressed Sema3A-induced growth cone collapse. AR and expression of Axin-1 mutants did not have additive effects on preventing Sema3A-induced growth cone collapse. Data are shown as the mean ± SEM ($n = 6$). $^{*}p < 0.01$, by one-way ANOVA, compared with corresponding control. $^{\#}p < 0.01$, by one-way ANOVA, compared with DRG expressing Axin-1 wt treated with DMSO.

lapse in the DRG neurons. The introduction of EGFP alone did not affect the Sema3A response. The introduction of neither Flag-rAxin S322A/S326A/S330A nor T485A/S490A/S497A mutants rescued the responsiveness to Sema3A (Fig. 4*C*,*D*). These results demonstrate that the phosphorylation of Axin-1 at some of S322/S326/S330 and T485/S490/S497 residues regulates the responsiveness to Sema3A to induce growth cone collapse.

Furthermore, we examined whether inhibition of GSK3β and expression of Axin1 mutants have additive effects on preventing Sema3A-induced growth cone collapse. After introduction of Flag-Axin-1-wt, S322A/S326A/S330A or T485A/S490A/S497A mutants using HSV gene transfer, we investigated the effect of AR-A014418 on preventing Sema3A-induced growth cone collapse in cultured mouse DRG neurons (Fig. 5). AR-A014418 at 10 μM suppressed Sema3A-induced growth cone collapse in Flag-Axin-1-wt-expressing mouse DRG neurons, while DMSO had no effect (Eickholt et al., 2002). The expression of Axin-1 S322A/S326A/S330A or T485A/S490A/S497A mutants significantly inhibited the Sema3A response in the presence of either AR-A014418 or DMSO. However, inhibition of GSK3β and expression of the Axin1 mutants had no additive effects on preventing Sema3A-induced growth cone collapse (Fig. 5*A–C*). Thus, these results suggest that GSK3β activation acts upstream of phosphorylation of Axin-1 in Sema3A signaling.

β-Catenin is involved in Sema3A-induced growth cone collapse

The above finding raised the possibility that β-catenin also mediates intracellular signaling of Sema3A, because the phosphorylation of Axin-1 at T485/S490/S497 residues facilitates the interaction between Axin-1 and β-catenin (Jho et al., 1999; Willert et al., 1999). We investigated whether Sema3A alters distribution of β-catenin in cultured mouse DRG neurons. The distribution of β-catenin in the growth cones showed a high fluorescence dot-like staining pattern after Sema3A stimulation (Fig. 6*A*). Quantification of normalized fluorescent intensity revealed that Sema3A enhanced the intensity of β-catenin to the same extent of that of Axin-1 (Fig. 2), with the maximum effect of Sema3A being 1.4-fold increase over the basal level of unstimulated control. Immunocytochemical examination also revealed that spatial and temporal distribution pattern of β-catenin was altered in a similar fashion to that of Axin-1 in the growth cones in response to Sema3A (Figs. 2, 6*A*,*B*). To elucidate whether β-catenin is involved in Sema3A-induced growth cone collapse, we performed knockdown of β-catenin using β-catenin siRNAs in the DRG neurons. Transfection of β-catenin siRNAs into Neuro2A cells effectively reduced β-catenin expression level (Fig. 6*C*). The siRNA-mediated β-catenin knockdown in the DRG neurons suppressed responsiveness to Sema3A at the concentration range of 0.05 to 0.5 nM, while in control siRNA knockdown did not affect the Sema3A response (Fig. 6*D*,*E*). Furthermore, LPA similarly induced growth cones collapse in both control and β-catenin siRNA knockdown neurons (Fig. 6*F*,*G*), indicating that β-catenin is not involved in LPA signaling to induce growth cone collapse. These results suggest that, like Axin-1, β-catenin regulates the responsiveness to Sema3A signals.

Sema3A enhances colocalization of GSK3β, Axin-1, and β-catenin in growth cones

Our findings suggest that GSK3β, Axin-1, and β-catenin may form a functional complex in regulating the responsiveness to Sema3A signals. To test this possibility, we investigated whether the percentage of colocalization of GSK3β, Axin-1, and β-catenin is enhanced in response to Sema3A in cultured mouse DRG neurons. In control growth cones, most of β-catenin-positive clusters did not colocalize with GSK3β-positive clusters. Five minutes after Sema3A stimulation, the immunofluorescence sig-

