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FKBP133: A novel mouse FK506-binding protein homolog alters growth cone morphology

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Abstract

FK506-binding proteins are the peptidyl prolyl *cis–trans* isomerases that are involved in various intracellular events. We characterized a novel mouse FK506-binding protein homolog, FKBP133/KIAA0674, in the developing nervous system. FKBP133 contains a domain similar to Wiskott–Aldrich syndrome protein homology region 1 (WH1) and a domain homologous to FK506-binding protein motif. FKBP133 was predominantly expressed in cerebral cortex, hippocampus, and peripheral ganglia at embryonic day 18.5. FKBP133 protein was distributed in the axonal shafts and was partially co-localized with F-actin in the growth cones of dorsal root ganglion neurons (DRG). The number of filopodia was increased in the DRG neurons overexpressing FKBP133. In contrast, the overexpression of a mutant deleted the WH1 domain reduced the growth cone size and the number of filopodia. Furthermore, the neurons overexpressing FKBP133 became significantly resistant to Semaphorin-3A induced collapse response. These results suggest that FKBP133 modulates growth cone behavior with the WH1 domain.

Keywords: FK506-binding protein; PPIase; WH1; FKBP133; KIAA0674; Sema3A; Plexin; Nervous system; Development

FK506-binding protein (FKBP) family is firstly identified as immunophilins, targets for immunosuppressant FK506 and Rapamycin [1]. The members of FKBP family possess at least one peptidyl-prolyl isomerase (PPIase) homologous region, which is thought to catalyze *cis–trans* isomerization of X-proline peptide bonds [2]. To date, 17 distinct FKBP genes have been identified in human genome [3], however, half of the members have not been studied in detail their biochemical or biological role. Among the FKBP family, FKBP12 has been characterized extensively. FKBP12 interacts with calcineurin only in the presence of FK506 [4]. The immunosuppressive action of FK506 is exhibited through the inhibition of the calcineurin phosphatase activity by FKBP12-FK506 complex [5]. Rapamycin also forms tight complex with FKBP12 and binds to Target of Rapamycin (TOR) [6], and this leads to the suppression of S6 kinase-dependent protein synthesis and the inactivation of eukaryotic initiation factor 4E.

While FKBP12 and a related protein (FKBP12.6/FKBP12A) represent only an enzymatic domain in the primary structure, other FKBP proteins contain additional amino acid sequences [7]. FKBP13 and FKBP23 contain a secretion signal sequence at N-terminus and an endoplasmic reticulum-retention signal at C-terminus. In the case of

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FKBP25, insertion of basic amino acid stretch in the FKBP domain renders the protein nuclear localization. FKBP38 contains a FKBP, three tetratricopeptide repeats (TPR), and a calmodulin-binding domain [8]. FKBP51 and FKBP52, which have been found in steroid hormone receptor complexes, contain two tandem FKBP domains, three TPR, and a calmodulin-binding consensus sequence [9]. The accessory sequences determine the subcellular localization of each member of FKBP and contribute to the protein–protein interaction.

Several members of FKBP family are more abundant in the nervous system than in the immune system [1]. Furthermore, FK506 and various non-immunosuppressive analogs of FK506 have been shown to exert neuroregenerative and neuroprotective action on both central and peripheral nervous systems [1]. It has been shown that FKBP52 is responsible for the FK506-stimulated neurite outgrowth of hippocampal neurons [10]. FKBP38 has been characterized by predominant expression in human brain [8]. Recently, calcium–calmodulin–FKBP38 complex was shown to create an active FKBP and to promote apoptosis in neuronal tissues [11].

Neuronal growth cones receive axon guidance molecules and alter the growing direction and speed of neurites through the rearrangement of cytoskeletons in the growth cones [12]. Semaphorin-3A (Sema3A), one of the repulsive axon guidance molecules, induces F-actin depolymerization in the growth cones of the embryonic neurons of peripheral sensory ganglia [13]. These neurons express the Sema3A receptor complex, Neuropilin-1 and Plexin-A [14]. It has been shown that the cytoplasmic tail of Plexin-A interacts with numerous proteins, such as MICAL, Collapsin Response Mediator Protein, and small G-proteins, and mediates Sema3A-intracellular signaling [15–17]. However, the molecular mechanism that links these proteins and the cytoskeletons remains to be determined.

We recently found a novel FK506 binding protein homolog, FKBP133/KIAA0674, which was directly immunoprecipitated with anti-Plexin-A4 polyclonal antibody [18]. To elucidate the function of FKBP133 in the nervous system, we exploited the dominant-negative effect of FKBP133 mutants on cultured neurons. Here we present that the overexpression of FKBP133 alters the growth cone morphology through the Wiskott–Aldrich syndrome homology region 1 (WH1) of FKBP133.

Experimental procedures

Materials. Antibodies were used: a rabbit polyclonal anti-HA antibody Y-11 from Santa Cruz Biotech (Santa Cruz, CA, US), anti-α-tubulin mAb (DM1A) from ICN (Costa Mesa, CA, US), secondary antibodies conjugated Alexa 488 or 594 and Rhodamine–Phalloidin from Invitrogen-Molecular Probes (Eugene, OR, US), and Horseradish peroxidase-conjugated secondary antibodies from Amersham (Arlington Heights, IL, US).

Plasmid construction. To construct mouse FKBP133 expression vector, the coding region of BC060651 (44–3692 bp) was PCR-amplified from reverse transcriptional mouse embryonic day 18.5 brain cDNA library with 2 primers (5′-tttggatccTTCGGTGCCGGAGACGAGGATGACACC-3′, 5′-ttttctagattaCCCCAGCCAGCCAATGTCATCGTCATCA-3′). The underlines indicate the introduced *Bam*HI and *Xba*I sites, respectively. The amplified fragment was subcloned into pcDNA3.1 with a N-terminal HA epitope tag using *Bam*HI and *Xba*I sites. N-terminal EGFP-tagged FKBP133 was also generated. The coding fragment of mouse FKBP12 or FKBP133-FKBP domain (183–298 aa) was cloned into *Bam*HI and *Xho*I sites of pGEX-6p-2 (Amersham). The EGFP-tagged FKBP133 was used as a template for deletion mutants. WH1 domain deleted mutant EGFP-FKBP133ΔWH1 (Δ70–167 aa) was generated with two primers (5′-ttcGTGGCCAAGTGCAATAGCATCTCTTCC-3′, 5′-ttcGCCTGTGGTAGCTGGGGCTGGCTTTGG-3′) by PCR method. FKBP domain deleted mutant EGFP-FKBP133ΔFKBP (Δ191–286 aa) was generated with two primers (5′-ttcCGGGTGAAGTTTGCCAGAGATTCTGGCT-3′, 5′-ttcCTCTGCTGCAACCAGGTCCTGACACA-3′). All PCR products were verified by sequencing. The coding regions of various mutants were excised and transferred to pHSV vector to generate recombinant herpes simplex virus as previously described [19].

