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Midline crossing and Slit responsiveness of commissural axons require USP33

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Abstract

Commissural axons cross the ventral midline of the neural tube in a Slit-dependent manner. The underlying molecular mechanisms remain to be elucidated. Here we report that the deubiquitinating enzyme USP33 interacts with the Robo1 receptor. USP33 is essential for midline crossing by commissural axons and for their response to Slit. Our results reveal a previously unknown role of USP33 in vertebrate commissural axon guidance and in Slit signaling.

Developing axons are guided by multiple molecular cues to navigate toward their targets. Slit proteins are a family of guidance cues critical for axon pathfinding, especially, at the ventral midline of the central nervous system[1–3]. To identify regulatory components in the Slit-Roundabout (Robo) pathway, we searched for proteins interacting with the intracellular domain of Robo1[4]. By screening a mouse brain cDNA library using the yeast two-hybrid strategy, we isolated multiple clones encoding ubiquitin-specific protease 33 (USP33) [also known as von Hippel-Lindau protein-interacting deubiquitinating enzyme 1 (VDU1)][5]. USP33 is widely expressed in the brain and other tissues[5]. Our Western blotting and immunostaining experiments show that USP33 is expressed in commissural neurons of the neural tube (Supplementary Fig. 1).

Robo1-USP33 interaction was confirmed in transfected cells and in embryonic neurons. Robo1 and USP33 were co-immunoprecipitated in lysates of HEK293 cells expressing hemagglutinin (HA)-tagged Robo1 (Robo1-HA) and green fluorescent protein (GFP)-tagged USP33 (GFP-USP33) (Fig. 1a). Slit treatment did not affect Robo1-USP33 interaction. To test the specificity of Robo1-USP33 interaction, we examined whether Robo1 was co-

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AUTHOR CONTRIBUTIONS

J.Y.-K. and M. K.-K. carried out the experiments. J. Y.-K., M. K.-K., J.Y.W. and Y.R. designed the experiments, analyzed the data and wrote the manuscript. G.W. provided the GFP-USP33 construct.

immunoprecipitated with USP20, a homolog sharing similar domain organization and 59% amino acid identity with USP33[6]. A stronger signal was detected in co-immunoprecipitation using cell lysates containing USP33 than those expressing USP20, (Fig. 1b). We confirmed that the endogenously expressed Robo1 and USP33 interacted with each other in the mouse brain (Fig. 1c). Co-immunoprecipitation using deletion mutants of Robo1 and USP33 showed that the Robo1 CC3 motif interacted with USP33 in the region containing the catalytic USP and substrate-recognizing DUSP domains[7] (Supplementary Figs. 2 and 3).

To examine the potential role of USP33 in Slit-Robo function, we used commissural neurons dissociated from developing dorsal spinal cords. We first examined growth cone behavior of these primary neurons using time-lapse microscopy. The majority of growth cones of commissural neurons prepared from embryonic day 11.5 (E11.5) mice showed collapse response to Slit treatment (Fig. 2a). We further characterized Slit responsiveness of commissural neurons isolated from E9.5 and E11.5 embryos. Deleted in Colorectal Cancer (DCC) was used as a commissural neuron marker[8,9]. A significant fraction of E11.5 commissural growth cones collapsed in response to Slit treatment (30 min stimulation; control: 17.6 ± 1.3%, Slit: 60.0 ± 0.7%; 4 independent experiments), whereas E9.5 neurons showed no obvious response (control: 14.2 ± 1.3%, Slit: 12.9 ± 2.5%; 3 independent experiments; Fig. 2b,d). In E11.5 neurons, the growth cone morphology recovered to the normal appearance approximately 30 min after Slit was washed out (control: 17.6%, Slit: 71.5%, Slit & wash-out: 20.0%; 2 independent experiments). Thus, using dissociated commissural neurons of different embryonic stages, in the absence of floor-plate cells, we have established an *in vitro* assay that recapitulates the *in vivo* change in Slit responsiveness of commissural axons.

