hop* mice display synchronous hindlimb locomotion and a ventrally fused lumbar spinal cord caused by a point mutation in *Ttc26

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1 ***hop* mice display synchronous hindlimb locomotion and a**
2 **ventrally fused lumbar spinal cord caused by a point mutation**
3 **in *Ttc26***

4 Short Title: Spinal CPG fusion causes synchronous gait in *hop* mice.

5
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25

26

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35 **Abstract**

36 Identifying the spinal circuits controlling locomotion is critical for unravelling the
37 mechanisms controlling the production of gaits. Development of the circuits governing left-
38 right coordination relies on axon guidance molecules such as ephrins and netrins. To date, no
39 other class of proteins have been shown to play a role during this process. Here we have
40 analyzed *hop* mice, which walk with a characteristic hopping gait using their hindlimbs in
41 synchrony. Fictive locomotion experiments suggest that a local defect in the ventral spinal
42 cord contributes to the aberrant locomotor phenotype. *Hop* mutant spinal cords had severe
43 morphological defects, including the absence of the ventral midline and a poorly defined
44 border between white and grey matter. The *hop* mice represent the first model where,
45 exclusively found in the lumbar domain, the left and right components of the central pattern
46 generators (CPGs) are fused with a synchronous hindlimb gait as a functional consequence.
47 These defects were associated with abnormal developmental processes, including a misplaced
48 notochord and reduced induction of ventral progenitor domains. Whereas the underlying
49 mutation in *hop* mice has been suggested to lie within the *Ttc26* gene, other genes in close
50 vicinity have been associated with gait defects. Mouse embryos carrying a CRISPR replicated
51 point mutation within *Ttc26* displayed an identical morphological phenotype. Thus, our data
52 suggest that the assembly of the lumbar CPG network is dependent on fully functional TTC26
53 protein.

54

55 **Significance statement**

56 Our work reveals novel developmental defects in hop mice affecting nervous system
57 development and the assembly of local locomotor circuits. The hop mouse mutant appeared
58 spontaneously in the 1960s but the underlying cause of the unnatural synchronously hopping
59 gate has not yet been revealed. Altered functionality in hop mutant mice originates from an early
60 developmental defect in the lumbar spinal cord, resulting in a fused ventral midline. The hop
61 mouse represents an animal model harboring a very particular locomotor style that can help us
62 understand the assembly and properties of locomotor networks and how they function to
63 coordinate motor behavior.

64 **Introduction**

65 In the early 20th century, Thomas Graham-Brown demonstrated that the spinal cord could
66 generate the basic pattern for stepping without descending or peripheral input (Graham-
67 Brown T, 1911). Within his model of spinal locomotor control, he implemented Sherrington's
68 term “half-center” for two groups of reciprocally organized neurons mutually inhibiting each
69 other to provide a coordinated pattern for stepping. Such local spinal networks, capable of
70 generating rhythmic motor output, are referred to as central pattern generators (CPGs).
71 Studies on the spinal cord locomotor CPG have identified key principles for the development
72 and function of neuronal circuitry. For example, mouse mutants with abnormal locomotor
73 coordination have been informative for understanding CPG organization and function
74 (Kullander et al., 2001, 2003; Lanuza et al., 2004; Akay et al., 2006; Lundfald et al., 2007;
75 Crone et al., 2008, 2009; Zhang et al., 2008; Rabe et al., 2009; Zagoraiou et al., 2009;