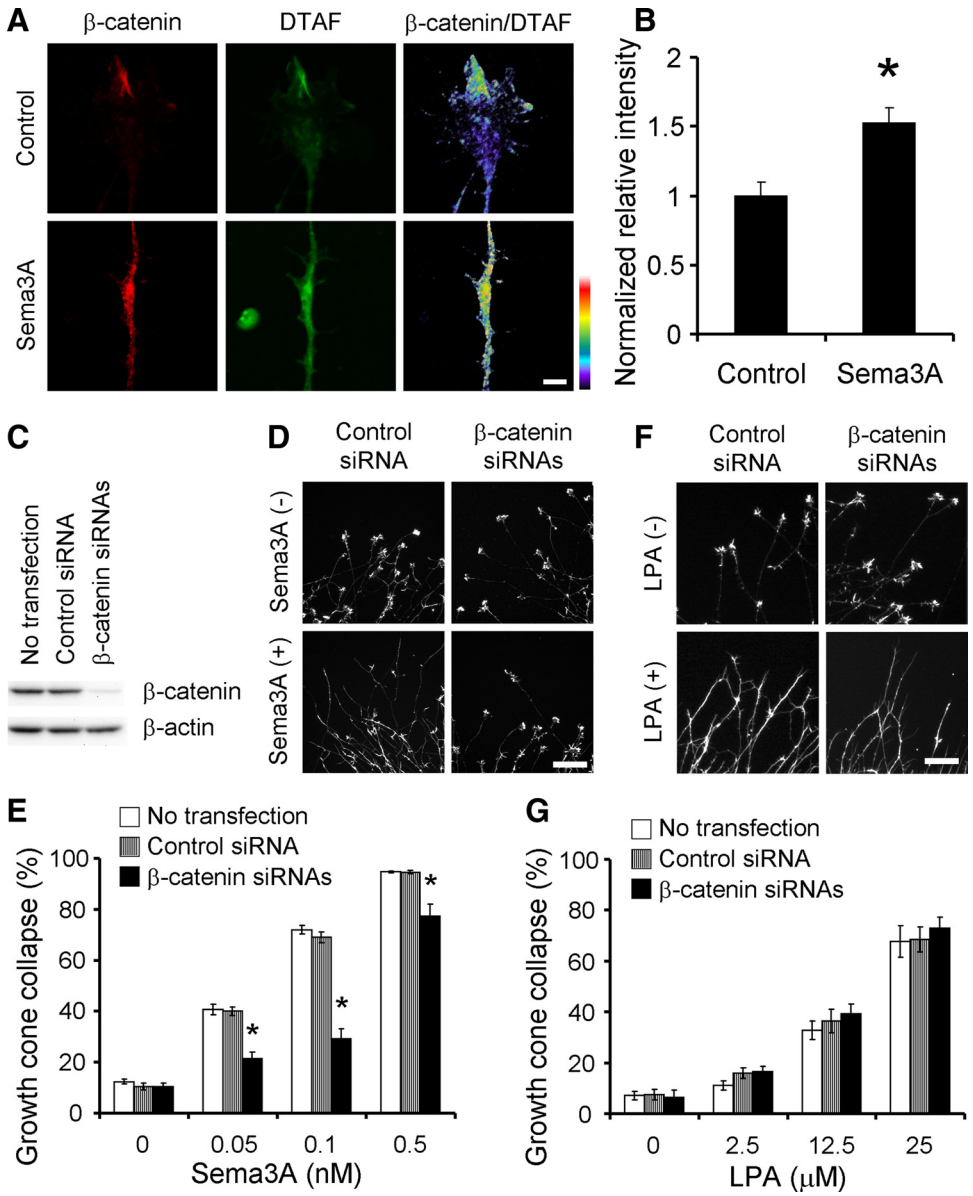

Figure 6. β-Catenin is involved in Sema3A-induced growth cone collapse. ***A***, Cultured mouse E12.5 DRG neurons were treated with 0.1 nM Sema3A for 5 min. The cultures were stained with anti-β-catenin antibody (red) and DTAF (green). Scale bar, 5 μm. ***B***, Normalized relative intensity of β-catenin immunofluorescence signals at a single growth cone after control or Sema3A application. Data are shown as the mean ± SEM ($n = 42$). *$p < 0.01$, by Student's *t* test, compared with control neurons. ***C***, Neuro2A cells were transfected with control or β-catenin siRNAs. The cell lysates were separated by SDS-PAGE and immunoblotted with anti-β-catenin and anti-β-actin antibody. ***D***, Cultured DRG neurons were transfected with the indicated siRNA. Transfection of β-catenin siRNAs suppressed Sema3A-induced growth cone collapse. Scale bar, 50 μm. ***E***, Quantitative analysis of growth cone collapse response to Sema3A in nontransfected, control, and β-catenin siRNA-transfected DRG neurons. ***F***, Cultured DRG neurons were transfected with the indicated siRNA. Transfection of β-catenin siRNAs has no effect on LPA-induced growth cone collapse. Scale bar, 50 μm. ***G***, Quantitative analysis of growth cone collapse response to LPA in nontransfected, control, and β-catenin siRNA-transfected DRG neurons. Data are shown as the mean ± SEM ($n = 6$). *$p < 0.01$, by one-way ANOVA, compared with nontreated or control siRNA-treated neurons.

nal of β-catenin was colocalized with that of GSK3β in the growth cones (Fig. 7*A*,*B*). Sema3A stimulation also enhanced the colocalization of Axin-1 with GSK3β in the growth cones (Fig. 7*C*,*D*). Again, Sema3A stimulation induced the colocalization of Axin-1 with β-catenin in the growth cones (Fig. 7*E*,*F*). The colocalization signals of these three molecules were highly distributed at the transition zone between growth cone and axonal shaft 5 min after the stimulation (Fig. 7*A*, *C*,*E*). The increased colocalization of these signals was also observed in axonal shafts. These results suggest that Axin-1 plays a role as a scaffolding protein in forming a complex with GSK3β and β-catenin in DRG growth cones and axons.

