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Midline crossing and Slit responsiveness of commissural axons require USP33

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Commissural axons cross the ventral midline of the neural tube in a Slit-dependent manner. The underlying molecular mechanisms remain unclear. We found that the deubiquitinating enzyme USP33 interacts with the Robo1 receptor. USP33 was essential for midline crossing by commissural axons and for their response to Slit. Our results reveal a previously unknown role for 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 that are critical for axon pathfinding, especially at the ventral midline of the CNS[1–3]. To identify regulatory components in the Slit-Roundabout (Robo) pathway, we searched for proteins interacting with the intracellular domain of Robo1 (ref. 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]. We found via western blotting and immunostaining experiments that USP33 was expressed in commissural neurons of the neural tube (**Supplementary Fig. 1**).

We confirmed the Robo1-USP33 interaction in transfected cells and in embryonic neurons. Robo1 and USP33 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 the Robo1-USP33 interaction. To test the specificity of Robo1-USP33 interaction, we examined whether Robo1 was co-immunoprecipitated with USP20, a homolog sharing similar domain organization and 59% amino acid identity with (ref. 6). We detected more USP33 than USP20 coimmunoprecipitating with Robo1 (**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 ('domain present in ubiquitin-specific proteases') 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 (**Fig. 2**). We first examined the 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 collapsed in response to Slit treatment (**Fig. 2a**). We further characterized the Slit responsiveness of commissural neurons isolated from E9.5 and E11.5 embryos. DCC (deleted in colorectal cancer) was used as a commissural neuron marker[8,9]. A substantial 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%; four independent experiments), whereas E9.5 neurons showed no obvious response (control, 14.2 ± 1.3%; Slit, 12.9 ± 2.5%; three independent experiments; **Fig. 2b**,**d**). In E11.5 neurons, growth cone morphology recovered approximately 30 min after Slit was washed out (control, 17.6%; Slit, 71.5%; Slit and wash-out, 20.0%; two independent experiments). Thus, using dissociated commissural neurons of different embryonic stages, in the absence of floor plate cells, we created 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 determine the role of USP33 in mediating Slit-induced growth cone collapse, we examined the Slit responsiveness of E11.5 commissural neurons when USP33 expression was downregulated by specific small

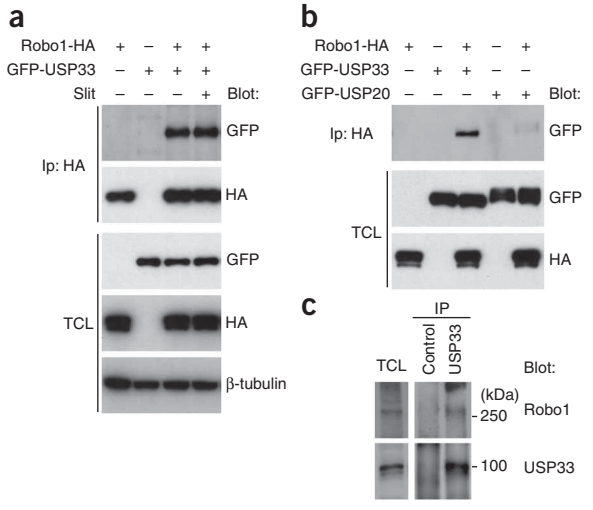

Figure 1 USP33 interacts with Robo1. (**a**,**b**) Co-immunoprecipitation of Robo1-HA and GFP-USP33 (**a**,**b**) or GFP-USP20 (**b**) in HEK293 cells. Cells were stimulated with control (−) or Slit (+) preparations for 10 min (**a**). Immunoprecipitates and total cell lysates (TCL) were immunoblotted with antibodies as indicated. (**c**) Interaction of the endogenous Robo1 and USP33 proteins in the cell extract prepared from E17 mouse cerebral cortex. IP, immunoprecipitation.