In situ hybridization of fkbp133. Digoxigenin-labeled cRNA probes for mouse fkbp133 were transcribed from pBluescript KS vector containing BC060651 fragment (150–1133 bp) with either T3 or T7 RNA polymerase (Roche, Meylan, France). C57BL/6J mice (Charles River, Japan) were deeply anesthetized with diethyl ether and perfused intracardially with 4% paraformaldehyde in PBS. The brains were removed and post-fixed in 4% paraformaldehyde at 4 °C for overnight. After cryoprotected with 30% sucrose, the brains were embedded in Tissue-Tek O.C.T Compound (Sakura, Tokyo, Japan). Frontal (embryonic day 18.5) or sagittal (postnatal day 15) cryostat sections (16 μm thickness) were collected on Superfrost slides (Matsunami, Tokyo, Japan) and stored at −80 °C before use. The brain sections were re-fixed with 4% paraformaldehyde for 30 min and were hybridized with anti-sense or sense probes at 65 °C for overnight. The final concentration of the probes diluted in the hybridization solution (containing 50% formamide, 5× SSC, 1% SDS, 50 mg/ml yeast tRNA, and 50 mg/ml heparin) was 4 μg/ml. The slides were washed at 65 °C in 50% formamide, 5× SSC, 1% SDS, and then at 65 °C in 50% formamide, 2× SSC twice. The slides were incubated for 1 h at room temperature with 10% BSA, then overnight at 4 °C with alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche). Bound probes were visualized with BCIP and NBT substrates.

Immunoblot analysis and immunohistochemistry. Rabbit polyclonal anti-FKBP133 antibody was raised against the N-terminal region of mouse FKBP133 (2–363 aa). Anti-FKBP133 antiserum was subjected to the affinity purification. Various tissues from E18.5 ICR mouse (SLC, Japan) were homogenized in 10 volumes of 20 mM Tris–HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 4 mM EDTA, 50 μM p-APMSF, and 1 mM sodium orthovanadate. The solubilized fractions (6 μg) were analyzed by immunoblot with anti-FKBP133 antibody. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and Immobilon Western kit (Millipore, Bedford, MA, US). For immunohistochemistry, the coronal section of mouse E18.5 brain was sequentially incubated with anti-FKBP133 antibody and with HRP-conjugated anti-rabbit IgG secondary antibody. The bound antibodies were visualized with DAB staining. Nuclei were counterstained with hematoxylin.

Mouse E12 DRG explants were prepared as described previously [20]. The cells were fixed with 2% paraformaldehyde/PBS for 30 min. The cells were blocked in the PBS buffer supplemented with 5% donkey serum and 0.1% Triton X-100. The cells were reacted with anti-FKBP133 rabbit pAb and with anti-α-tubulin mouse mAb. The bound primary antibodies were visualized with Alexa 488-conjugated anti-rabbit IgG antibody and Alexa 594-labeled anti-mouse IgG antibody. F-actin was stained with rhodamine-phalloidin.

Growth cone size measurement and collapse assay. Recombinant herpes simplex virus (HSV) preparations and infections of mouse E12 DRG explants were performed as previously described [21]. Recombinant viruses possessing EGFP, EGFP-FKBP133, EGFP-FKBP133ΔWH1, or EGFP-FKBP133ΔFKBP were added to the explants at a concentration of 10^6 PFU/ml. The percentage of axons expressing EGFP fluorescence

ranged 60–80% in the cultures. For the measurement of growth cone size, the neurites stained with rhodamine-phalloidin were taken with CCD camera (Spot) at 8 bit B/W range through 40× object lens mounted on Olympus IX70 inverted microscope, then the size of each growth cone was quantified with ImageJ program (NIH). In each construct, 60–100 growth cones were randomly selected and the average of the size was scored. The number of filopodia ($\geqslant 3$ μm) in each growth cone was also counted. Growth cone collapse assays were performed with purified recombinant chick Sema3A (collapsin-His6) or alkaline phosphatase-fused mouse Sema3A as previously described [22]. All procedures were performed according to the guideline of Yokohama City University School of Medicine.

Results

FKBP133/KIAA0674 encodes a Wiskott–Aldrich syndrome protein homology region 1 and a FK506 binding motif

We have recently identified a mouse homolog of human KIAA0674 protein (AB014574) as an antigen cross-reacting with anti-Plexin-A4 polyclonal antibody [18]. The mouse Riken cDNA C430014M02 (GenBank Accession No. BC060651) encodes the full length of the protein. We assigned the open reading frame of BC060651 from 41 to 3691 bp. The coding region encodes 1216 amino acid polypeptides and the calculated molecular weight is 133-kDa (Fig. 1A). Due to the acidic isoelectric point ($pK_i = 4.8$) of the polypeptides, the protein may exhibit higher molecular weight on SDS–PAGE analysis than the estimated mass. The coding region of BC060651 is homologous (94% identity) to that of mouse KIAA0674 (AK122343) [23]. Six percent difference reflects the diversity of 3′ exons that encode the last 27 (BC060651) and 66 (AK122343) amino acids of C termini, suggesting alternative splicing (Fig. 1B). We assigned the former and the latter as A- and B-isoform, respectively. The predicted molecular mass of the B-isoform is 137-kDa. The deduced amino acid sequences of BC060651 and of human KIAA0674 share 79.7% identity. The orthologs of BC060651 were found in rat (XM_342846), chick (XM_415541), and zebra fish (XM_695432) but not in invertebrates.