Axin-1 is required for the increase in the intensity levels of β-catenin in the growth cones in response to Sema3A

To investigate whether Axin-1 plays a role in regulating the Sema3A-induced increase in the intensity of β-catenin, we examined the effect of siRNA knockdown of Axin-1 on the increase of β-catenin in cultured mouse DRG growth cones. In Axin-1 siRNAs knockdown DRG neurons, Sema3A failed to increase the normalized fluorescent intensity levels of β-catenin in the growth cones. In the neurons transfected with control siRNA, Sema3A increased the intensity level of β-catenin by 1.7-fold in the growth cones over unstimulated control level (Fig. 8*A*, *B*). This result indicates that Axin-1 is required for

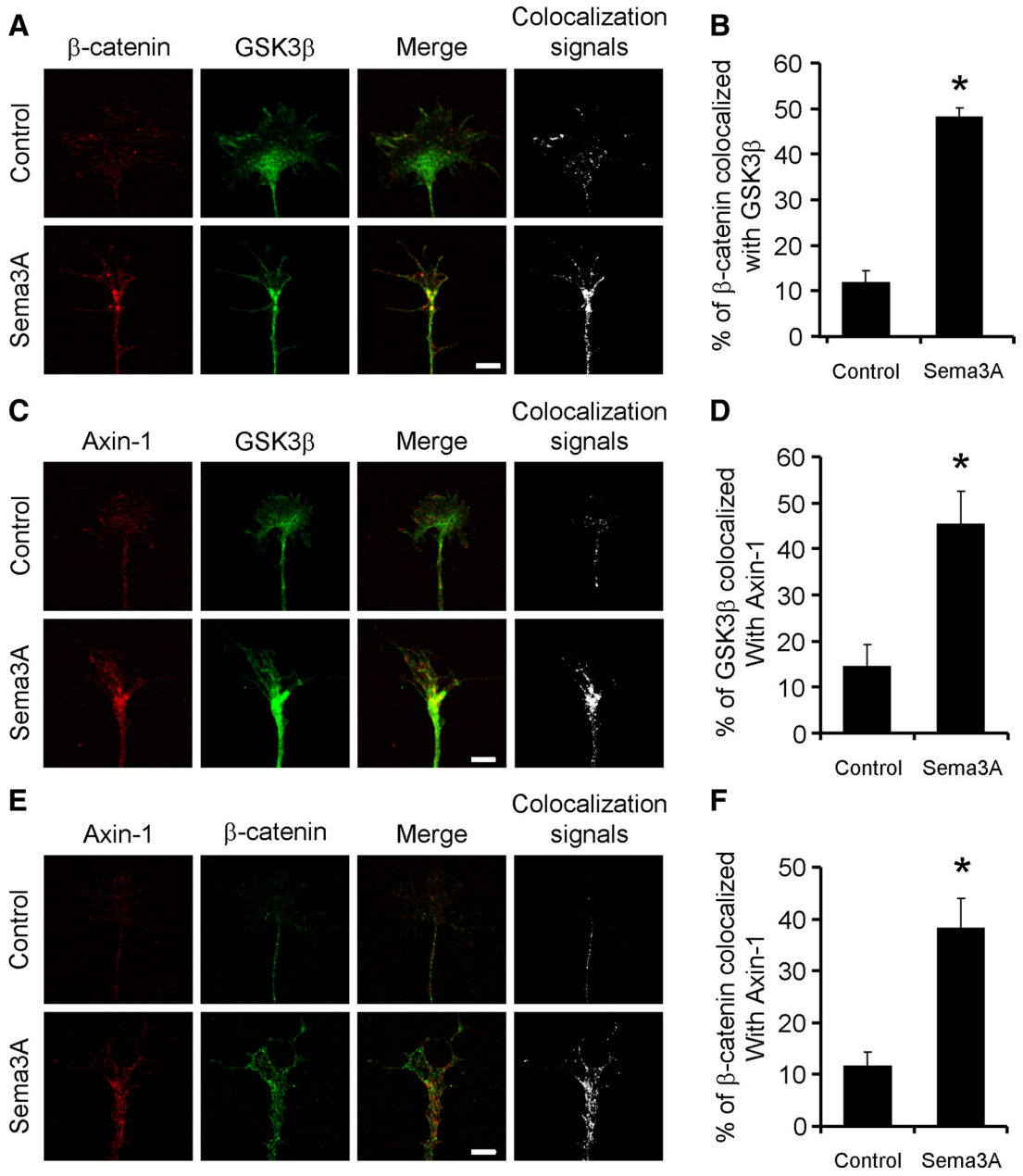

Figure 7. Sema3A enhances colocalization of β-catenin, GSK3β, and Axin-1 in DRG neurons. ***A***, ***C***, ***E***, An increase of colocalization of β-catenin, GSK3β, and Axin-1 in growth cone in response to Sema3A. Cultured mouse E12.5 DRG neurons were treated with 0.1 nM Sema3A for 5 min. The cultures were stained with the indicated antibody. Scale bar, 5 μm. ***B***, ***D***, ***F***, Quantitative analysis of the percentage of colocalization of β-catenin, GSK3β, and Axin-1 at a single growth cone as described in text. Data are shown as the mean ± SEM ($n = 21$). *$p < 0.01$, by Student's *t* test, compared with control neurons.

the increase in the intensity levels of β-catenin in the growth cones induced by Sema3A stimulation.