Immunocytochemistry showed that USP33 protein was expressed in E9.5 and E11.5 commissural neurons (Supplementary Fig. 4). To test the role of USP33 in mediating Slit-induced growth cone collapse, we examined Slit responsiveness of E11.5 commissural neurons when USP33 expression was down-regulated by specific small interfering RNAs (siRNAs). Transfection of dorsal spinal cord neurons with two different siRNAs against the mouse *USP33* (siUSP33#2, 3) reduced the level of endogenous USP33 protein (Supplementary Fig. 1). The growth cone morphology was examined in DCC^+ commissural neurons with fluorescence of transfected Alexa555–conjugated siRNA (see Supplementary Fig. 7), revealing that RNAi-mediated downregulation of USP33 expression was specific and efficient (Supplementary Fig. 1). Similar to untransfected neurons, control siRNA (siCtl)–transfected neurons showed growth cone collapse 30 min after Slit stimulation (control: 19.6 ± 2.2%, Slit: 60.1 ± 2.8%; 7 independent experiments; Fig. 2c). USP33 knockdown abolished growth cone collapse in response to Slit (siUSP33#2, control: 21.0 ± 4.2%, Slit: 17.3 ± 2.4%; 4 independent experiments; siUSP33#3, control: 17.8 ± 2.2%, Slit: 25.5 ± 4.5%; 3 independent experiments) but not to Sema3F, another guidance cue for commissural axons[10] (untransfected neurons, control: 12.2 ± 4.0%, Sema3F: 54.4 ± 2.2%; siCtl-received neurons, control: 15.5 ± 1.1%, Sema3F: 62.2 ± 2.9%; siUSP33#2-received neurons, control: 21.1 ± 2.9%, Sema3F: 60.0 ± 5.1%; 3 independent experiments; Fig. 2c,e). These results indicate that USP33 is required for Slit-induced growth cone collapse in commissural neurons.

We next examined whether Robo1 protein could be ubiquitinated. FLAG-tagged ubiquitin (FLAG-Ub) was transfected into HEK293 cells expressing Robo1-HA[11]. Immunoprecipitation was performed using anti-HA, and Robo1 was detectable by Western blotting using anti-FLAG or anti-ubiquitin, suggesting that Robo1 could be ubiquitinated (Supplementary Fig. 5a). Treatment of the transfected cells with MG132, a proteasome inhibitor, increased the ubiquitinated Robo1 level. To test whether USP33 was involved in Robo1 deubiquitination, we knocked down USP33 expression using siUSP33#1. USP33 knockdown increased the ubiquitinated Robo1 level, whereas overexpression of GFP-USP33 reduced the ubiquitinated Robo1 level (Supplementary Fig. 5b–e), suggesting that USP33 play a role in Robo1 deubiquitination.

We investigated whether USP33 influenced the subcellular distribution of Robo1 during Slit signaling in commissural neurons. To monitor the pool of Robo1 proteins initially located on the plasma membrane at the time of Slit treatment, we performed a fluorescence-based live cell antibody-feeding assay. We first labeled cell-surface Robo1 in cultured commissural neurons using an antibody (Ab) against the Robo1 extracellular domain (for the specificity of the antibody, see Supplementary Fig. 6 and previous studies[12,13]). The initial level of Ab-labeled axonal Robo1 (before stimulation) was indistinguishable between siCtl- and siUSP33- treated neurons (siCtl: 100.0 ± 14.7%; siUSP33: 108.4 ± 16.8%, n = 60; Supplementary Fig. 7a,b). In siCtl-transfected neurons, the level of Ab-labeled axonal Robo1 was drastically reduced during stimulation by the control preparation, suggesting a rapid turnover rate of axonal Robo1 (39.6 ± 5.6%, n = 60). However, Slit stimulation blocked the loss of Ab-labeled axonal Robo1 (75.3 ± 15.1%, n = 60; Supplementary Fig. 7). In siUSP33–treated neurons, the Ab-labeled axonal Robo1 level did not change in the absence of Slit stimulation (93.2 ± 17.6%, n = 60). Slit stimulation induced a significant loss of Ab-labeled axonal Robo1, as compared with siCtl-transfected, Slit-stimulated neurons (30.0 ± 5.8%, n = 60; Supplementary Fig. 7). Therefore, USP33 is required for maintaining the stability of axonal Robo1 after Slit stimulation.

