Conditional inactivation of Celsr3.

(a–c) Sections of lumbar spinal cord of Rosa26–Tomato;Isl1::Cre embryos at E12.5. Isl1::Cre (red) is expressed in all spinal motor neurons and DRG neurons. Foxp1 (green) and Isl1 (blue) antibodies were used to highlight LMC_M and LMC_L (the staining was performed 3 times).

(d–g) Cross sections of lumbar spinal cord of wild type (d), Celsr3f/–;Wnt1::Cre (e), Celsr3f/–;Isl1::Cre (f), and Celsr3f/–;Olig2::Cre (g) embryos at E12.5, hybridized with Celsr3 digoxigenin–labeled probe. Celsr3 expression is down-regulated in DRG and dorsal interneurons in Celsr3f/–;Wnt1::Cre (e); in motor columns and most DRG neurons in Celsr3f/–;Isl1::Cre (f) and in motor columns in Celsr3f/–;Olig2::Cre (g). The experiments were performed twice.

(h–k) Whole-mount neurofilament staining of hindlimbs from embryos at E12.5. Compared to wild type (h) and Celsr3f/–;Wnt1::Cre (i), the peroneal nerve (arrow) is reduced in Celsr3f/–;Isl1::Cre (j) and Celsr3f/–;Olig2::Cre (k) (mean thickness of the dPN = 41.4 µm in the wild type (n = 8); 40.9 µm in Celsr3f/–;Wnt1::Cre (n = 10); 27.4 µm in Celsr3f/–;Isl1::Cre (n = 12); and 20.7 µm in Celsr3f/–;Olig2::Cre (n = 12). p=0.8843 for Celsr3f/–;Wnt1::Cre vs wild type; p=0.0021 for Celsr3f/–;Isl1::Cre vs wild type; p<0.001 for Celsr3f/–;Olig2::Cre vs wild type; Mann–Whitney test)

DRG: dorsal root ganglia, LMC: lateral motor column, MMC: medial motor column, MC: Motor columns, PN: peroneal nerve (arrows), TN: tibial nerve (arrowhead), D and V: dorsal and ventral horns of the spinal cord.

Scale bar: 10 µm (a–c); 100 µm (d–f); 200 µm (g–j).
Supplementary Figure 2

Motor neurons are normally specified in Celsr3 mutant mice.

(a,b) Transverse section of the lumbar spinal cord at E11.5 from wild type (a) and Celsr3–/– (b) stained with Isl1 (green), Foxp1 (red) and cleaved caspase–3 (white) antibodies (the staining was performed at least 4 times).

(c,d) Quantification of LMC₅ and LMC₆ neurons expressed as LMC₅/LMC₆ ratio at E11.5 (c) and E13.5 (d). The LMC₅/LMC₆ ratio is calculated as the number of Foxp1–positive and Isl1–negative neurons divided by the number of Foxp1–positive and Isl1–positive neurons. This ratio is similar in both genotypes at E11.5 (c; mean number of LMC neurons is 4311 for wild type, 4452 for Celsr3–/– and 4377 for Celsr2–/–;Celsr3–/–; 3 embryos each genotype, \( p = 0.7000; p = 0.8971 \), Mann–Whitney test). It is significantly reduced in Celsr3–/– mutant at E13.5 (d; mean number of LMC neurons 3855 for wild type and 2776 for Celsr3–/–, 3 embryos each genotype; \( p = 0.0079 \), Mann–Whitney test).

(e,f) Transverse sections of the lumbar spinal cord at E13.5 from wild type (e) and Celsr2–/–;Celsr3–/– (f), stained for cleaved caspase 3 (green) and Isl1 (red). The experiments were repeated 3 times.

(g) Quantification of the number of cleaved caspase 3–positive cells. This number is significantly higher in Celsr3–/– and Celsr2–/–;Celsr3–/– mutants than in wild type (n = 4 embryos for each genotype; **: \( p = 0.0054 \); ***: \( p = 0.0002 \). Mann–Whitney test).

Error bars in c,d,g are mean ± s.e.m. Scale bar: 100 µm (a,b); 50 µm (e,f).
Supplementary Figure 3

Expression of axon guidance molecules in wild type and mutants.

(a–i) Transverse sections of lumbar spinal cord and hindlimb at E11.5 from wild type (a,d,g), Celsr3<sup>−/−</sup> (b,e,h) and Fzd3<sup>−/−</sup> (c,f,i) embryos, stained with EphA4 (a–c), Ret (d–f) and Neuropilin1 (g–i) antibodies. Dashed lines define boundary between ventral (V) and dorsal (D) limbs. The experiments were repeated 3 times.