Axin-1, β-catenin, and GSK3β are required for the internalization of venus-Sema3A in the growth cones

Sema3A induces repulsive responses of growth cones through various endocytic pathways (Fournier et al., 2000; Guirland et al., 2004; Piper et al., 2005; Kabayama et al., 2009; Carcea et al., 2010). To examine whether Axin-1 and β-catenin are involved in Sema3A-induced endocytosis, we evaluated the endocytosis by visualizing internalization of venus-Sema3A into growth cones. We first examined whether Axin-1 and β-catenin RNAi knockdown affect binding of venus-Sema3A with neuropilin-1 in mouse DRG neurons without permeabilization (Fig. 9*A*). In the neurons transfected with control siRNA, venus-Sema3A induced growth cone collapse and exhibited punctuate high fluorescence signals in the growth cones. In the neurons transfected with neuropilin-1 siRNA, venus-Sema3A failed to induce growth cone collapse and to exhibit apparent punctuate signals in the growth cones, suggesting that venus-Sema3A induces growth cone collapse through binding with neruopilin-1. However, in the neurons transfected with Axin-1 and β-catenin siRNA, venus-Sema3A did not induce growth cone collapse but exhibited apparent punctuate fluorescent signals in the growth cones. We next examined whether Axin-1 and β-catenin are involved in the internalization of venus-Sema3A (Fig. 9*B*). After incubation with venus-Sema3A for 3 min, the internalized venus-Sema3A was selectively visualized by stripping venus-Sema3A on cell surface with acidic buffer (Carcea et al., 2010). In the neurons transfected with control siRNA, venus-Sema3A exhibited punctuate

fluorescent signals in the growth cones without permeabilization. In the neurons transfected with neuropilin-1 siRNA, venus-Sema3A did not show punctuate signals in the growth cones, suggesting that venus-Sema3A is internalized through binding with neuropilin-1 (Fig 9*A*,*C*). In neurons transfected with control siRNA, venus-Sema3A also exhibited punctuate fluorescent spots in the growth cones with permeabilization. In the neurons transfected with either Axin-1 or β-catenin siRNA, venus-Sema3A did not exhibit punctuate fluorescent signals in the growth cones. Quantitative analysis revealed that Axin-1 and β-catenin RNAi knockdown increased the number of venus-Sema3A fluorescent spots on the growth cone surface, while they both suppressed the internalization of venus-Sema3A (Fig. 9*B*,*D*). These results suggest that Axin-1 and β-catenin are involved in growth cone collapse through regulation of endocytosis.

To further investigate whether GSK3β was involved in the internalization of Sema3A in growth cones, we examined the effect of AR-A014418 on venus-Sema3A fluorescence on growth cone surface of neurons fixed and stained without permeabilization (Fig. 10*A*). In the neurons treated with DMSO, venus-Sema3A induced growth cone collapse, and venus-Sema3A punctuate fluorescent signals were seen in the growth cones. However, in the neurons treated with AR-A014418, venus-Sema3A failed to induce growth cone collapse, but the punctuate fluorescence signals were still seen in the growth cones. This result suggests that AR-A014418 per se did not affect venus-Sema3A binding to growth cone neuropilin-1. We examined the effect of AR-A014418 on the internalization of Sema3A (Fig. 10*B*). In the neurons treated with DMSO, venus-Sema3A was visualized as punctuate fluorescent signals in the growth cones. However, in the neurons treated with AR-A014418, the punctuated signals were not seen in the growth cones. Quantitative analysis revealed that treatment with AR-A014418 increased the number of venus-Sema3A fluorescent spots on the growth cone surface, while it suppressed the internalized venus-Sema3A fluorescent spots (Fig. 10*C*,*D*). This result indicates that GSK3β activation is involved in Sema3A-induced endocytosis.