To test the role of USP33 in commissural axon midline crossing, we used *in ovo* RNAi electroporation in chick embryos. siCtl or siUSP33#4 were co-electroporated together with a *YFP* plasmid unilaterally into neural tubes at Hamburger-Hamilton stages 12–15. siUSP33#4, but not siCtl, suppressed USP33 expression in the spinal cord (Supplementary Fig. 1e). Using anterograde DiI labeling, we examined commissural axon trajectories in YFP/siRNA-expressing neurons in the dorsal regions of open-book stage 26 spinal cord preparations (Fig. 3a). Under normal conditions, almost all of dorsal commissural axons at the lumbosacral level crossed the midline and turn longitudinally by stage 26[14]. Similarly, almost all DiI-labeled axons from YFP-expressing regions extended ventrally and reached the midline in the siCtl–electroporated embryos, indicating that siCtl and electroporation per se did not affect midline-crossing behavior of commissural neurons (Fig. 3b). In siCtl-electroporated stage 26 spinal cords, DiI-labeled axons crossed the midline in a well-organized fashion, with only 1.4% of DiI-injection sites showing ε 10 axons stalled within the midline and 77.0% of DiI-injection sites showing 0–4 stalled axons (Fig. 3b,c). In siUSP33–electroporated stage 26 spinal cords, trajectories of pre-crossing axon segments were normal. However, a significant number of DiI-labeled axons were stalled within the

midline; 52.3% of DiI-injection sites had ε 10 axons stalled, and only 14.5% had 0–4 stalled axons (Fig. 3b,c). Similar axon stalling phenotype was observed in stages 27–28 embryos (Supplementary Fig. 8). The phenotype of axon stalling within the midline caused by USP33 knockdown was similar to those observed in *Slit1;Slit2;Slit3* triple knockout mice and in *Robo1* mutants[12].

The midline-crossing defect caused by siUSP33 was rescued by co-electroporation of a plasmid expressing human *USP33* that was resistant to siUSP33#4. Only 7.5% of DiI-injection sites showed ε 10 axons stalled within the midline, with 51.4% showing 0–4 stalled axons (Fig. 3c). In contrast, a USP33 mutant in which a highly conserved cysteine residue in the USP domain was replaced by alanine[7], failed to rescue the midline crossing phenotype caused by siUSP33, with 54.0% of DiI-injection sites showing ε 10 stalled axons within the midline, and only 6.6% had 0–4 stalled axons (siUSP33 + $USP33_{C163A}$).