76 Restrepo et al., 2009; Andersson et al., 2012; Talpalar et al., 2013; Lemieux et al., 2016;
 77 Thiry et al., 2018).

78 The CPGs controlling hindlimb muscle activity during locomotion are located in the
 79 ventral part of the lumbar spinal cord (Bracci et al., 1996; Kjaerulff and Kiehn, 1996) whereas
 80 CPGs controlling forelimb activity are located in the cervical spinal cord. Inhibitory actions
 81 between CPGs on different rostrocaudal levels of the spinal cord coordinate ipsilateral flexor
 82 and extensor muscles. Each side of the midline contains CPGs capable of rhythm generation
 83 where commissural inhibitory and excitatory projections ensure left-right coordination
 84 (Bannatyne et al., 2003; Butt and Kiehn, 2003; Hammar et al., 2004; Jankowska, 2008;
 85 Lanuza et al., 2004; Quinlan and Kiehn, 2007; Talpalar et al., 2013). Left-right activity
 86 persists when the dorsal spinal cord is removed, whereas left-right alternating fictive
 87 locomotion degrades after cutting the spinal cord ventral commissure. These experiments
 88 illustrate that ventromedial commissural interneurons (CINs) are critical for bilateral
 89 coordination (Andersson et al., 2012; Kjaerulff and Kiehn, 1996; Restrepo et al., 2009).

90 Neuronal connections form during embryonic development when differentiating
 91 neurons send their axons, navigating through the embryonic environment to synaptic targets.
 92 This process is mediated by conserved families of axon guidance proteins including netrins,
 93 ephrins, slits and semaphorins (Vallstedt and Kullander, 2013) and impaired axon guidance in
 94 the spinal cord results in locomotor coordination dysfunction (Kullander et al., 2001, 2003;
 95 Rabe et al., 2009; Rabe Bernhardt et al., 2012; Satoh et al., 2016; Wegmeyer et al., 2007).

96 In the present study, we analyzed *hop* mice that walk with a characteristic hopping
 97 gait using their hindlimbs in synchrony. Mice carrying the hop-sterile (*hop* or hydrocephalic-
 98 polydactyly, *hpy*) mutation appeared spontaneously in 1967 in the C57BL/10J strain, and the
 99 mutation was first found to be localized at the proximal end of chromosome 6 (Bryan et al.,
 100 1977; Hollander, 1976; Johnson and Hunt, 1971; Lyon, 1973). Later a point mutation within

101 the *Ttc26* gene was identified and suggested to be responsible for impaired hedgehog
102 signaling (Swiderski et al., 2014). However, since other genes in the close vicinity have been
103 associated with gait defects, it remained unclear whether the point mutation is causative for
104 the *hop* locomotor phenotype. Using CRISPR technology we introduced a single point
105 mutation within the *Ttc26* gene, which reproduced the anatomical phenotype observed in *hop*
106 mice. Further, we show that the synchronous gait in *hop* mice is the result of a left and right
107 CPG component fusion exclusively within the lumbar domain of the spinal cord. This is in
108 turn caused by abnormal notochordal and ventral spinal cord signaling during development,
109 resulting in migration defects and an absent ventral midline.

110

111 **Material and Methods**

112 **Animals**

113 *Mice*

114 CByJ.Cg-hop/J mice were imported from Jackson Laboratory (N12 on a backcross-intercross
115 to BALB/cByJ). The colony was kept on the BALB/cByJ background for analysis of the
116 locomotor phenotype and outcrossed 2-4 generations to the C57BL/6J background for
117 positional cloning. *Ttc26*^{Y430X} mutant mice were produced in the C57BL/6J background by
118 Siu-Pok Yee at the Gene Targeting and Transgenic Facility, Uconn Health Center. All
119 experiments involving animals were approved by the appropriate local Swedish ethical
120 committee (C147/7 or C79/9), and by the University of Calgary Health Sciences Animal Care
121 Committee.

123 **Gait study**

124 2-3 month old animals were trained to walk on a 1 meter long and 10 cm wide track the day
125 before the experiment. Mice were taken up and painting of the paws was mimicked with a
126 brush. Each mouse had to go to the end of the track at least 3 times without stopping. The day
127 of the experiment mice were handled exactly the same as during the training but this time the
128 paws were painted with two distinct colors for the forelimbs (red) and hindlimbs (blue).
129 Footprints were recorded on Whatmann paper placed at the bottom of the track. For each
130 animal, 3 runs were recorded and analyzed according to (Kullander et al., 2001).