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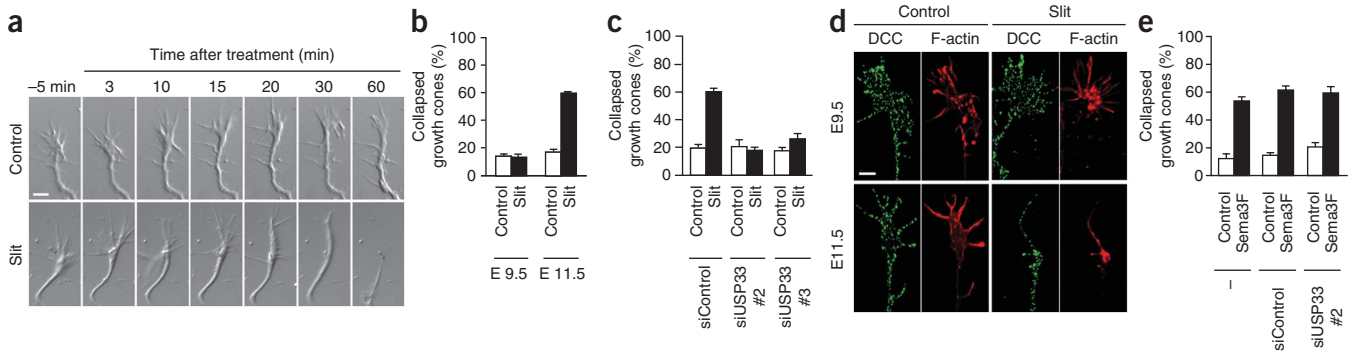

Figure 2 USP33 is required for Slit-induced growth cone collapse in commissural neurons. (**a**) Time-lapse images of growth cone behavior of E11.5 dorsal spinal cord neurons in response to Slit or control preparations. Scale bar represents 5 μm. (**b**) Quantification of growth cone collapse in response to control or Slit treatment in E9.5 and E11.5 DCC-positive commissural neurons (see also **d**; 30-min treatment). The percentage of collapsed growth cones is shown (mean ± s.e.m.). (**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 antibody to DCC (green) and Alexa 555–conjugated phalloidin (red). Scale bar represents 5 μm. (**e**) USP33 is not required for response to Sema3F in E11.5 neurons.

interfering RNAs (siRNAs). Transfection of dorsal spinal cord neurons with two different siRNAs to mouse *Usp33* (siUSP33 # 2 and 3) reduced the level of endogenous USP33 protein (**Supplementary Fig. 1**). We examined the growth cone morphology of DCC-positive commissural neurons using the fluorescence of transfected Alexa 555–conjugated siRNA; RNAi-mediated downregulation of USP33 expression was specific and efficient (**Supplementary Fig. 1**). Similar to non-transfected neurons, control siRNA (siControl)-transfected neurons showed growth cone collapse 30 min after Slit stimulation (control, 19.6 ± 2.2%; Slit, 60.1 ± 2.8%; seven 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%; four independent experiments; siUSP33 # 3: control, 17.8 ± 2.2%; Slit, 25.5 ± 4.5%; three independent experiments), but not in response to Sema3F, another guidance cue for commissural axons[10] (non-transfected neurons: control, 12.2 ± 4.0%; Sema3F, 54.4 ± 2.2%; siControl-transfected neurons: control, 15.5 ± 1.1%; Sema3F, 62.2 ± 2.9%; siUSP33 # 2-transfected neurons: control, 21.1 ± 2.9%; Sema3F, 60.0 ± 5.1%; three 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 antibody to HA and Robo1 was detectable by western blotting using antibodies to FLAG or ubiquitin, suggesting that Robo1 could be ubiquitinated (**Supplementary Fig. 5**). 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 amount of ubiquitinated Robo1, whereas overexpression of GFP-USP33 reduced the amount of ubiquitinated Robo1 (**Supplementary Fig. 5**), suggesting that USP33 is involved 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 to the Robo1 extracellular domain (for the specificity of the antibody, see **Supplementary Fig. 6** and refs. 12,13). The initial level of antibody-labeled axonal Robo1 (before stimulation) was indistinguishable between siControl- and siUSP33-transfected neurons (siControl,