BC060651 possesses two conserved domains: a Wiskott–Aldrich syndrome protein (WASP) homology region 1 (WH1) (70–168 aa) and an FKBP-type peptidyl-prolyl *cis–trans* isomerase domain (FKBP) (178–289 aa). A partially homologous region (571–781 aa) to Band4.1-Ezrin-Radixin-Moesin (FERM) domain is also present. The amino acid identity of WH1 domain between BC060651 and WASP (42–147 aa) is 22% and of FKBP domain between BC060651 and FKBP12 is 30% (Fig. 1C and D). Regardless of PPIase activity or FK506 binding ability, the proteins containing a FKBP domain have been classified as FKBP protein family. Thus, we designated the protein FKBP133 according to the nomenclature of FKBP proteins [3,7]. FKBP133 possesses a phosphorylation consensus motif of cAMP/cGMP kinase (KKKS, 745–748 aa) in the FERM domain.

We expressed and purified GST-fused FKBP domain of FKBP133 to examine enzymatic property. In so far, we have not been able to detect PPIase activity or [^{3}H]dihydro-FK506 binding of the protein, while GST-FKBP12 showed significant activities in the same condition (not shown).

The mRNA of fkbp133 predominantly expressed in the developing nervous system

To examine expression patterns of fkbp133 mRNA, we performed *in situ* hybridization analysis in the developing mouse nervous system at embryonic day 18.5 (E18.5). A strong signal for fkbp133 transcripts was detected in the dorsal root ganglia (DRG) and sympathetic ganglia (SG) (Fig. 2A), while no signal was detected using the sense probes (Fig. 2B). The transcripts of fkbp133 were expressed in the spinal cord (Fig. 2A). In the neocortex of embryos, a strong signal for fkbp133 transcripts was detected in the cortical plate and a modest signal was in subventricular and ventricular zones, while the expression in intermediate zone was barely detectable (Fig. 2C). In the hippocampus, fkbp133 was strongly expressed in the pyramidal cell layer of CA1–CA3 fields and in the dentate gyrus (not shown).

We next surveyed the expression of fkbp133 in the nervous system at postnatal day 15 (P15). The fkbp133 signal in the neocortex was downregulated and a weak signal was detected in the 2/3 layers (Fig. 2D). In the hippocampus, the expression of fkbp133 was prominent in the granule cell layer of dentate gyrus and was modest in the pyramidal cell layer in CA1 to CA3 fields (Fig. 2E). Interestingly, the granular layer of cerebellum exhibited strong fkbp133 signal (Fig. 2F). A modest signal was also observed in the Purkinje cell layer (not shown).

FKBP133 protein distributes in the developing axons and growth cones

To confirm the protein expression of FKBP133 in mouse embryo, we performed immunoblot analysis on mouse E18.5 tissues. As shown in Fig. 3A, a 180-kDa signal was blotted with anti-FKBP133 antibody in the lanes of brain, heart, lung, kidney, and thymus but not in the lane of liver. Transiently expressed HA-FKBP133 in HEK293T cells was also blotted at the same size, suggesting that the assigned coding frame represents full length FKBP133 protein. The specific-reactivity of anti-FKBP133 antibody with a 180-kDa polypeptide was confirmed by the preincubation of the antibody with the antigen (not shown).

We next examined immunostaining in the hippocampal formation using the anti-FKBP133 antibody where we previously observed strong mRNA signal in the pyramidal cell layer [18]. As shown in Fig. 3B and C, hippocampal alveus, stratum radiatum of CA1–CA3 fields, and fimbria were stained with anti-FKBP133 antibody (Fig. 3B) but not with control rabbit IgG (Fig. 3C). The signal was also present in the pyramidal cell layer and dentate gyrus. These results suggest that FKBP133 protein distributes in the axonal and/or den-

A

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   1 MFGAGDEDDT DFLSPSGGAK LASLFGLDQA TMGHGNEFFQ YTAPKQPKKG QGTAAGNQTA
  61 PKPAPATTGT SSVLFATAVH AYRYINGQYA KQGKFGAAVL GNHTSREYRI LLYISQQQPV
 121 TVATIHLNFE LMVRPNNYST FYDDQRQNWS IMFESEKAAV SFNKQVCVAK CNSISSLDAV
 181 LCQDLVAAEG PAVETGDSLE VAYTGWLLQN HVLGQVFDST ANKDKPLRLK LGSGKVVKGL
 241 EDGLLGMKKG GKRLIITPSA CAAGSEGVIG WTQPTDSILV FEVEVRRVKF ARDSGSDGHS
 301 VSSRDSAAPS PIPASDSLSA DPVVTPLPLP LKPGEPGLRS KSNSLSEQLT VNSNPDTVKA
 361 KLISRMAKMG QPMLPILPPQ LDSNDSETED ATVLRGAGQS LVTPSIQPSL QPAHPVLPQM
 421 ASQAPQPSGS GLQTPSAALM QAVSLDSHSA VSGNAQNFQP YAGVQAYAYP QTPSVTSQLQ
 481 PVRPLYPAPL SQAPHFQGSG DMMSFLMTEA RQHNTEIRMA VNKVADKMDH LMTKVEELQK
 541 HSSGNSMLLP SMSVTMETSM IMSNIQRIIQ ENERLKQELL EKSSRIEEQN DKISDLIERN
 601 QRYVEQSNLM MEKRNNSLQT ATENTQARIL HAEQEKAKVT EELAAATAQV SHLQLKMTAH
 661 QKKETELQLQ LTDNLKETDL LRGHVTRLQA DLSELREASE QTQTKFKSEK QSRRQLELKV
 721 TSLEEELTDL RAEKTSLEKN LSERKKKSAQ ERCQAEAEMD EIRKSHQEEL DRLRQLLKKA
 781 RVSTDQAAAE QLTLAQAELQ SQWEAKCEQL LASARDEHLQ QYREVCAQRD AHQQKLALLQ
 841 DECLALQAQI AAFTEQKEHM QRLEKTKSQA PAGRAAADPS EKVKKIMNQV FQSLRGEFEL
 901 EESYDGGTIL RTIMHTIKMV TLQLLNHQEE EEEEEEEEEE EKKPLRPSLE QPGPATPGMP
 961 PAPPSGETQE APEVLPEQVV GETTPLPLQA LPTPENGAQT RKGEPAEAEV PSEIKDSSLP
1021 PQPAGIPAHR VLGPPTSIPP KPPGPVTMDS ESEEMLAADQ RTVQPNGLLG EEHVREVATD
1081 GLLQGNSRRL SLTPDPEKGE PPALDPESQG GEAQPPECKQ AEDVSSSGPR ETLLDTELAS
1141 AAAGTSLRHN QDSQHCSLSG DEEDELFKGA TLKVPRPTAQ PEEEDEDEVS MKGRPPPTPL
1201 FGDDDDDDDD DIGWLG
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B