131

132 **Electrophysiology**

133 Experiments were performed on 0-3 days old (P0-P3, weight 1.16 g to 4.05 g; n = 62) *hop*
 134 homozygote, heterozygote and wild type control mice. Mutant mice were identified by
 135 preaxial polydactyly of the hindfeet (Fig. 1-1) and the synchronous activity in their hindlimbs
 136 when suspended in the air with their tails lightly pinched. There were no obvious deficits in
 137 gait in the *hop* heterozygote and wild type control mice.

138 For in vivo experiments, animals were anaesthetized by hypothermia and then suspended in a
 139 sling such that the fore- and hindlimbs moved freely (Norreel et al., 2003). Electromyographic
 140 (EMG) electrodes were inserted into the left and right tibialis anterior muscles parallel to the
 141 muscle fibres, and a grounding electrode was inserted subcutaneously into the back. EMG
 142 electrodes were made of 75 mm Teflon-coated platinum-iridium wires (A-M Systems Inc.). A
 143 heat lamp was used to keep the air temperature at 30°C. Recordings were amplified (1000
 144 times), bandpass filtered (100 – 1 kHz), and digitized at 3 kHz (Axon Digidata 1322A) for
 145 future analysis. 5 – 10 minutes after the EMG wires were inserted, air-stepping sequences
 146 were elicited by pinching the tail with forceps and the activity was recorded.

147 For in vitro experiments, the animals were anaesthetized by hypothermia. Animals were
 148 rapidly decapitated, eviscerated and the remaining tissue was placed in a dissection chamber
 149 filled with oxygenated (95% O₂ – 5% CO₂) artificial cerebrospinal fluid (ACSF:
 150 concentrations in mM: 128 NaCl, 4 KCl, 0.1 CaCl₂, 2 MgSO₄, 0.5 Na₂HPO₄, 21 NaHCO₃, 30
 151 D-glucose). A ventral laminectomy exposed the cord, and the ventral and dorsal roots were
 152 cut. The spinal cord was transected at thoracic 1 – 3 (T₁₋₃) to sacral 2 – 3 (S₂₋₃) and carefully
 153 removed from the vertebral column. After 30 minutes, the preparation was transferred to the
 154 recording chamber and superfused with oxygenated ACSF (concentrations in mM: 128 NaCl,
 155 4 KCl, 1.5 CaCl₂, 1 MgSO₄, 0.5 Na₂HPO₄, 21 NaHCO₃, 30 D-glucose). The bath solution was

156 then heated gradually from room temperature to 27°C. The preparation was allowed to
 157 acclimate for one hour thereafter. Population motoneuron bursting activity was recorded using
 158 suction electrodes into which segmental ventral roots from the left and right lumbar L₂ and L₅
 159 segments were drawn (Whelan et al., 2000). In some experiments we found that the L₂ ventral
 160 root was not well formed and in these cases we recorded from the L₃ ventral root. Neonatal
 161 mice show synchrony between L₁, L₂, and L₃ ventral root neurograms (Whelan et al 2000).
 162 The resultant neurograms were amplified (100 – 20,000 times), band pass filtered (100 Hz – 1
 163 kHz) and digitized at 2 – 5 kHz (Axon Digidata 1320) for future analysis. Ten minutes of
 164 control baseline activity was recorded prior to adding drugs. 5 μ M N-methyl-DL-aspartic acid
 165 (NMA, Sigma-Aldrich), 10 – 20 μ M Serotonin (5-HT, Sigma-Aldrich) and 50 – 75 μ M
 166 dopamine (DA, Sigma-Aldrich) were added to the bath and the rhythm stabilized for 10 – 30
 167 minutes. In some experiments, a GABA uptake inhibitor (0.1 – 1 mM nipecotic acid or 50 –
 168 100 μ M NO-711, Sigma-Aldrich) and/or a glycine uptake inhibitor (100 μ M sarcosine,
 169 Sigma-Aldrich) was added to the bath solution. In some experiments, we examined whether a
 170 hemisected spinal cord was capable of generating coordinated rhythmic activity. In these
 171 experiments, we first examined the activity patterns in the presence of rhythmogenic drugs.
 172 We then removed the recording electrodes and using a pair of micro-clippers completely mid-
 173 sagittally hemisected the spinal cord. The suction electrodes were then reattached to the
 174 ventral roots.
 175 Data were digitally rectified, integrated and then analyzed using custom written programs
 176 (MatLab, MathWorks, Natick, MA). Locomotor-like activity was quantified using time series
 177 analysis. Time series analysis was performed by taking intervals of 60 seconds of raw data,
 178 rectifying the data, applying a low-pass filter and resampling at 100 Hz. Means were
 179 subtracted from the processed data and further smoothed using a digital filter (Savitzky-
 180 Golay, 3rd order polynomial operating over 13 points). Cross and auto-correlograms were then

