Midline crossing and Slit responsiveness of commissural axons require USP33

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Supplementary Information
a

siRNA - | siCtl | siUSP3 #1 #2
GFP-USP33 - | + | + + +
Blot:
GFP
β-tubulin
HEK cells

b

siRNA - | siCtl | Blot:
USP33
HA
β-tubulin
Robo1-HA-expressing HEK cells

c

siRNA - | siCtl | siUSP33#2
Blot:
USP33
Robo1
β-tubulin
Mouse dorsal spinal cord neurons

Mouse dorsal spinal cord neurons

d

siRNA - | siCtl | Blot:
USP33
Robo1
β-tubulin

Mouse dorsal spinal cord neurons

e

siRNA - | siCtl | siUSP33 #4
Blot:
USP33
YFP
β-tubulin
Chick spinal cords (in ovo)

f

| siCtl | siUSP33#2 |
|-------|-----------|
| USP33 | DCC       |
| DIC   | USP33     |
| DCC   | DIC       |
**Supplementary Figure 1.** siRNA-mediated knockdown of USP33. (a) Western blot analysis showing that siRNAs targeting USP33 (#1: specific for human USP33; #2: for human and mouse USP33) suppress the expression of GFP-tagged human USP33 in HEK293 cells. (b) The expression of endogenous USP33 and Robo1-HA in siUSP33#1–transfected, Robo1-HA-expressing HEK293 cells. (c) The levels of the endogenous USP33 and Robo1 proteins in dorsal spinal cord neurons isolated from E11.5 mouse embryos following transfection with siUSP33#2. The level of USP33 protein in siCtl or siUSP33–transfected cells was quantified relative to that in untransfected cells (−) and normalized to β-tubulin in three independent experiments. Data are presented as the mean ± SEM. (d) The level of the endogenous USP33 and Robo1 proteins in dorsal spinal cord neurons prepared from E11.5 mouse embryos after siUSP33#3 transfection. The level of USP33 protein was quantified as in panel c. (e) The level of the endogenous USP33 protein in stage 26 chick spinal cords after in ovo electroporation with siUSP33#4 and YFP. The level of USP33 protein in siCtl or siUSP33–electroporated spinal cords was quantified relative to that in unelectroporated spinal cords (−) and normalized to β-tubulin in three independent embryos. Data are presented as the mean ± SEM. (f) Immunostaining with anti-USP33 showing that expression of USP33 protein (green) in DCC-positive (cyan) commissural neurons was suppressed by transfection with siUSP33#2. Maximal projections of Z-stacks together with images of differential interference contrast (DIC) are shown. The efficient introduction of siRNAs into the neurons was visualized by fluorescence of Alexa555–conjugated siRNA (red signals in the insets), which accumulated in the soma. Scale bar, 10 µm.
Supplementary Figure 2. USP33-interacting domains in Robo1. Immunoprecipitation was carried out using lysates of cells co-transfected with HA-tagged Robo1 (full-length or deletion mutants) and GFP-USP33. Lysates of transfected HEK293 cells were immunoprecipitated with anti-HA. The immunoprecipitates and lysates were probed with anti-GFP or anti-HA in Western blotting. FL: full-length Robo1. Residues 1099 to 1657 were deleted in ΔCC23; residues 1455–1657 were deleted in ΔCC3; residues 930–1098 and 1455–1657 were deleted in ΔCC013; residues 930–1098 were deleted in ΔCC01; residues 930–1454 were deleted in ΔCC012. (a) Schematic illustration of full-length Robo1 and its deletion mutants. (b) Deletion of the CC3 motif significantly reduced Robo1–USP33 interaction.
Supplementary Figure 3. Robo1-interacting domains in USP33. HEK293 Cells expressing Robo1-HA together with either full-length USP33 or its deletion mutants were subjected to co-immunoprecipitation using anti-HA antibody followed by Western blotting using anti-GFP or anti-HA. Both the USP and DUSP domains of USP33 contribute to interaction with Robo1. Open and closed arrowheads show signals of immunoglobulin heavy chain and a non-specific band, respectively. FL: full-length USP33; #1 + 2 comprises residues 1 to 696; #2 + 3 comprises residues 151–911; #1 comprises residues 1–158; #2 comprises residues 151–696; #3 comprises residues 676–911. (a) Schematic illustration of full-length USP33 and its deletion mutants. (b) The precipitated proteins and the total cell lysates were analyzed by Western blotting. The total amount of cell lysates used was adjusted to similar expression level among different mutants.
Supplementary Figure 4. Expression of the endogenous USP33 protein (in green) in DCC\(^+\) (red) commissural neurons prepared from E9.5 and E11.5 mouse spinal cords.