In summary, our experiments demonstrate that USP33 interacts with the intracellular domain of Robo1 and that USP33 is required for Slit responsiveness of commissural neurons. The live-cell antibody-feeding experiment suggests that USP33 may protect signal-competent Robo1 receptor complex from degradation, and/or facilitate its recycling by deubiquitinating Robo1 protein after Slit stimulation. Interestingly, a recent study suggests that proteasomal degradation of ubiquitinated proteins is not essential for Slit signaling[15]. Further study will be required to elucidate roles of ubiquitination/deubiquitination in regulating axonal sensitivity to guidance cues. Our work has provided insights into the involvement of USP33 in vertebrate axon guidance and revealed a new component in Slit signaling pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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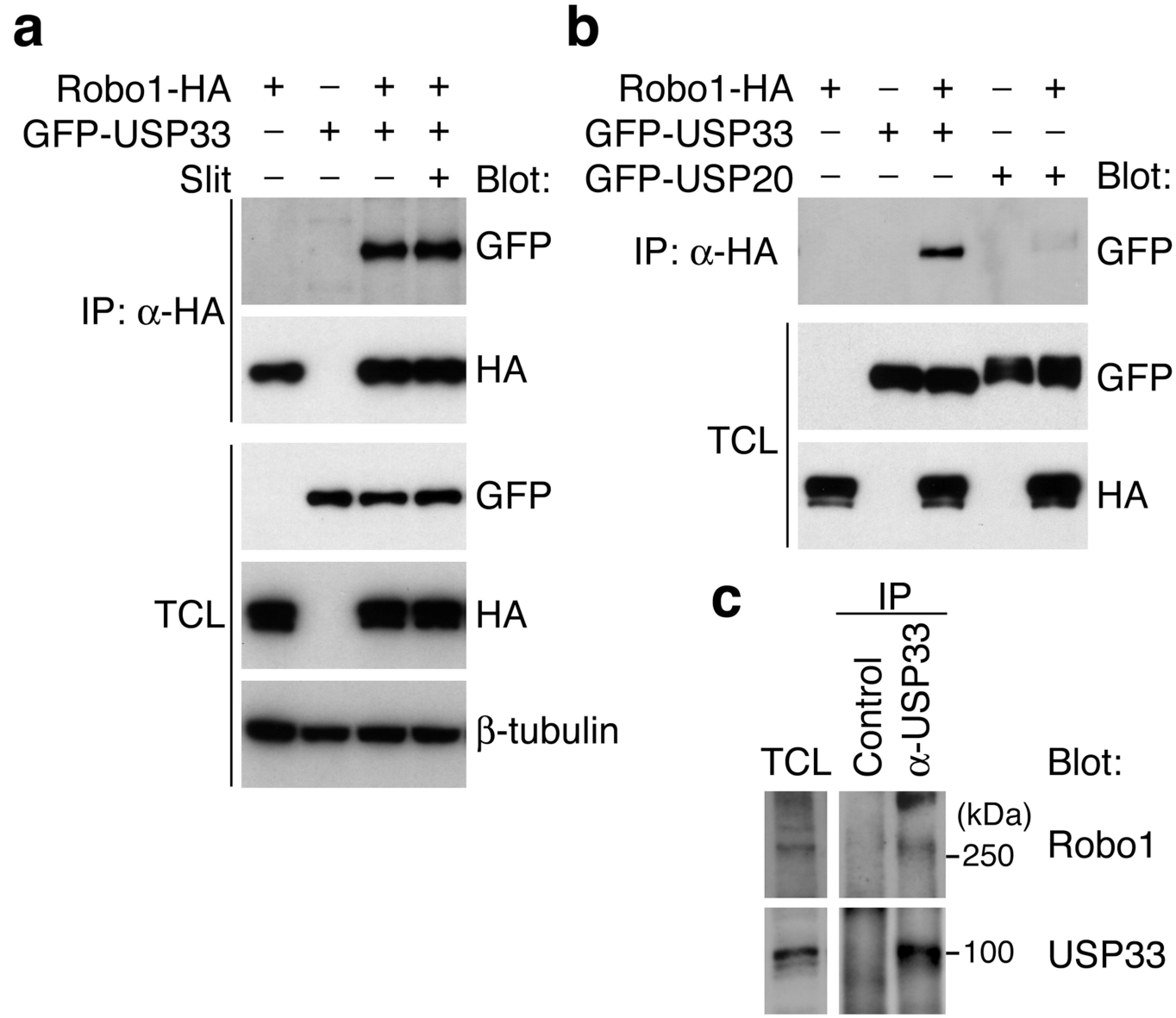

Figure 1.

USP33 interacts with Robo1. (**a** and **b**) Co-immunoprecipitation of Robo1-HA and GFP-USP33 (**a** and **b**) or GFP-USP20 (**b**) in HEK293 cells. In **a**, cells were stimulated with control (−) or Slit (+) preparations for 10 min. Immunoprecipitates and total cell lysates (TCL) were immunoblotted with antibodies as indicated. (**c**) Interaction of the endogenous Robo1 and USP33 proteins in the cell extract prepared from E17 mouse cerebral cortex.