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1190                                                          V RRPQALLSPH
1201 TTTWGLFPAG SETRKGVCWW LVSWTLCTGP CGSKLGAQSG QLCKHDGDGQ LPLYS
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C

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WASP      42 GRKCWTLATTVVQLYLALPPGAEHWTMEHCGAVCFVKDNPQKSYFIRLY-GLQAGRLL
                 |   | | |      |          ||            | | ||    |
FKBP133   70 -TSS-VLFATAVHAYRYI-NG-QYAKQGKFGA-AVLGNHTSREYRILLYISQQQPVTV

WASP      99 WEQELYSQLVYLTPTPFFHTFAGDDCQV-GLNFADESEAQAFRALVQEKI
               |    |     |      ||  |  |      |  |  |  |     |
FKBP133  123 ATIHLNFELM-VRPNN-YSTFYDDQRQNWSIMFESEKAAVSFNK--QVCV
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D

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                                        *             **        *      *
FKBP12     1 -GVQVETISPGDGRTFPKRGQTCVVHYTG-MLED---GKKFDSSRDRNKPFKFTLGKQ
              |         |      |    | |||  |      |  |||     ||     ||
FKBP133  178 DAVLCQDLVAAEGPA-VETGDSLEVAYTGWLLQNHVLGQVFDSTANKDKPLRLKLGSG

             ***  *                       *    *    *       *
FKBP12    54 EVIRGWEEGVAQMSVGQRAKLIISSDYAYGATGHPG-IIPPHATLVFDVELLKLE
              |  | | |    |  |      |  |  | |  |  |    |     ||| ||
FKBP133  235 KVVKGLEDGLLGMKKGGKRLIITPSACAAGSEGVIGWTQPTDSILVFEVEVRRVK
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Fig. 1. Primary structure of mouse FKBP133. (A) Deduced amino acid sequence of mouse FKBP133. WASP homology region 1 (WH1) is shown by underline. FKBP-type peptidyl-prolyl *cis–trans* isomerase is shown by bold. Double underline indicates A-isoform specific sequence. (B) B-isoform specific amino acid sequence. (C) Alignment of the WH1 domain of FKBP133 with that of WASP. The WH1-domain of mouse FKBP133 (residues 70–168) is shown aligned with the domain (residues 42–147) of mouse WASP. Vertical lines indicate identical amino acids. (D) Alignment of the FKBP domain of FKBP133 with FKBP12. The FKBP-domain of mouse FKBP133 (residues 178–289) is shown aligned with mouse FKBP12. Identical amino acids are indicated by vertical lines. Asterisk (*) indicates the residues of FKBP12 involved in FK506 binding. The aligned FKBP12 is mature form of which initiation Met is cleaved. The alignment was performed using the PAM250 score.

dritic shaft of the developing neurons. Then, we examined the subcellular localization of FKBP133 in cultured neurons by immunocytochemistry. As shown in Fig. 3D and E, the axonal shafts of mouse E14.5 DRG neurons were strongly stained with anti-FKBP133 antibody. Moderate distribution of FKBP133 in the growth cones was also observed. Half of the growth cones exhibited the preferred distribution of FKBP133 in the peripheral domain (Fig. 3D). The localization was partially overlapped with F-actin in the growth cone (Fig. 3F and H). FKBP133 distribution in the axonal shaft was merged with α-tubulin, while the FKBP133 and α-tubulin were not co-localized in the growth cones (Fig. 3G and I).