Scale bar, 10 \(\mu\)m.
Supplementary Figure 5. USP33-mediated deubiquitination of Robo1. (a)

Ubiquitination of Robo1. HEK293 cells expressing corresponding proteins were incubated in the absence or presence of 5 µM MG132 for 6 h. Immunoprecipitated Robo1-HA was used in Western blotting using corresponding antibodies as indicated. Arrows in a–c mark the positions of ubiquitinated Robo1. (b) Knockdown of USP33 increases Robo1 ubiquitination. Robo1-HA-expressing, siRNA-transfected HEK293 cells
were stimulated with Slit or control for 30 or 60 min. For quantification, see panel d. (c) Overexpression of USP33 reduces Robo1 ubiquitination. Robo1-HA-expressing cells were transfected with GFP or GFP-USP33, and stimulated with Slit for 60 min. For quantification, see panel e. (d) The levels of ubiquitinated Robo1 in HEK293 cells stimulated with Slit or control for 60 min were quantified relative to control-stimulated, siCtl-transfected cells expressing Robo1-HA and normalized to total levels of Robo1-HA in three independent experiments. Data are represented as the mean ± SEM. (e) The levels of ubiquitinated Robo1 in HEK293 cells stimulated with Slit or control for 60 min were quantified relative to control-stimulated, GFP and Robo1-HA-coexpressing cells and normalized to total levels of Robo1-HA in three independent experiments.
Supplementary Figure 6. The specificity of the anti-Robo1 antibody used in this study. (a) Co-immunostaining of parental and Robo1-HA-expressing HEK293 cells with anti-Robo1 (green) and anti-HA (red) antibodies. Scale bar, 10 µm. (b) Western blot analysis showing the endogenous Robo1 and USP33 protein levels in primary dorsal spinal cord neurons from E11.5 mouse embryos after transfection with siCtl, siRobo1#1, or siRobo1#2. (c) Immunostaining of siCtl or siRobo1 (#1 or #2)–transfected E11.5 DCC⁺ (cyan) commissural neurons with the anti-Robo1 antibody (green). Effective transfection of siRNA into the neurons is visualized by fluorescence emitted by Alexa555–conjugated siRNA (red signals in the insets), which accumulates in the soma. Only weak fluorescence signals of Robo1 are detected in siRobo1–transfected neurons. Scale bar, 10 µm.
a

Before stimulation  | Control  | Slit
---|---|---
Robo1 | DCC | Robo1 | DCC | Robo1 | DCC

siCtI

siUSP33

b

Axonal Robo1 (%)

Before stimulation | Control | Slit | Before stimulation | Control | Slit
---|---|---|---|---|---
siCtI | siUSP33

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Supplementary Figure 7. USP33 is required for maintaining the stability of axonal Robo1 after Slit stimulation. (a) Robo1 in the distal axon. Commissural neurons were incubated with anti-Robo1 (green) and stimulated with Slit or control for 10 min before fixation and permeabilization. DCC expression is shown in cyan. The efficient introduction of siRNAs into the neurons was visualized by fluorescence of Alexa555–conjugated siRNA (red in the cell body). Scale bars, 5µm. (b) Quantification of relative Robo1 protein levels in the distal part of axons of commissural neurons. The Robo1 level in the distal-most 30 µm of axons was normalized to that of siCtl-transfected neurons before stimulation. Three independent experiments were performed, with 20 neurons per group analyzed in each experiment. Data are represented as the mean ± SEM. **p < 0.01 by Mann-Whitney test.
**Supplementary Figure 8.** Quantification of axon stalling within the floor plate of later-stage chick embryos (Hamburger-Hamilton stages 27–28). Trajectories of DiI-labeled axons in spinal cords at lower thoracic and lumbosacral levels were examined. ***p < 0.0001 by chi-square test.
SUPPLEMENTARY METHODS

Antibodies, reagents and plasmids

Affinity-purified anti-USP33 antisera were generated by immunizing rabbits with peptides corresponding to residues 896–911 of the type II sequence of human USP33 (Operon, Japan). Immunoblotting confirmed that the antibody reacted with USP33 but not its closely related homolog USP20. A rabbit polyclonal antibody against the extracellular domain of Robo1 was a kind gift from Dr. F. Murakami. Mouse monoclonal antibodies (mAbs), such as anti-FLAG (M2) and anti-β-tubulin (TUB2.1) from Sigma-Aldrich, anti-HA.11 (16B12) and anti-myc (9E10) from Covance, anti-Ub (P4D1) from Santa Cruz Biotechnology, and anti-DCC (AF5) from Calbiochem, and rabbit anti-GFP antibody from Torrey Pines Biolabs were used. Alexa-conjugated secondary antibodies and phalloidin were purchased from Invitrogen. Sema3F protein was obtained from R&D.