181 calculated and the quality of the rhythm was assessed by measuring the correlation
 182 coefficients for the segmental L₂ ventral root bursts and the left L₂ and left L₅ ventral root
 183 bursts. To measure rhythm stability, the peak-to-trough correlation coefficient (PTCC) was
 184 calculated from the cross correlogram by subtracting the minimum negative value of the
 185 correlation coefficient from the maximum of the first positive peak over the first 75 lags (each
 186 lag = 50 ms). Stable synchronous rhythms typically have high positive correlation coefficients
 187 at zero phase lag. The cycle periods for the resultant rhythm were calculated by measuring the
 188 number of lags from one peak to the next from the auto-correlogram. The phase lag between
 189 ventral root bursts was obtained from the cross correlogram and was defined as the distance
 190 from the minimum trough around lag 0 to the next peak divided by the cycle period
 191 (Madriaga et al., 2004). Data are expressed as mean $\pm$ standard error of the mean and
 192 significance was analyzed using paired and unpaired Student's t-tests if the data were
 193 normally distributed ($P < 0.05$). Data that were not normally distributed were analyzed using a
 194 Wilcoxon-Signed Rank test. Multiple data points were analyzed using a one-way ANOVA
 195 followed by a Tukey post hoc test to detect significant differences. To analyze *in vivo* EMG
 196 data and to illustrate phase relationships for selected *in vitro* experiments, we used circular
 197 statistics, in which the phase was normalized from 0 to 1 (Drew and Doucet, 1991). If the
 198 length of the arrow is large this suggests a tendency for the rhythms represented by the two
 199 neurograms to be coupled. Significance was computed using Rayleigh's test ($P < 0.05$).
 200

201 **Tracing of commissural interneurons**

202 Fluorescent dextran-amines 3,000 MW rhodamine-dextran-amine (RDA) and 3,000 MW
 203 fluorescein-dextran-amine (FDA) (Invitrogen, Sweden) were used for retrograde tracing of
 204 commissural interneurons (CINs) as described previously (Eide and Glover, 1995). P0–P3
 205 mice spinal cords were prepared as described earlier (Wegmeyer et al., 2007). Tracings on
 10

206 E12.5 embryos were performed essentially the same way, but the spinal cord remained within
 207 the vertebral column during tracer application and incubation. Preparations were incubated for
 208 12–16 h and then fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline
 209 (PBS), pH 7.4 and stored dark at 4°C for 1 week. Spinal cords were cut into 60 mm thick
 210 transverse sections on a vibratome (Leica, Germany) and stored in the dark at –20°C until
 211 analysis. Embryonic tracings were fixed for 2 hours, transferred to 30% sucrose in PBS at 4°C
 212 over-night, embedded in OCT and 12 mm sections were cut using a cryostat (CM1800,
 213 Leica), collected onto Superfrost slides (Menzel-Gläser, Germany)