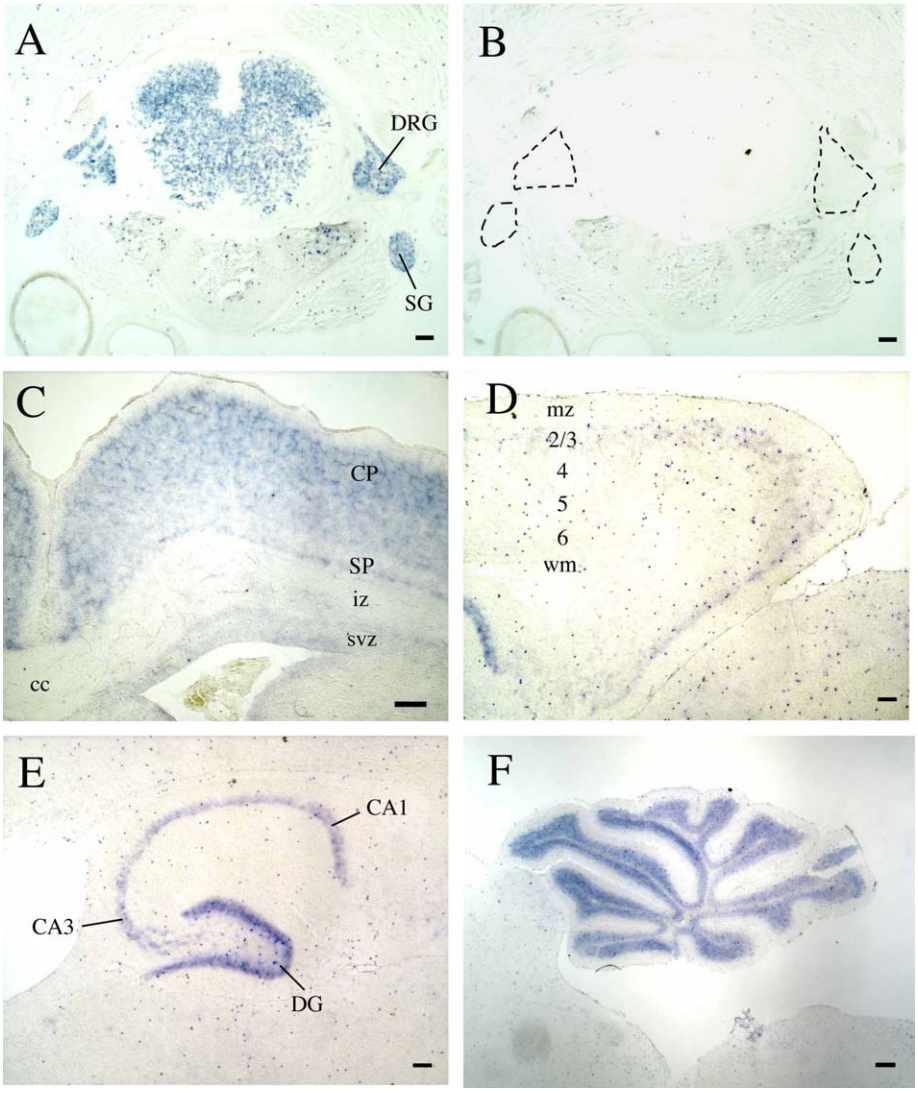

Fig. 2. Expression of FKBP133 mRNA in the mouse nervous system. (A) Distribution of fkbp133 transcripts in dorsal root ganglia, sympathetic ganglia, and spinal cord of embryos at E18.5. (B) None of the sense probes showed any labeling in the same region of (A). Dashed lines indicate the border of the ganglia. (C) fkbp133 transcripts in the neocortex of coronal section at E18.5. Note that cortical plate and subventricular zone express the mRNA. (D,E,F) fkbp133 mRNA in the central nervous system of sagittal section at P15. (D) Neocortex, (E) hippocampus, and (F) cerebellum. Abbreviations are used as follows: 2–6, cortical layers; CA1 and CA3, CA1 and CA3 fields of the hippocampus, respectively; cc, corpus callosum; CP, cortical plate; DG, dentate gyrus; DRG, dorsal root ganglion; iz, intermediate zone; mz, marginal zone; SG, sympathetic ganglion; SP, subplate; svz, subventricular zone. Scale bar: (A)–(E) 100 μm, (F) 250 μm.

Overexpression of FKBP133 in DRG neurons alters growth cone morphology

To evaluate the function of FKBP133 in the growth cones, we assessed the morphology of the growth cones overexpressing a series of deletion mutants of FKBP133. Fig. 4A illustrates the constructed FKBP133 mutants: EGFP-FKBP133, EGFP-FKBP133ΔFKBP, and EGFP-KFBP133ΔWH1. Fig. 4B indicates the immunoblot analysis of transiently expressed these constructs in COS-7 cells.

We next introduced these constructs in mouse E12 DRG neurons. The localization of the EGFP-fusion proteins in the axons was confirmed by EGFP fluorescence. The overexpression of EGFP, EGFP-FKBP133, or EGFP-FKBP133ΔFKBP did not modulate the rate of axon outgrowth, while EGFP-FKBP133ΔWH1 slightly inhibited the outgrowth. Focused on the growth cone morphology, the overexpression of EGFP-FKBP133ΔWH1 (Fig. 4E) reduced the size of growth cone approximately half comparing to the size in other preparation (Fig. 4G). The overexpression of FKBP133ΔWH1 also decreased the number of filopodia while the overexpression of FKBP133 or FKBP133ΔFKBP exhibited opposite effect (Fig. 4H). These results suggest that FKBP133 may be involved in the cytoskeletal organization of the growth cone via the WH1 domain.