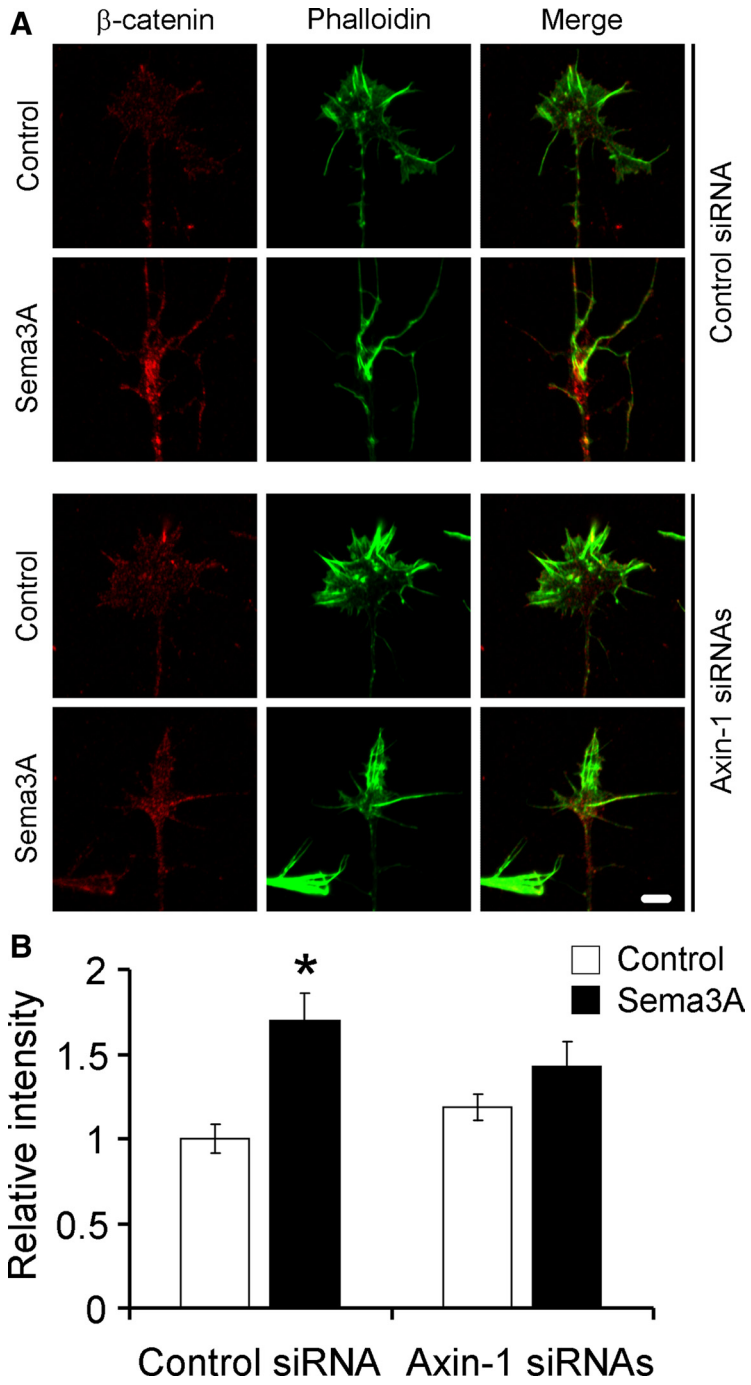

Figure 8. Axin-1 is required for Sema3A-induced increase in the intensity levels of β-catenin in the growth cones. ***A***, Cultured mouse DRG neurons were transfected with control or Axin-1 siRNAs. After transfection, the DRG neurons were treated with 0.5 nM Sema3A for 5 min. The cultures were stained with anti-β-catenin antibody (red) and Alexa 488-phalloidin (green). Scale bar, 5 μm. ***B***, Quantitative analysis of the average intensity levels of β-catenin immunofluorescence signal at a single growth cone. Data are shown as the mean ± SEM ($n = 26$). *$p < 0.01$, by Student's *t* test, compared with control siRNA-treated neurons without Sema3A.

Discussion

In this study, we identify Axin-1 as an unprimed substrate of GSK3β participating in the intracellular signaling of Sema3A. The phosphorylation of S322/S326/S330 and T485/S490/S497 residues in Axin-1 is necessary for the regulation of the responsiveness to repulsive Sema3A signals. We identify β-catenin and GSK3β as Axin-1 binding proteins, which are also involved in Sema3A signaling. Axin-1, β-catenin, and GSK3β are necessary for internalization of Sema3A. These results suggest that GSK3β, Axin-1, and β-catenin coordinately play an important role in Sema3A-induced endocytosis and probably other signaling events.

The involvement of Axin-1 as a unprimed substrate of GSK3β participating in intracellular signaling of Sema3A

GSK3β is a multifunctional serine/threonine kinase, and numerous GSK3β substrates have been classified into two categories:

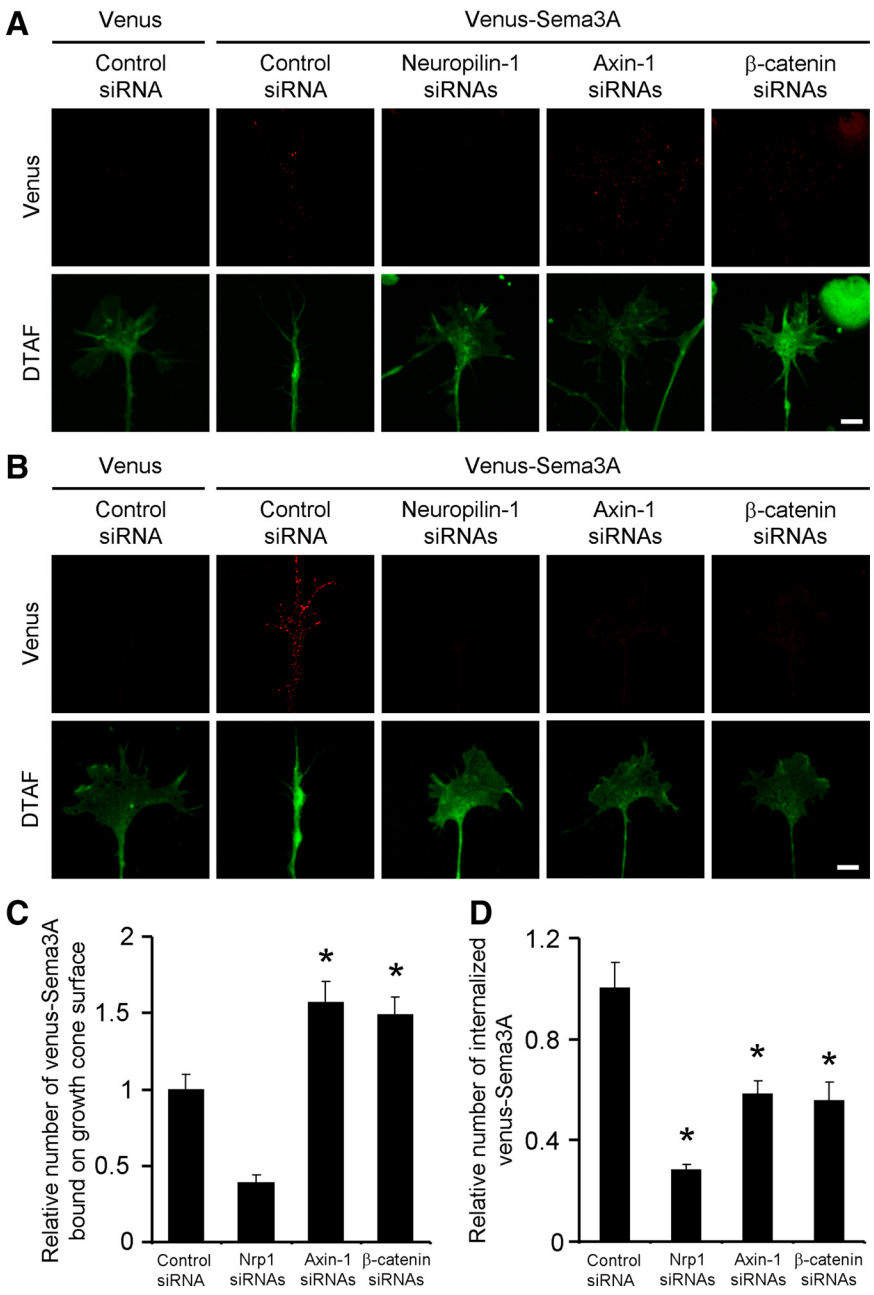

Figure 9. Axin-1 and β-catenin are involved in the internalization of venus-Sema3A. Cultured mouse DRG neurons were transfected with control, neuropilin-1, Axin-1, and β-catenin siRNAs. After transfection, the DRG neurons were treated with 0.5 nM venus-Sema3A for 3 min. ***A***, The cultures were stained with anti-GFP antibody (red) and DTAF (green) without permeabilization. Scale bar, 5 μm. ***B***, To remove the venus-Sema3A on cell surface, the DRG neurons were treated with acidic buffer. The cultures were fixed, permeabilized, and stained with anti-GFP antibody (red) and DTAF (green). Scale bar, 5 μm. ***C***, Quantitative analysis of the number of venus-Sema3A spots on a single growth cone surface. ***D***, Quantitative analysis of the number of fluorescent spots of venus-Sema3A internalized into a single growth cone. Data are shown as the mean ± SEM ($n = 24$). *$p < 0.01$, by one-way ANOVA, compared with control siRNA-treated neurons.

primed substrates, such as CRMP2, and unprimed substrates, such as Axin-1 (Hur and Zhou, 2010; Sutherland, 2011). We previously showed that phosphorylation of CRMP2 by Cdk5/GSK3β is necessary to induce growth cone collapse by Sema3A. However, the involvement of unprimed substrate remains unknown. Axin was originally identified from the characterization of the Fused locus, the disruption of which leads to duplication of axis and embryonic lethality (Zeng et al., 1997). Extensive studies have revealed that Axin serves as negative regulator of canonical Wnt/β-catenin signaling, which forms a complex with β-catenin and GSK-3β, leading to β-catenin degradation (Willert et al., 1999; MacDonald et al., 2009). In addition, Axin-1 has emerged as a major protein for regulating a variety of signaling pathway, including TGF and SAPK (stress-activated protein kinase)/JNK signaling (Luo and Lin, 2004). The involvement of Axin-1 in axon guidance remains unknown, although Axin has recently been reported to play a critical role in axon formation during cerebral cortex development (Fang et al., 2011). Here, we demonstrated that Axin-1 is required for the intracellular singling of Sema3A to induce growth cone collapse (Figs. 3, 4). Importantly, Axin-1 RNAi knockdown has no effect on LPA-induced growth cone collapse (Fig. 3). This suggests that Axin-1 is specifically involved in the signaling cascade of Sema3A. In addition, introduction of nonphosphorylated mutant forms of Axin-1 suppressed the Sema3A response, and these mutant forms of Axin-1 did not rescue the responsiveness to Sema3A in Axin-1 knocked down neurons (Fig. 4). Furthermore, inhibition of the Sema3A response by these mutant forms of Axin-1 showed no additive effect with AR-A014418 (Fig. 5). These results suggest that Axin-1 plays an important role as an unprimed substrate of GSK3β in repulsive intracellular signaling of Sema3A in DRG neurons.