214 ***In situ* hybridization**

215 Free floating vibratome sections were rehydrated in consecutive washes for 10 min in 75%,
 216 50% and 25% methanol in PBT, bleached in 6% hydrogen peroxide in PBT for 15 min and
 217 treated with 0.5% Triton X-100 for 5 min. The sections were digested with proteinase K (10
 218 µg/ml) in PBT for 15 min. The digestion was stopped with a wash in glycine (Scharlau
 219 Chimie, Spain; 2mg/ml) in PBT for 5 min, and sections were postfixated in 4% formaldehyde
 220 for 20 min. The sections were prehybridized at 65°C in hybridization buffer (50% formamide,
 221 5xSSC pH 4.5, 1% SDS, 50 µg/ml tRNA (Sigma) and 50 µg/ml heparin (Sigma)) for two
 222 hours prior to addition of probe. 1 µg/ml probe was added to the hybridization buffer and
 223 sections were hybridized over-night at 65°C. Excess probe was removed by washes in wash
 224 buffers (50% formamide, 5xSSC pH 4.5 and 1% SDS; 50% formamide, 2xSSC pH 4.5 and
 225 0.1% tween-20) at 65°C for 3 times 30 min each. The sections were transferred to blocking
 226 solution (1% blocking reagent in TBST) for 2 h before addition of anti-DIG AP (1:5000)
 227 diluted in blocking solution and incubated over-night at 4°C. The sections were treated with
 228 levamisole (0.5 mg/ml) in TBST and levamisole (0.5mg/ml) in NTMT (100 mM NaCl, 10
 229

230 mM Tris-HCl pH 9.5, 50 mM MgCl₂ and 0.1% Tween-20) before developing in BM purple
 231 AP substrate (Roche) at 37°C 1h – 4 days. Between additions of new chemicals, sections were
 232 washed with either PBT (prior to addition of probe) or TBST (after addition of probe). In *situ*
 233 hybridization on cryosections was performed essentially as described (Schaeren-Wiemers and
 234 Gerfin-Moser, 1993). The VIAAT probe covers nucleotides 588-2072, the Vglut2 probe 1616
 235 –2203 used as described earlier (Wallén-Mackenzie et al., 2006), the VACHT probe 1534 –
 236 2413, Netrin-1 and EphrinB3 probe 1-1021.

237

238 **Immunohistochemistry**

239 The tissue was cryoprotected in 30% sucrose in PBS, embedded in OCT medium and
 240 sectioned at a thickness of 12-14 µm on a cryostat (CM1800, Leica). The sections were
 241 washed in PBS and pre-blocked in blocking solution - 5% goat serum, 0.3% Bovine Serum
 242 Albumin (BSA, Sigma Aldrich) and 0.1% Triton®X-100 (Sigma Aldrich) in PBS. Primary
 243 antibodies were diluted in blocking solution, added to the sections and incubated over night at
 244 4°C. The following dilutions were used: mNkx2.2 1:100 (Hybridoma bank), mEvx1 1:50
 245 (Hybridoma bank), gpLbx1 1:10000 (kind gift from C. Birchmeyer), rPax2 1:1000 (Covance),
 246 mBrn3a 1:500 (Chemicon), mIsl1/2 1:100 (Hybridoma bank), mLhx1/5 1:500 (Hybridoma
 247 bank), Lhx2/9 1:8000 (kind gift from Dr. T.Jessell), mShh 1:100 (Hybridoma bank), mMap2
 248 1:500 (Chemicon, Sweden), rbNeuN 1:1000 (Chemicon), MBP 1:500 (AbCam), rbTtc26
 249 1:1000 (Novus Biologicals), mNeuN 1:500 (Chemicon), gpVACHT 1:500 (Millipore), gChAT
 250 1:250 (Millipore), mParvalbumin 1:1000 (Sigma) and gpDmrt3 1:10 000 (custom made using
 251 the immunizing peptide CKQSIYTEDDYDERS-amide).