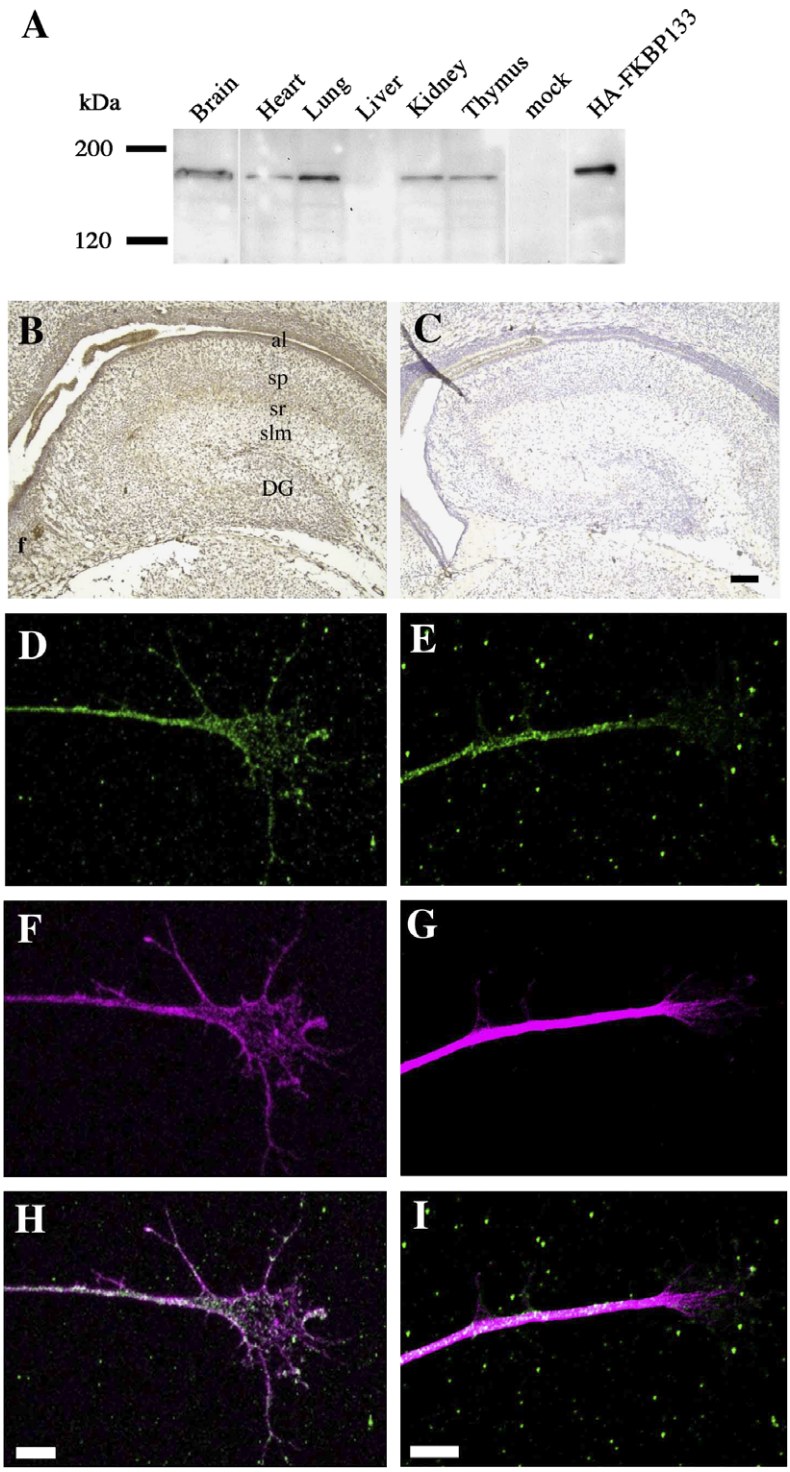

Fig. 3. FKBP133 protein expression and the subcellular localization in the cultured neurons. (A) Immunoblot analysis of FKBP133 in various tissues. Lysates of above indicated organs (6 μg) were examined with anti-FKBP133 antibody. HEK293T cells transfected with HA-FKBP133 or with mock vector were also examined. The immunoreactive signal of 180-kDa was apparent in brain, heart, lung, kidney, and thymus but not in liver. Transiently expressed HA-FKBP133 exhibited same size as the endogenous protein in the tissues. (B,C) Immunohistochemistry of anti-FKBP133. Mouse E18.5 hippocampal region was stained with anti-FKBP133 pAb (B) or with rabbit control IgG (C). Bound antibodies were visualized with DAB (brown). Nuclei were counterstained with hematoxylin (purple). Note that immunoreactive signal is detected in the hippocampal alveus, the stratum radiatum in CA1-3 fields, and fimbria. Scale bar, 100 μm. al, alveus; DG, dentate gyrus; f, fimbria; slm, stratum lacunosum-moleculare; sp, stratum pyramidale; sr, stratum radiatum. (D,F,H) Localization of FKBP133 and F-actin in the neurons. (D) Anti-FKBP133 staining (green); (F) F-actin staining with rhodamine-phalloidin (magenta); (H) merged (D) and (F). FKBP133 (green) was strongly localized in the axonal shaft and moderately distributed in the growth cone. In the growth cone, FKBP133 was partially aligned with F-actin in the peripheral region (white in H). Scale bar, 10 μm. (E,G,I) Distribution of FKBP133 and α-tubulin in the neurons. (E) anti-FKBP133 staining (green); (G) α-tubulin staining (magenta); (I) merged (E) and (G). FKBP133 (green) and α-tubulin (magenta) were strongly stained in the axonal shaft. FKBP133 and α-tubulin were not co-localized in the growth cone. Scale bar, 10 μm.