Rat Robo1 tagged with an HA epitope at the C terminus (Robo1-HA) was described previously\(^1\). Human type II USP33 tagged with GFP at the N terminus (GFP-USP33) and GFP-USP20 (human) constructs were generated as described\(^2\). Rat Robo1 deletion mutants were made as previously described\(^3\), and USP33 deletion mutants were generated from full-length GFP-USP33. The GFP-USP33\(_{C163A}\) mutant was generated by site-directed mutagenesis (QuikChange II XL, Stratagene).

Cell culture

Primary cultures were harvested from embryonic dorsal spinal cords of staged pregnant CD1 (ICR) mice obtained from Charles River. The day of vaginal plug was dated as E0.5,
and embryos were staged according to Bard et al. (http://genex.hgu.mrc.ac.uk/Databases/Anatomy/MAstaging.shtml). The dorsal half (E9.5) or dorsal third (E11.5) of spinal cords were removed and trypsinized at 37°C (for E9.5, 0.025%, 10 min; for E11.5, 0.25%, 15 min) in the presence of DNase I (Roche Applied Science) in Ca^{2+}/Mg^{2+}-free Hanks’ solution. Dissociated cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin /streptomycin, and 2 mM L-glutamine on coverslips precoated with poly-D-lysine (50 µg/ml, Sigma) and laminin (5 µg/ml, Roche) for 48 h before stimulation (cell density: 5.0 x 10^4 cells/cm^2).

HEK293 cells stably expressing rat Robo1-HA or human Slit2-myc were described previously^1^,^4^. Stimulation of spinal neurons or HEK293 cells with Slit was as described previously^3^,^4^; in the other experiments, Slit-conditioned media were used. Control conditioned media or preparations, which were made from parental HEK293 cells by employing the same procedure as for Slit, were used for the assays in parallel. In all experiments, Slit was added to the medium to give a final working concentration of ~25 pM. We used either calcium phosphate method^1^ or Lipofectamine 2000 (Invitrogen) to transiently transfect HEK293 cells with plasmid DNAs.

**Immunoprecipitation and Western blotting**

Forty-eight h (for plasmids), or 72 h (for siRNAs) after transfection, HEK293 cells were stimulated and then lysed. Immunoprecipitation was performed as described^1^.

For co-immunoprecipitation of endogenous Robo1 and USP33 proteins, lysates prepared from
E16–17 mouse cerebral cortices were incubated with control rabbit IgG or anti-USP33 antibody.

Proteins were resolved on 8.0% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes using a semi-dry blotting apparatus (ATTO, Japan). The blots were blocked with 0.1% Tween20/phosphate-buffered saline (PBST) containing 1% skim milk or 2% ECL Advance blocking agent (GE Healthcare), and reacted with primary antibodies as indicated in the figures for 3 h and then with horseradish peroxidase-linked secondary antibodies (1:5,000 to 1:12,000 with the blocking buffer) for 2 h at room temperature. The signals were visualized using ECL plus or Advance kits, according to the manufacturer’s instructions (GE Healthcare).

**RNAi**

Duplex siRNAs were purchased from Ambion or Dharmacon, they were: control (siCtl: 5’-CGUACGCGGAAUACUUCGATT-3’ and 5’-UCGAAGUAUUCGCCGUCGTTT-3’); si-human USP33 (siUSP33#1: 5’-UCUCGACAGUGCGUAAUAA-3’ and 5’-UAUUAAGCCACUGUCGAAA-3’); si-mouse/human USP33 (siUSP33#2: 5’-GGAUUCAGUUGGUGAAUUC-3’ and 5’-UAUUUCACCAACUGAAUCAA-3’); si-mouse USP33 (siUSP33#3: J-048337-12 from Dharmacon); si-chicken USP33 (siUSP33#4: 5’-UGAUGACCUUGAUAGUGU-3’ and 5’-UUCUAUAUCAAGGUCAUCAA-3’); si-mouse Robo1 (siRobo1#1 and #2: J-046944-10 and -11, respectively, from Dharmacon). Elbashir et al. designed the sequences of the control siRNA, which targets GL2 luciferase.5
To knockdown USP33 in dorsal spinal cord neurons (1.0 x 10^6 cells per reaction) of mouse embryos with RNAi, 40 pmol of Block-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen) was mixed with 90 pmol of non-labeled siCtl or siUSP33, and transfected into dissociated neurons using 2 µl of Lipofectamine RNAiMAX (Invitrogen) at 37°C for 1 h and washed once before plating. The neurons were cultured for 48 h before Slit stimulation, as described above. HEK293 cells were transfected with siRNAs using Lipofectamine 2000.