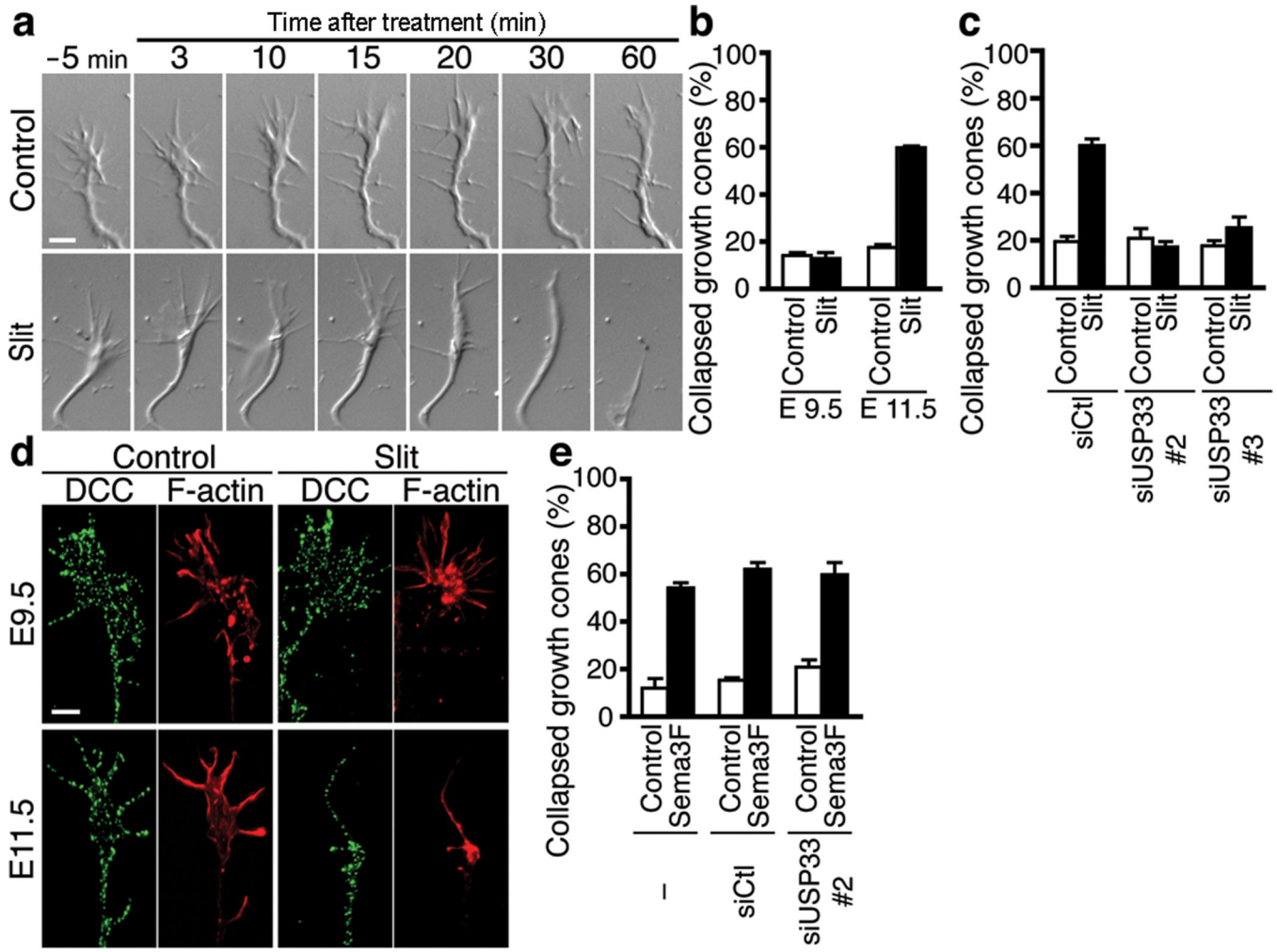

Figure 2.

USP33 is required for Slit-induced growth cone collapse in commissural neurons. (**a**) Time-lapse images of growth cone behavior of E11.5 dorsal spinal cord neurons in response to Slit or control preparations. Scale bar, 5 μm. (**b**) Quantification of growth cone collapse in response to control or Slit treatment in E9.5 and E11.5 DCC^{+} commissural neurons (see also panel **d**; 30 min treatment). The percentage of collapsed growth cones with the mean ± SEM is shown. (**c**) Slit-induced growth cone collapse in E11.5 siRNA-transfected neurons. (**d**) Images of Slit-induced growth cone collapse. Growth cones were visualized by staining with anti-DCC (green) and Alexa555–conjugated phalloidin (red). Scale bar, 5 μm. (**e**) USP33 is not required for response to Sema3F in E11.5 neurons.