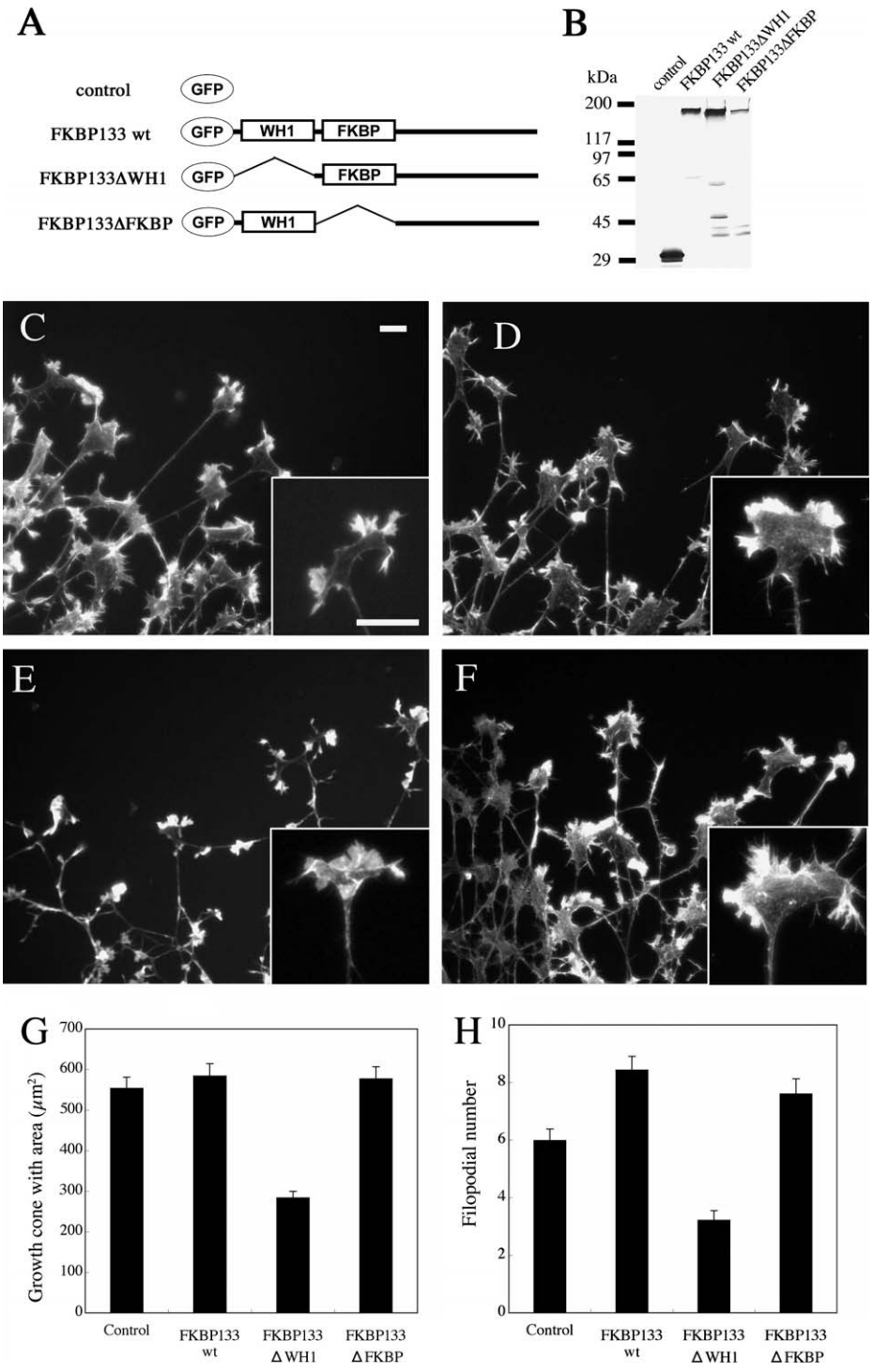

Fig. 4. Overexpression of FKBP133 in DRG neurons alters growth cone morphology. (A) The major features of the FKBP133 derivatives used in this study are illustrated. Note the two major domains of FKBP133: the WH1 domain and FKBP domain. EGFP tag was attached to the N-terminus. Control, FKBP133 wt, FKBP133ΔWH1, and FKBP133ΔFKBP represent EGFP, EGFP-FKBP133, EGFP-FKBP133ΔWH1, and EGFP-FKBP133ΔFKBP, respectively. (B) Immunoblot analysis with anti-EGFP antibody demonstrates the expression of proteins. The apparent molecular sizes of EGFP, EGFP-FKBP133, EGFP-FKBP133ΔWH1, and EGFP-FKBP133ΔFKBP on the immunoblot are 30, 190, 180, and 180, respectively. (C–F) Rhodamine–phalloidin staining of the DRG neurons overexpressing EGFP-FKBP133 protein or EGFP-FKBP133 deletion mutants. (C) EGFP, (D) EGFP-FKBP133, (E) EGFP-FKBP133ΔWH1, and (F) EGFP-FKBP133-ΔFKBP. (Inset) Higher magnification of the growth cones overexpressing each construct. Scale bars, 20 μm. (G) The growth cone size of EGFP-FKBP133ΔWH1 expressing cells was reduced. 60–100 of the growth cones were randomly selected from three independent experiments and the area size was measured with computer aid. The bar graph represents the average ± SEM (μm^2) of the growth cone size. The growth cones expressing ΔWH1 mutant reduced the size approximately half comparing to the growth cones of other preparations. (H) The number of filopodia was increased in the neurons overexpressing EGFP-FKBP133 or EGFP-FKBP133ΔFKBP. The filopodia longer than 3 μm in a growth cone were scored. At least 50 growth cones were analyzed in each preparation. The filopodial number was increased in the cells overexpressing FKBP133 or FKBP133ΔFKBP while the number was decreased in the cells expressing FKBP133ΔWH1. Data are means ± SEM for $n = 3$.

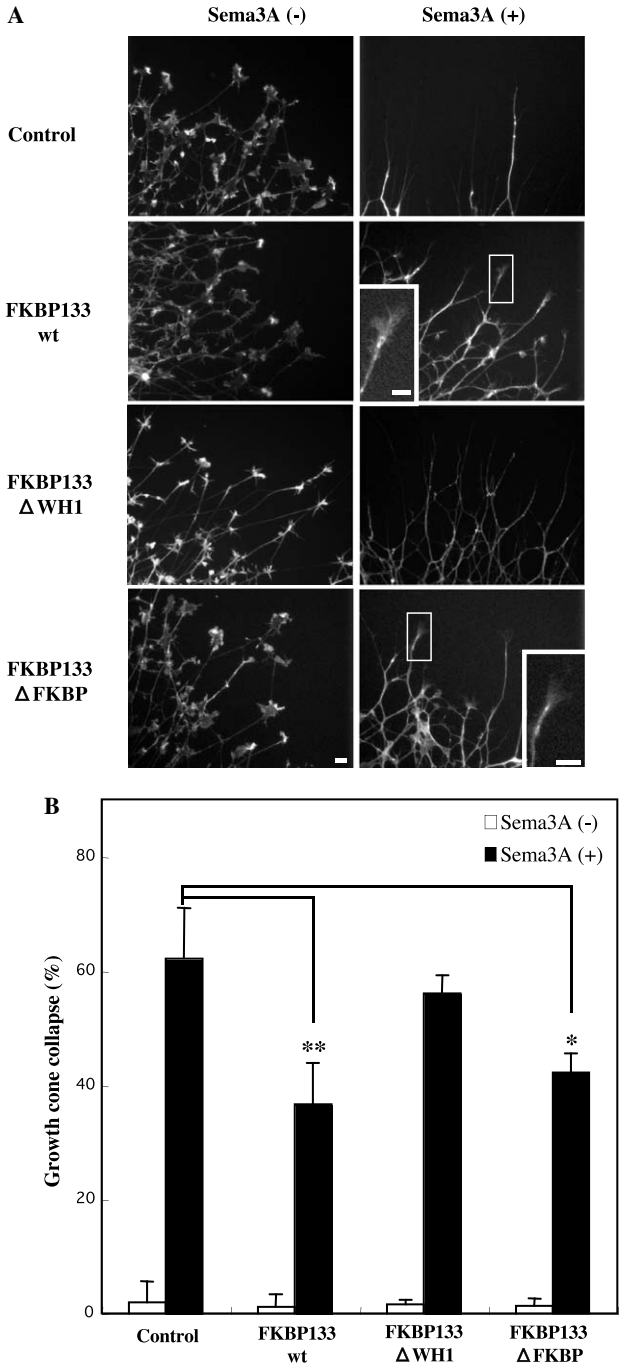

Fig. 5. Overexpression of FKBP133 attenuates Sema3A-induced collapse response. (A) Rhodamine–phalloidin staining of the DRG neurons expressing FKBP133 and the deletion mutants with or without Sema3A stimulation. The expression of recombinant proteins from the virus was confirmed by EGFP fluorescence (not shown). Cultures were incubated with or without Sema3A (1 nM) for 30 min. The cells were fixed and stained with rhodamine–phalloidin. Inset shows higher magnification of the marked growth cones. Note that the growth cones expressing FKBP133 and FKBP133ΔFKBP but not FKBP133ΔWH1 were partially protected from Sema3A-induced collapse response. Scale bars, 10 μm. (B) Sema3A response of the DRG growth cones. Growth cone collapse was determined after infection with the indicated virus preparations and stimulation with 1 nM Sema3A. The white-bar represents the percentage of collapsed growth cones without stimulation and black-bar represents with Sema3A stimulation. Data are means ± SEM for $n = 3$. *$P < 0.01$, **$P < 0.05$ (Student's t-test).