(j–r) Transverse sections of lumbar spinal cord at E11.5 from wild type (j,m,p), Celsr3<sup>−/−</sup> (k,n,q) and Fzd3<sup>−/−</sup> (l,o,r) embryos, hybridized with ephrinA2 (j–l), ephrinA5 (m–o) and Semaphorin 3A (p–r) digoxigenine–labelled probes. Expression of these cues is not perturbed in absence of Celsr3 or Fzd3. The experiments were repeated twice.

Scale bar: 100 µm.
**Supplementary Figure 4**

Lack of Celsr3 does not affect survival or axon growth of motor neuron.

(a,b) Illustration of LMC neurons from wild type (a) and Celsr3−/− (b) after 3 days *in vitro*.

(c) Quantification of motor neuron survival. *Hb9::GFP*–positive LMC neurons were dissociated from E12.5 embryos and plated as duplicates. Motor neurons were counted after 5 hours and 3 days. The percentage of survival was 56.4% in wild type and 56.8% in Celsr3 mutants (n = 1464 neurons for wild type and 1630 Celsr3 mutants; p = 0.8971, 3 embryos for each genotype, Mann–Whitney test)

(d) Mean axon length after 3 days in culture (n = 64 neurons for wild type and 72 neurons for Celsr3 mutants; p = 0.6665, Mann–Whitney test)

(e,f) LMC explants from wild type (e) and Celsr3−/− (f) cultured on laminin coated coverslips (100 µg/ml).

(g) Quantification of neurite growth (GFP pixels; n = 32 explants for the wild type and 36 for Celsr3 mutants; p = 0.7642; 3 embryos for each genotype, Mann–Whitney test)

Error bars in c,d,g are mean ± s.e.m. Scale bar: 50 µm (a,b); 100 µm (e,f).
Supplementary Figure 5

Normal innervation of axial muscles in Celsr3−/− mice.

(a,b) Whole mount views of axon projections labeled by Hb9::GFP in the back muscles of wild type (WT, a) and Celsr3−/− (b) embryos at E12.5. The medial anterior thoracic nerve (N. cut) innervates the superficial cutaneous maximus, and the thoracodorsalis nerve (N. th, arrow) innervates the latissimus dorsi. Both genotypes have a similar innervation pattern.

Scale bar: 200 µm.
**Supplementary Figure 6**

Celsr3 mutant axons respond to HGF in the turning assay.

(a–d) Scatter plots of turned angles $\beta$ versus initial angles $\alpha$ ($5^\circ<\alpha<175^\circ$) in the indicated conditions. *Celsr3*–/– motor axons in EphA7–Fc (n = 46); GDNF (n = 44); HGF (n = 68), Wild type in HGF (n = 67). 3 independent experiments were performed for each condition.

(e) Mean angle turned $\beta$ (mean ± SEM) of wild type and *Celsr3*–/– motor axons in different conditions (ns: $p>0.05$; **: $p<0.01$; $p = 0.0086$ for wild type in HGF; 0.6851 for *Celsr3*–/– in EphA7; 0.7906 for *Celsr3*–/– in GDNF; and 0.0094 for *Celsr3*–/– in HGF; unpaired t–test versus wild type in IgG–Fc gradient; error bars are mean ± s.e.m).

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Supplementary Figure 7

Genetic interaction between Celsr3 and Epha4.

(a–d) Lateral views of sciatic nerves stained by anti–neurofilament 160 at E12.5 in wild type (a), Celsr2+/–; Celsr3+/–; Epha4+/– (b), Celsr3–/– (c) and Celsr3–/–; Epha4+/– (d) embryos. Peroneal nerve (arrows), Tibial nerve (arrowhead).

(e) Quantification of the deep peroneal nerve width at E12.5. Mean thickness of the dPN = 47.6 µm in the wild type (n = 12), 47.4 µm in Celsr2+/–; Celsr3+/–; Epha4+/– (n = 14), 31.6 µm in Epha4–/– (n = 22), 16.5 µm in Celsr3–/– (n = 20), 12.8 µm in Celsr3–/–; Epha4+/– (n = 18) and 10.2 µm in Celsr3–/–; Epha4–/– (n = 12) embryos. ns: not significant, p = 0.8637; *: p = 0.026; **: p = 0.008; ***: p < 0.0001, unpaired t–test. Error bars are mean ± s.e.m.

Scale bar: 200 µm.

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Supplementary Figure 8

Generation of Fzd3 mutant mice.