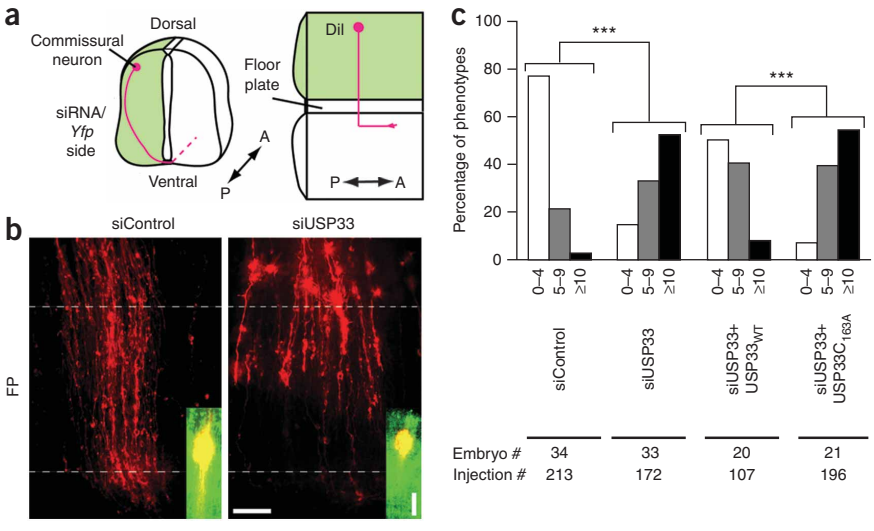

Figure 3 USP33 is required for commissural axons to cross the midline. (**a**) Schematics of axon trajectories (red) of commissural neurons in stage 26 chick spinal cords. Left, a transverse section showing axon midline crossing and anterior turning. Right, anterograde DiI-labeling of the axons in the open-book spinal cord preparation electroporated with siRNA and *yfp* (green). A, anterior; P, posterior. (**b**) Trajectories of DiI-labeled commissural axons in spinal cords electroporated with *yfp* and siControl or siUSP33 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 represents 20 μm (200 μm for the insets). (**c**) Quantification of axon stalling in the midline. *** $P < 0.0001$ by χ^2 test.

100.0 ± 14.7%; siUSP33, 108.4 ± 16.8%; $n = 60$; **Supplementary Fig. 7**). In siControl-transfected neurons, the level of antibody-labeled axonal Robo1 was markedly reduced during stimulation by the control preparation, suggesting that there was a rapid turnover rate of axonal Robo1 (39.6 ± 5.6%, $n = 60$). However, Slit stimulation blocked the loss of antibody-labeled axonal Robo1 (75.3 ± 15.1%, $n = 60$; **Supplementary Fig. 7**). In siUSP33-transfected neurons, the amount of antibody-labeled axonal Robo1 did not change in the absence of Slit stimulation (93.2 ± 17.6%, $n = 60$). Slit stimulation induced a significant loss of antibody-labeled axonal Robo1, as compared with siControl-transfected, Slit-stimulated neurons (30.0 ± 5.8%, $n = 60$, $P < 0.01$; **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. siControl or siUSP33 # 4 were co-electroporated together with a *yfp* (yellow fluorescent protein) plasmid unilaterally into neural tubes at Hamburger-Hamilton stages 12–15. siUSP33 # 4, but not siControl, suppressed USP33 expression in the spinal cord (**Supplementary Fig. 1**). Using anterograde labeling with the lipophilic fluorescent carbocyanine dye DiI, we examined commissural axon trajectories in neurons expressing both YFP and siRNA in the dorsal regions of open-book stage 26 spinal cord preparations (**Fig. 3a**). Under normal conditions, almost all of the dorsal commissural axons at the lumbosacral level crossed the midline and turn longitudinally by stage 26 (ref. 14). Similarly, almost all of the DiI-labeled axons from YFP-expressing regions extended ventrally and reached the midline in the siControl-electroporated embryos, indicating that siControl and electroporation *per se* did not affect the midline-crossing behavior of commissural neurons (**Fig. 3b**). In siControl-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 more than ten axons stalled in the midline and 77.0% of DiI-injection sites showing 0–4 stalled axons (**Fig. 3b,c**). In siUSP33-electroporated stage 26 spinal cords, the trajectories of precrossing axon segments were normal. However, a significant number of DiI-labeled axons were stalled in the midline; 52.3% of DiI-injection sites had more than ten axons stalled and only 14.5% had 0–4 stalled axons ($P < 0.0001$ versus siControl; **Fig. 3b,c**). We observed a similar axon stalling phenotype in stages 27–28 embryos (**Supplementary Fig. 8**). The phenotype of axon stalling in 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 more than ten axons stalled in 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 more than ten stalled axons in the midline and only 6.6% had 0–4 stalled axons (siUSP33 + USP33$_{C163A}$).

In summary, our experiments indicate 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. Notably, a recent study suggests that proteasomal degradation of ubiquitinated proteins is not essential for Slit signaling[15]. Further study will be required to elucidate the roles of ubiquitination/deubiquitination in regulating axonal sensitivity to guidance cues. Our work provides insights into the involvement of USP33 in vertebrate axon guidance and reveals a new component in the Slit signaling pathway.

Note: Supplementary information is available on the Nature Neuroscience website.

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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.

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