Overexpression of FKBP133 attenuates Sema3A-induced growth cone collapse

Co-localization of FKBP133 and F-actin in the growth cones and the morphological alteration by the overexpression of FKBP133 prompted us to examine the role of FKBP133 in the downstream of axon guidance molecules at the growth cone. It has been known that Sema3A induces growth cone collapse response of DRG neurites through the depolymerization of F-actin [13]. We examined Sema3A-induced collapse response of the DRG neurons overexpressing either EGFP-FKBP133 wild type or mutants. We did not observe significant difference of Sema3A-sensitivity between non-infected DRG neurons and EGFP-expressing neurons, indicating that EGFP overexpression does not affect Sema3A signaling (not shown). In contrast, the expression of EGFP-FKBP133 and EGFP-FKBP133ΔFKBP in the neurons significantly suppressed Sema3A-induced collapse response comparing to the control neurons expressing EGFP (Fig. 5A and B). The overexpression of EGFP-FKBP133ΔWH1 did not alter Sema3A-sensitivity in growth cone collapse response. To eliminate the possibility if the overexpression of FKBP133 attenuates cell surface expression of Sema3A receptor components, we immunostained the neurons with anti-Plexin-A4 mAb prior to fixation and found no significant difference between the cells overexpressing FKBP133 and untreated neurons (not shown).

Discussion

In the present study, we demonstrate predominant expression of FKBP133 in the developing nervous system (Figs. 2 and 3) and subcellular localization of the protein in the axons and in the growth cones (Fig. 3). The signal of *in situ* hybridization at E18.5 is thought to reflect FKBP133 mRNA expression in neuronal cells, because small numbers of glial cells have been generated at the stage. FKBP133 mRNA exhibited limited expression in the neuronal cell bodies of the hippocampus and of the cerebellum at postnatal day 15. These fkbp133 signal-positive neurons are thought to extend the axons and dendrites to form synaptic connection with other neurons and/or target organs. Indeed, the immunohistochemistry demonstrates the axon-rich regions, such as alveus and fimbria, are stained with anti-FKBP133 antibody (Fig. 3B). In the cultured DRG neurons, FKBP133 protein was present in the axons and partially co-localized with F-actin cytoskeleton in the growth cones (Fig. 3). These results lead to the idea that FKBP133 may participate in the process of axon outgrowth, axon guidance, and/or synapse formation.

Our data of the overexpression experiments suggest that FKBP133 participate in the cytoskeletal regulation of the growth cones. The dominant-interfering experiments suggest that the WH1 domain but not the FKBP domain is the indispensable region of FKBP133 for the alteration of growth cone morphology. The WH1 domain of FKBP133

may serve as a molecular conformational switch of FKBP133 and a regulatory region of F-actin cytoskeleton. It has been shown that the WH1 domain and the G-protein binding domain of WASP bind to the VCA (Verpolin homology, Cofilin homology, and Acidic sequence) region of the C-terminus at inactive state [24]. Rac1/Cdc42 binding to the G-protein binding domain unlocks the auto-inhibited structure of WASP. Then, the WH1 domain and the VCA region stimulate Arp2/3 complex to promote the nucleation of new actin filaments [25]. Interestingly, FKBP133 A-isoform possesses a C-terminal acidic amino acid sequence similar to that of WASP. FKBP133 was relatively localized in peripheral region of the growth cones where F-actin is the major cytoskeleton (Fig. 3D and H). Overexpression of EGFP-FKBP133 induced filopodial formation of the growth cones (Fig. 4D) while EGFP-FKBP133ΔWH1 mutant reduced the growth cone size and the numbers of filopodia (Fig. 4E). Furthermore, overexpression of FKBP133 but not of FKBP133ΔWH1 rendered the growth cones less sensitive to Sema3A-induced collapse response (Fig. 5). Taken together, FKBP133 is thought to stabilize F-actin cytoskeleton in the growth cones through the WH1 domain and to antagonize Sema3A-induced F-actin depolymerization. Alternatively, FKBP133 may modulate a downstream molecule of Sema3A-Plexin intracellular signaling. It remains to be clarified whether FKBP133 selectively attenuates Sema3A action or it exerts over broad range of guidance molecules.

Considering the process of axon guidance, the growth cones are continuously exposed to extracellular cues and are navigated to the target organs. If Sema3A is presented as a gradient, the growth cones can steer away from Sema3A. In such process, attenuating mechanisms must be required to restrict Sema3A-Plexin signaling in the limited area of the growth cones. FKBP133 may contribute to the process by stabilizing F-actin cytoskeleton in the growth cones. Other molecules, such as RhoD and CRMP5 (CRAM), have been shown to play similar role [26,27]. The relevance of this assumption will be tested by the turning assay of the growth cones with the combination of FKBP133 mutants and Sema3A gradient. Analyzing neural projection in the *fkbp133−/−* mutant mouse will also provide further insights into FKBP133 function.

The role of the FKBP domain of FKBP133 remains to be clarified. The phenotype of ectopic expression of EGFP-FKBP133ΔFKBP in the neurons was indistinguishable from that of EGFP-FKBP133. We have not been able to detect apparent FK506 binding or PPIase activity of the FKBP domain of FKBP133. The enzymatic inactivity may be explained by poor conservation (5 residues) of the 13 critical residues [28,29] in the domain (Fig. 1D). However, FKBP38 initially reported as an inactive PPIase [8] and FKBP38 conserves only five out of the 13 residues but lacks three catalytic residues corresponding to Phe36, Asp37, and Trp59 of FKBP12. Despite these facts, it has recently shown that calcium–calmodulin complex binds to FKBP38 and activates the PPIase [11]. Therefore, full length FKBP133 may exhibit enzymatic activity aided by other domains or by other binding proteins.

In summary, we characterized a novel member of FKBP family, FKBP133, predominantly expressed in the developing nervous system. The protein was distributed in the axons and partially co-localized with F-actin in the growth cones. Ectopic expression of FKBP133 mutants revealed that FKBP133 modulates the growth cone morphology through the WH1 domain. Furthermore, the growth cones overexpressing FKBP133 is partially resistant to the Sema3A-induced collapse response. Revealing the function of FKBP133 will open a new avenue to manipulate the action of repulsive guidance molecules in the nervous system and to regenerate damaged central nervous system.

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