252 For some antibodies, antigen retrieval was necessary in order to get good
 253 immunohistochemical staining. The sections were then washed with PBS and thereafter
 254 placed in a metal rack inside a beaker filled with preheated citrate buffer (9 ml 0.1 M citric
 12

acid, 41 ml 0.1 M trisodium citrate dehydrate and 450 ml dH₂O – pH6.0). After 30 minutes at 98°C, the beaker was left to cool down at room temperature for 30 minutes. Subsequently the sections were washed with dH₂O for 1 minute and pre-blocked in 1% normal goat serum and 0.1% Triton[®]X-100 in PBS for 1 hour. Finally, the primary antibodies were diluted in blocking solution (1% normal goat serum and 0.1 % Triton[®]X-100 in PBS), added to the sections and incubated over night at 4°C. The following day, the sections were washed with PBS and incubated with DAPI (Sigma Aldrich, 200ng/ml) and the secondary antibodies (diluted 1:500 in blocking solution) for 2-3 hours at room temperature. After washing with PBS, the sections were mounted in Mowiol[®] 4-88 (Sigma Aldrich). Secondary antibodies used: goat anti-mouse conjugated with FITC 488 (F(ab')₂Fragment, 111-096-003, Jackson Immunoresearch Labs), donkey anti-guinea pig conjugated with Alexa 594 (Jackson Immunoresearch Labs), donkey anti-rabbit conjugated with Alexa 488 (Invitrogen) and donkey anti-goat conjugated with Alexa 488 (Jackson Immunoresearch Labs).

Ttc26^{Y430X} mice embryos (E16.5) were euthanized, genotyped, fixed in 4% paraformaldehyde for 4 hours on a rocking table at room temperature and then washed in 0.1 M phosphate buffered saline (PBS - Sigma Aldrich, Stockholm, Sweden) for 30 minutes before being cryoprotected in 30% sucrose in PBS. The tissue was shipped in 30% sucrose in PBS (in a falcon tube placed inside a Styrofoam box with cool-packs) to Uppsala University. Upon arrival 5 days later the embryos were frozen in OCT medium (Richard Allan Scientific NEG50TM) on dry ice. Cryostat sections were cut (cryostat CM1800, Leica) at a thickness of 14µm and placed on Superfrost slides (Menzel-Gläser, Germany). For the immunohistochemistry experiment, postnatal P2 wild type rabbits from the Sauteur d'Alfort breed were obtained from the Fédération Française de Cuniculture. They were anesthetized with a mix of medetomidine (Dormilan[®] 0,1ml/kg) and ketamine (Ketamidor[®] 0,15 ml/kg) and cardiac perfusion was performed with PBS followed by formaldehyde 4% in

280 PBS. The spinal cords were dissected and postfixed overnight at 4°C in formaldehyde 4% in
281 PBS.

282

283

284 **Histology staining**

285 For haematoxylin and Eosin staining, cryosections of spinal cord were rehydrated in dH₂O for
286 1 minute, stained with progressive haematoxylin (Mayer's) for 90 seconds, washed twice in
287 dH₂O for 1 minute, dipped in Scott's tap water (20 g MgSO₄·7H₂O and 2 g NaHCO₃ in 1 L
288 tap water), dehydrated in 96% Ethanol in dH₂O for 1 minute and finally counter-stained with
289 0.5% Eosin (Sigma Aldrich) in dH₂O for 20 seconds. Afterwards the sections were
290 dehydrated with successive washes in 70% and 96 % Ethanol in dH₂O for 1 minute and
291 treated with X-tra Solve[®](MEDITE) for 4 minutes before being mounted in Pertex[®]
292 (Histolab[®]). 3,3'-diaminobenzidinetetrahydrochloride (DAB) (Sigma, St Louis, MO, USA)
293 was used on free-floating spinal cord sections to visualize white matter (dark brown) and
294 Luxol fast blue (LFB) was used to analyze paraffin embedded brain sections staining myelin
295 and phospholipids (blue-green) counterstained with cresyl violet as previously described
296 (Kullander et al., 2001).