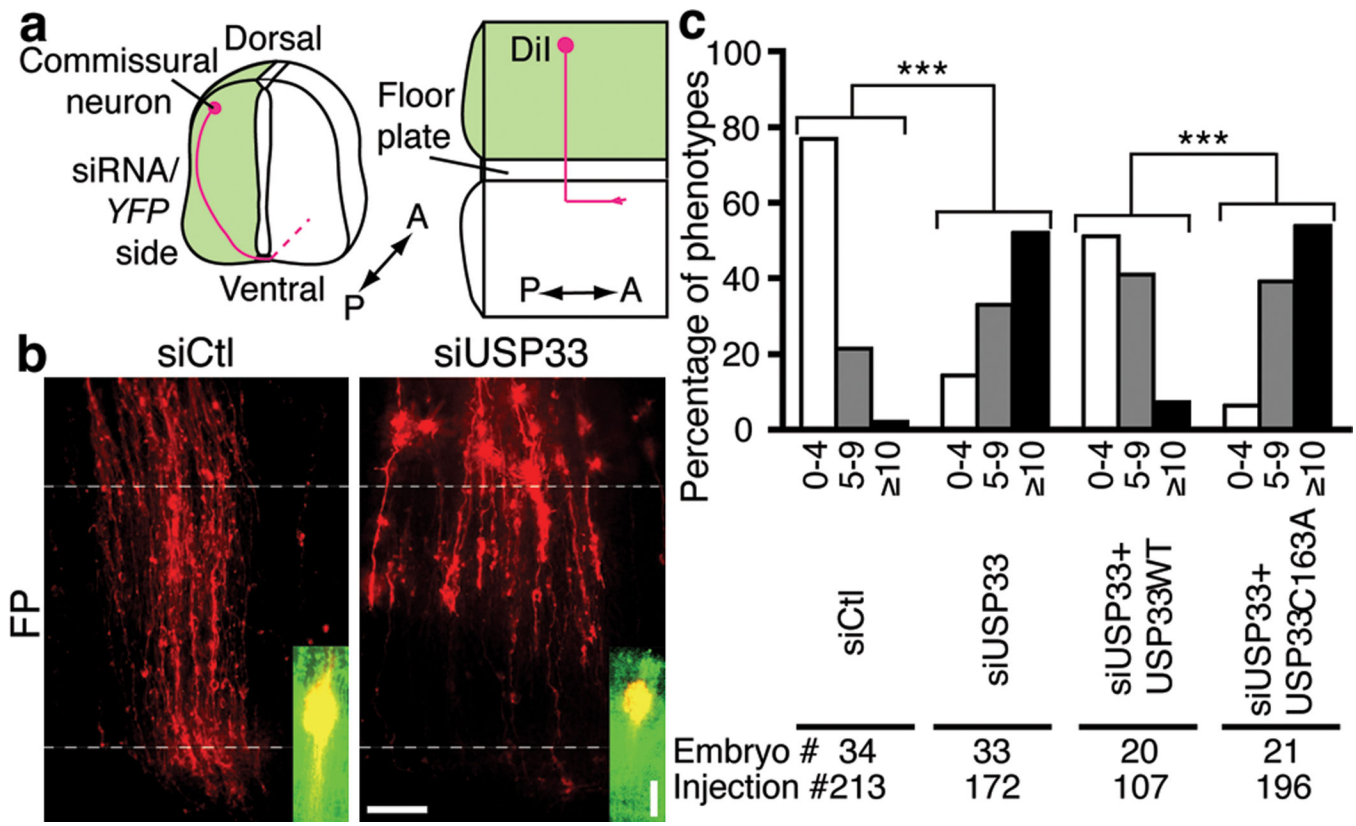

Figure 3.

USP33 is required for commissural axons to cross the midline. (**a**) Schematics of axon trajectories (red) of commissural neurons in stage 26 chick spinal cords. Left: a transverse section showing axon midline crossing and anterior turning. Right: anterograde DiI-labeling of the axons in the open-book spinal-cord preparation electroporated with siRNA/*YFP* (green). A: anterior; P: posterior. (**b**) Trajectories of DiI-labeled commissural axons in siCtl or siUSP33/*YFP*-electroporated spinal cords at the lumbosacral level. The longitudinal tract is out of focus from the image planes shown. Insets show the overlay of DiI (injection site) and YFP signals. FP: floor plate. Scale bar, 20 μm (200 μm for the insets). (**c**) Quantification of axon stalling within the midline. ***$p < 0.0001$ by chi-square test.