**Growth cone collapse assay and immunocytochemistry**

Dorsal spinal cord neurons were stimulated with Slit (~25 pM), Sema3F (R&D; 100 µg/ml), or control for 30 min and fixed with 4% paraformaldehyde (PFA)/10% sucrose in phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100 in PBS for 2 min. The use of conditioned media made from Sema3F-expressing HEK293 cells gave equivalent results (the Sema3F construct that was transfected into HEK293 cells was a kind gift from Dr. Y. Zou). The neurons were then stained with anti-DCC, Alexa555 or 647-conjugated secondary antibodies, and phalloidin (to show filamentous actin). Samples were mounted in Permafluor (Beckman Coulter; Thermo Fisher Scientific). The growth cone morphology was examined in randomly selected DCC^+ commissural neurons. Growth cones were defined by the presence of lamellipodia and/or filopodia. In each experimental group, at least three independent experiments were performed (35 or more neurons per group were scored in each experiment). For the growth cone collapse assay combined with RNAi, the growth cone morphology of DCC^+ commissural neurons exhibiting red fluorescence of Alexa555–conjugated siRNA in the cell body, which
revealed efficient transfection, was examined. Z-stacks and time-lapse recordings were acquired under a BX61i microscope (Olympus) equipped with a CoolSNAP HQ CCD camera (Roper Scientific) and controlled by MetaMorph software (version 6.2r5; Molecular Devices). Z-stack images were obtained with a PlanApo 60 X/NA1.40 oil objective (Olympus) and further processed with Leica Deblur deconvolution software. All of fluorescent images shown in this study represent maximum projections of the deconvoluted Z-stacks. MetaMorph and Prism 4.0 (GraphPad) softwares were used for quantitative and statistical analyses.

Because our cultures contained heterogeneous populations of neurons, it was important to identify commissural neurons. In this study, we mainly used DCC, an established marker for commissural neurons\textsuperscript{6,7}. DCC distributed almost uniformly within expressing neurons, allowing us to definitely commissural axons along their entire length. Although TAG-1 is also a marker of commissural neurons\textsuperscript{8}, we found that, in dissociated neurons from E11.5 embryos, TAG-1 expression was markedly reduced and restricted to the cell body.

**Live-cell antibody-feeding assay**

Surface Robo1 was labeled with an anti-Robo1 antibody (2 µg/ml; a kind gift from Dr. F. Murakami) in fresh culture media supplemented with 20 mM HEPES (Invitrogen) for 30 min at room temperature. After extensive washing with fresh media, the neurons were stimulated with Slit or control for 10 min, and fixed with PFA/sucrose. Samples were then permeabilized with 0.2% Triton X-100 in PBS for 2 min and immunostained with anti-DCC and Alexa-conjugated secondary antibodies.
**In ovo RNAi and axon labeling**

Chick embryos were staged according to Hamburger and Hamilton\(^9\). Neural tubes at stages 12–15 (E2) were injected with a solution containing duplex siRNA (4 µg/µl siUSP33#4 or the control siRNA#1 [siCtl] from Ambion), plasmid encoding *Venus YFP*\(^{10}\) (under the control of the CMV promoter; 0.4 µg/µl) and 0.05% Fast Green. siUSP33#4 is specific against chick *USP33*. Immediately thereafter, embryos were electroporated *in ovo* with four 50 msec pulses of 25 V, 100 msec apart using CUY21SC (Nepa Gene). For the rescue experiment, a plasmid encoding human *USP33* (wild type or C163A mutant) that was resistant to siUSP33#4 was co-electroporated with siRNA and the *YFP* plasmid. Embryos were sacrificed at stages 26–28 (E5–6), spinal cords were then dissected out and fixed in an open-book configuration with 4% PFA in PBS at 4°C overnight as described\(^11\).

For fluorescent dye tracing of commissural axons, small crystals of DiI (Invitrogen) were implanted with tungsten needles into the dorsal region of the spinal cord, as described previously\(^12\). DiI labeling was performed after inspecting *YFP/siRNA*-transfected areas with a binocular epifluorescence microscope (FLIII, Leica). Prior to mounting and observation, dyes were given two days at room temperature to diffuse along the entire length of axons. Z-stack images were obtained and further processed with the deconvolution software. Trajectories of DiI-labeled axons in spinal cords at lower thoracic and lumbosacral levels were examined. Prism 4.0 software was used for quantitative and statistical analyses.
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