297 For the histology experiment, wild adult rabbits were euthanized in Stockholm by Stockholms
298 Vilt- och Skadedjursgrupp and rabbit tissue was shipped on ice to Uppsala University within a
299 few hours. Upon arrival, the vertebral column was placed in 4% paraformaldehyde in PBS for
300 two weeks, before the spinal cord was dissected. Thereafter the tissue was cryoprotected in
301 30% sucrose in PBS for 60 hours and frozen in OCT medium (Richard Allan Scientific
302 NEG50TM) on dry ice.

303

304

305

306 **CRISPR mutagenesis**

307 Based on previously published data (32) and our independent exome sequencing validation
 308 (Fig. 7-1), a point mutation located at Chr6:38,362,071 (C>A) and resulting in a nonsense
 309 mutation (Y430X) within the *Tetratricopeptide repeat protein 26* (*Ttc26*) gene was identified
 310 in *hop* mice. We used CRISPR/Cas9-mediated genome editing technology with the help from
 311 Siu-Pok Yee, Ph.D. at the Gene Targeting and Transgenic Facility, Uconn Health Center. A
 312 C/A transversion at position 38412065 in exon 15 of the gene *Ttc26* on chromosome 6 was
 313 created (Inui et al., 2015) to replace a TAC codon in the amino acid chain for tyrosine 430
 314 with a TAA to create a premature STOP codon. A silent point mutation was also introduced
 315 to create a novel PvuII site, 3' of the knock-in termination codon in *Ttc26* to allow for
 316 straightforward genotyping of the mice by PCR followed by a PvuII digestion. The PvuII site
 317 also affected the PAM sequence of the CRISPR so re-digestion of the KI allele was avoided.
 318 CAS nuclease, a guide RNA and an oligonucleotide carrying the desired mutation and
 319 homology arms were co-injected into the nuclei of fertilized oocytes, followed by
 320 implantation of the eggs into surrogate mothers to obtain offspring. An allele-specific primer
 321 was developed for genotyping of the pups. We obtained one *Ttc26* Y430X founder female
 322 that appeared to be heterozygous for the mutation (1:1 peak ratio from sequencing, Fig 5A).
 323 From F1 pups from the founder female, we bred heterozygous males and females to obtain
 324 homozygous *Ttc26*^{Y430X} mouse mutants.

325

326 **Imaging and picture processing**

327 Fluorescent and bright field images were acquired either on an Olympus BX61WI microscope
 328 (Olympus, Sweden) using the Volocity software (Improvision, Lexington, USA) or on a
 329 MZ16F dissection microscope with a DFC300FX camera and FireCam software (Leica).

330 Close-ups were taken using the OptiGrid Grid Scan Confocal Unit (Qioptiq, Rochester, USA)
 331 or a confocal microscope (Zeiss LSM 510 Meta, Oberkochen, Germany). For illustrations,
 332 captured images were auto-leveled using Adobe Photoshop software. Quantitative image
 333 analysis was performed using the Fiji processing suite (REF). For each image, region of
 334 interests were manually defined. For quantification of fluorescence, a global threshold was
 335 applied before relevant object information was extracted. Distribution plots were obtained
 336 using R Studio Version 4.0.4 (R Core Team (2021).) and the spatstat package (Baddeley and
 337 Turner, 2005).

338

339 **Multiple sequence alignments of TTC26**

340 An amino acid sequence analysis of TTC26 was performed to generate multiple sequence
 341 alignment. In order to perform this analysis, we first downloaded the protein sequences from
 342 the National Center for Biotechnology Information (NCBI) for multiple species (Human,
 343 Rabbit, Dog, Cow, Pig, Chimpanzee, Mouse and Rat) with the accession number
 344 (NP_079202.2, XP_002722121.2, XP_003639595.2, XP_002687045.1, XP_003134668.2,
 345 XP_527905.3, NP_705828.2, NP_001020216.1) respectively. Multiple sequence alignments
 346 was performed using MUSCLE software (Edgar 2004, PMID: 15034147) with default
 347 parameters, in the multiple sequence alignment editor SEASView (Galtier 1996, PMID:
 348 9021275) version 3.2 by setting the human sequence as the reference with the block size of
 349 10.