Interestingly, Sema3A increased the normalized fluorescent intensity levels of Axin-1 in DRG growth cones (Fig. 2), suggesting that Sema3A induces accumulation of Axin-1 in the growth cones. Sema3A triggers growth cone collapse via regulation of cytoskeletal and membrane dynamics (Mann and Rougon, 2007; Tran et al., 2007). The increase in the levels of Axin-1 may reflect change in localization of Axin-1 via these dynamics occurring in the growth cones. In addition, inhibition of GSK3β activity suppressed the increase of Axin-1. GSK3β at the leading edge of extending growth cone is maintained in its inactive form, and Sema3A activates it (Eickholt et al., 2002; Zhou et al., 2004). Since phosphorylation of S322/S326/S330 residues in Axin-1 by GSK3β increases Axin-1 stability (Yamamoto et al., 1999), the increase in the levels of Axin-1 may reflect the Axin-1 phosphorylated and stabilized by the activated GSK3β in the growth cones. Further studies are required to evaluate whether Axin-1 phosphorylation by GSK3β is involved in Sema3A-induced increase in the levels of Axin-1.

The involvement of Axin-1 as a scaffolding protein in intracellular signaling of Sema3A

Axin-1 forms a complex with various molecules and plays an important role in regulation of diverse signaling transduction

(Luo and Lin, 2004). Axin-1 might possibly function as a scaffolding protein in repulsive intracellular signaling of Sema3A. GSK3β is an Axin-binding protein and phosphorylation of Axin-1 by GSK3β is required for interaction of GSK3β with Axin-1 (Thomas et al., 1999). We showed that introduction of FRATtide, which blocks interaction of Axin-1 with GSK3β, suppressed Sema3A-induced growth cone collapse (Fig. 1). In addition, Sema3A enhanced the colocalization of Axin-1 with GSK3β in the growth cones (Fig. 7). These results suggest that the interaction of Axin-1 with GSK3β is necessary for repulsive Sema3A signaling. This idea is also consistent with the recent finding that Cdk5-mediated phosphorylation of Axin-1 increases its interaction with GSK3β (Fang et al., 2011). Since Cdk5 is required for repulsive Sema3A signaling (Sasaki et al., 2002), Cdk5-mediated phosphorylation of Axin-1 may play an important role for the interaction of Axin-1 with GSK3β.

We further demonstrated that β-catenin is involved in the intracellular signaling of Sema3A to induce growth cone collapse in the DRG neurons (Fig. 6). Again, Sema3A promoted the colocalization of Axin-1 with β-catenin in the growth cones (Fig. 7). Consistently, we showed evidence for the involvement of phosphorylation of Axin-1 T485/S490/S497 by GSK3β, which increases the interaction with β-catenin (Jho et al., 1999), in Sema3A signaling (Figs. 4, 5). These results suggest that the interaction of β-catenin with Axin-1 is involved in repulsive Sema3A signaling. Sema3A facilitated the colocalization of GSK3β with β-catenin in the growth cones (Fig. 7). Formation of GSK3β/Axin-1/β-catenin complex could be associated with phosphorylation of β-catenin by GSK3β (Willert et al., 1999). At present, we have not determined whether phosphorylation of β-catenin by GSK3β was induced by Sema3A stimulation. Phosphorylation of β-catenin by GSK3β in GSK3β/Axin-1/β-catenin complex requires priming phosphorylation by casein kinase 1 (Amit et al., 2002). Further studies are required to determine whether sequential casein kinase 1 and GSK3β phosphorylation of β-catenin is involved in Sema3A signaling. Axin-1 also interacts with CRMP1 (Stelzl et al., 2005). Therefore, Axin-1 may play an important role in forming a large complex containing GSK3β, β-catenin, CRMP1, CRMP2, and other related molecules to regulate the responsiveness to Sema3A in axon guidance mechanism (Eickholt et al., 2002; Brown et al., 2004; Cole et al., 2004; Uchida et al., 2005). Recently, asymmetrical membrane trafficking and accumulation of signaling components in growth cones occurring in the process of axon guidance attracted attention (Quinn and Wadsworth, 2008; Tojima et al., 2010). Axin-1 may contribute to axon guidance via regulation of asymmetrical clustering of signaling components containing GSK3β and β-catenin.