(a) Schematic representation of the knockout first allele (modified from EUCOMM). A cassette containing FTR–Engrailed–2 exon–IRES–LacZ–loxP–neo–FRT–loxP was inserted in intron 2. In Fzd3\textsuperscript{ko/ko} mice, three mRNAs isoforms are produced. In the first, the engrailed–2 splice acceptor is “ignored” leading to the production of the wild type transcript. In the second, the engrailed–2–LacZ fusion cassette is transcribed as expected. In the third, a cryptic splice donor in the engrailed–2 exon results in a premature and aberrant splicing of the engrailed–2–LacZ cassette with the insertion of 115 nucleotides between exons 2 and 3 of the wild type mRNA (red box).

(b) The conditional allele was obtained by removal of the FTR–Engrailed–2–IRES–LacZ–loxP–neo–FRT cassette upon crossing with ROSA26–Flp. Exon 3 is flanked by two loxP sites thereby allowing its conditional excision.

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(c) In the null allele, exon 3 was deleted in the germline by crossing the conditional allele with PGK::Cre.

(d) Sequence of the 115 nucleotides found in transcript 3.

(e–h) Lateral views of sciatic nerves stained by anti–neurofilament 160 at E12.5 in wild type (e), Celsr3+/–;Fzd3+/– (f), Celsr2+/–;Celsr3+/–;Fzd3+/– (g) and Fzd3f/–;Isl1::Cre (h) embryos. Peroneal nerve (arrows), Tibial nerve (arrowhead).

(i) Quantification of the deep peroneal nerve width at E12.5. Mean thickness of the dPN = 43.1 µm in the wild type (n = 10); 43.8 µm in Celsr3+/–;Fzd3+/– (n = 16); and 44.4 µm in Celsr2+/–;Celsr3+/–;Fzd3+/– (n = 10). ns: not significant, p=0.6337 for Celsr3+/–;Fzd3+/– vs wild type; p=0.2250 for Celsr2+/–;Celsr3+/–;Fzd3+/– vs wild type, unpaired t–test. Error bars are mean ± s.e.m.

(j) Incidence of different phenotypes in wild–type and mutant mice (green: normal; blue: thinning; red: severe reduction). Wild type: n = 32; Celsr3+/–;Fzd3+/–: n = 16; Celsr2+/–;Celsr3+/–;Fzd3+/–: n = 10; Fzd3f/–;Isl1::Cre: n = 12.


Scale bar: 200 µm.
Supplementary Figure 9

Celsr3 and Fzd3 interact with GFRα1, Ret and ephrinA5.


(b,c) Co–IP assays between Myc–GFRα1, Ret, ephrinA5 and Celsr3–eGFP (b) or Fzd3–eGFP (c) in transfected HEK293T cells. Myc–GFRα1, Ret, ephrinA5 can interact with Celsr3–eGFP and Fzd3–eGFP.

(d,e) Co–IP assays between Myc–EphA7 and Celsr3–eGFP (d) or Fzd3–eGFP (e) in transfected HEK293T cells. Myc–EphA7 does not interact with Celsr3–eGFP and Fzd3–eGFP.

Full length blots are shown in Supplementary Fig. 11.
Supplementary Figure 10

Schematic summary of the function of Celsr3 in pathfinding of LMC\(_L\) axons.

(a,b) In the wild type (a), LMC\(_L\) axons are repelled by EphA forward signaling from ventral limb and attracted dorsally by GDNF and ephrinA reverse signaling. In Celsr3 mutants (b), LMC\(_L\) axons are still able to respond to EphA forward and GDNF, but not to attractive ephrinA signaling. Hence they segregate from axons innervating the ventral limb, engage in the common peroneal nerve, but fail to progress beyond the location where the peroneal nerve branches.

(c) A model for the interaction between Celsr3/Fzd3 and ephrinA reverse signaling. EphAs in dorsal mesenchymal cells bind to ephrinAs in axon growth cones, which recruits Ret, Celsr3, and Fzd3, and attracts axons into the dorsal limb.
Supplementary Figure 11

Full-length pictures of western blots.

(a) Full length blots for Fig. 6f. The membrane was cut according to the molecular weight and incubated with indicated antibodies. (b) Full length blots for Fig. 7j. Fzd3–eGFP was further detected by a chick α–GFP antibody due to a high background generated by the same rabbit α–GFP antibody used in IP. (c) Full length blots for Supplementary Fig. 7k. (d) Full length blots for Supplementary Fig. 9a. (e) Full length blots for Supplementary Fig. 9b. (f) Full length blots for Supplementary Fig. 9c. (g) Full length blots for Supplementary Fig. 9d. (h) Full length blots for Supplementary Fig. 9e.