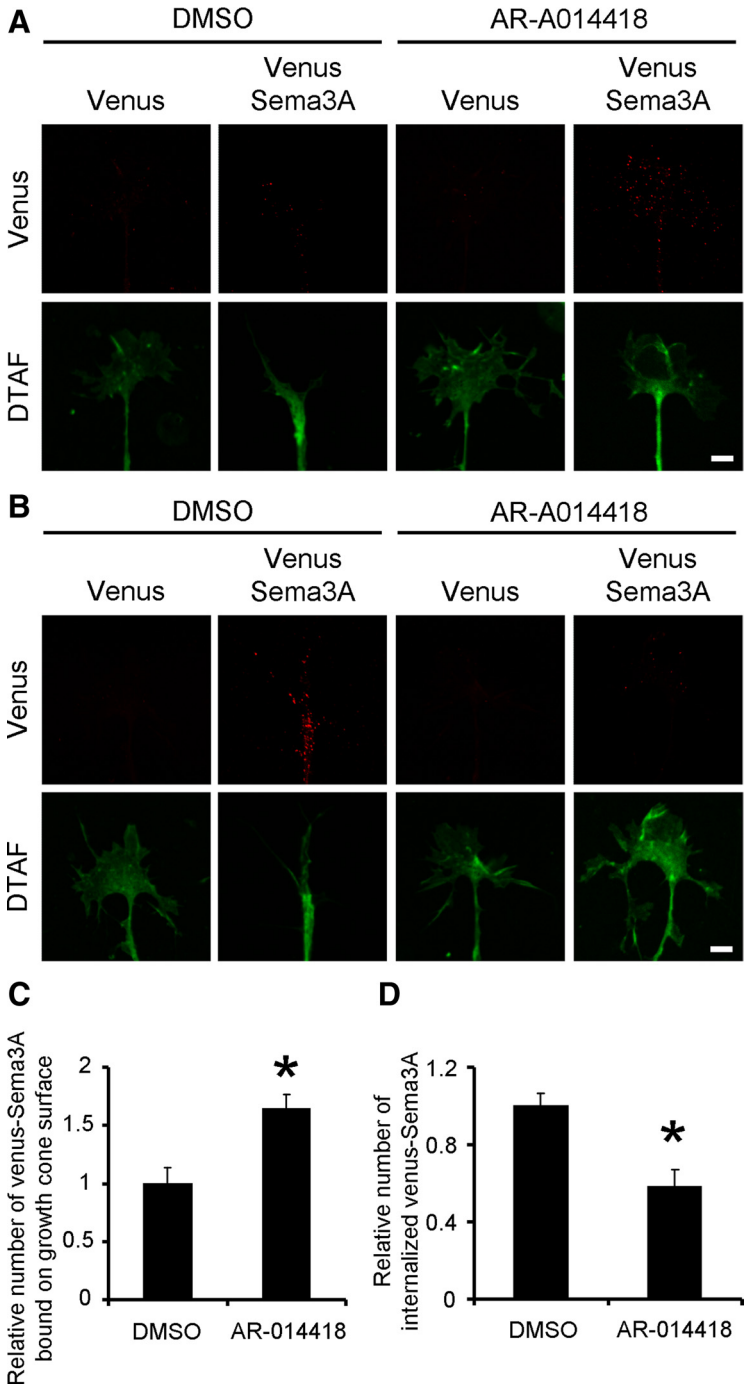

Figure 10. GSK3β is involved in the internalization of venus-Sema3A. Cultured mouse DRG neurons were treated with DMSO or AR-A014418 at 10 μM for 30 min, and then treated with 0.5 nM venus-Sema3A for 3 min. ***A***, The cultures were stained with anti-GFP antibody (red) and DTAF (green) without permeabilization. Scale bar, 5 μm. ***B***, To remove venus-Sema3A on cell surface, the DRG neurons were treated with acidic buffer, fixed, permeabilized, and stained with anti-GFP antibody (red) and DTAF (green). Scale bar, 5 μm. ***C***, Quantitative analysis of the number of venus-Sema3A fluorescent spots on a single growth cone surface. ***D***, Quantitative analysis of the number of fluorescent spots of venus-Sema3A internalized into a single growth cone. Data are shown as the mean $\pm$ SEM ($n = 20$). *$p < 0.01$, by Student's t test, compared with treatment with DMSO.

Surprisingly, Sema3A increased the normalized fluorescent intensity levels of β-catenin in the growth cones, and the increase was suppressed by Axin-1 knockdown in neurons (Figs. 6, 8). These results suggest that Axin-1 is required for Sema3A-induced increase in the levels of β-catenin in the growth cones. These

findings also suggest that Sema3A signaling induces the increase of β-catenin with a mechanism different from the canonical Wnt/β-catenin signaling that requires Axin degradation for promoting accumulation of β-catenin in cytoplasm and nucleus (Willert et al., 1999; Yamamoto et al., 1999). We observed that Sema3A increased the intensity of β-catenin in axonal shafts (Fig. 6), which is reminiscent of a facilitation of axonal transport elicited by Sema3A (Goshima et al., 1997). Therefore, the increase of β-catenin in growth cones may reflect the β-catenin transported from axon via the facilitation of axonal transport, and the β-catenin may transmit repulsive Sema3A signaling via formation of a complex with GSK3β and Axin-1 in growth cones.

The involvement of Axin-1, β-catenin, and GSK3β in internalization of Sema3A

How do Axin-1, β-catenin, and GSK3β act downstream of Sema3A signaling? Axin is involved in the internalization of LRP6 through caveolin-mediated endocytosis in Wnt/β-catenin signaling (Yamamoto et al., 2006). In addition, Wnt signaling induces formation of multivesicular bodies containing Wnt receptor complex including GSK3β, Axin-1, and β-catenin (Taelman et al., 2010). Although it is unknown whether or not similar mechanisms underlie the Sema3A signaling, we found that Axin-1, β-catenin, and GSK3β are involved in the internalization of venus-Sema3A (Figs. 7, 9, 10). In fact, Sema3A induces repulsive response through clathrin-dependent endocytosis, lipid raft-dependent endocytosis and macropinocytosis (Fournier et al., 2000; Guirland et al., 2004; Piper et al., 2005; Kabayama et al., 2009, Carcea et al., 2010, Tojima et al., 2010), and internalization of Sema3A via raft-mediated endocytosis is required for repulsive responsiveness to Sema3A (Carcea et al., 2010).

In summary, the GSK3β/Axin-1/β-catenin complex may play an important role in Sema3A signaling via an endocytic mechanism, which regulates axon repulsion, attraction, synapse maturation, and neural polarity. Future studies are required to elucidate the possibility of cross talk between signaling of these important axon guidance and/or morphogen molecules.

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