350

351 **Statistical analysis**

352 Simple effects of genotype (*crtl*, *hop*) were analyzed using independent t-test (two-tailed) or
 353 Mann-Whitney U test dependent on data distribution. Analyses were performed using MS
 354 Excel 2016 or SPSS Statistics (version 27.0, IBM, Armonk, NY, USA). Data was plotted

355 using R (<https://www.r-project.org>) and the package shiny and vioplot. Statistical significance
 356 was set at $p < .05$, effect sizes are reported with Cohen's d or r (rank-biserial correlation) for
 357 non-parametric testing.

358 **Results**

359 **Aberrant synchronous hindlimb coordination in *hop* mice correlates to local spinal cord** 360 **neuronal circuitry**

361 Homozygous *hop* mutants show distinct phenotypes such as preaxial polydactyly of all feet,
 362 sperm tail deficiency resulting in male sterility, and hydrocephalus (Hollander, 1976; Johnson
 363 and Hunt, 1971; Swiderski et al., 2014). Although there have been no detailed reports on the
 364 locomotor pattern produced by *hop* mutant animals, it has been described that they exhibit a
 365 characteristic hopping gait (Johnson and Hunt, 1971; Wojnowski et al., 1998). We confirmed
 366 and extended these observations by conducting gait analysis and electrophysiological
 367 experiments. Gait analysis in adults revealed that during locomotion, mice homozygous for
 368 the *hop* mutation, herein referred to as *hop* mice, alternated their forelimbs while their
 369 hindlimbs moved in synchrony (Fig. 1A, B). To assess the onset of this hopping pattern, we
 370 evoked locomotor activity by pinching the tail, followed by electromyographic (EMG)
 371 recordings from the *tibialis anterior* muscles in conscious early postnatal (P0-P5) *hop* and
 372 control animals. We found that *hop* mice produced an air locomotor pattern of synchronous
 373 hindlimb movements. As expected, control neonatal mice produced a typical left-right
 374 alternating pattern (Fig. 1C-E).

375 To determine whether deficiencies in the spinal cord CPG are the sole cause of the locomotor
 376 phenotype, we performed experiments using isolated spinal cord preparations. Following bath
 377 application of 5-HT, DA and NMDA, we found that in the majority of *hop* mutants (20/28), a

378 synchronous fictive locomotor rhythm developed between left-right ventral roots. In contrast,
 379 the alternating ipsilateral flexion-extension like pattern between the L2 and L5 roots was
 380 preserved (Fig. 1F-H). We observed that in contrast to control mice the L5 burst duration was
 381 extended and an asymmetry was observed between the L2 and L5 burst duration in 13/20 *hop*
 382 mutants. The data represents an average for 5 min following the development of a sustained
 383 rhythm (L2-L2: 1.10 ± 0.06 (PTCC), 5.61 ± 0.68 s (cycle period), 0.05 ± 0.01 (phase lag);
 384 L2-L5: -1.04 ± 0.06 (PTCC), 0.52 ± 0.03 (phase lag), $n=20$). Although the dominant rhythm
 385 observed was a synchronous left-right and an ipsilateral alternating pattern, it was not the only
 386 one. 5/28 *hop* mutant mice exhibited a left-right alternating pattern consistent with a normal
 387 locomotor-like pattern. In 3/28 *hop* mutant mice, we observed a synchronous pattern between
 388 all roots (L₂-L₂: 1.26 ± 0.10 (PTCC), 11.78 ± 3.76 s (cycle period), 0.01 ± 0.01 (phase lag);
 389 L₂-L₅: 1.14 ± 0.13 (PTCC), 0.01 ± 0.003 (phase lag)). Moreover, the data indicate that the
 390 fictive locomotion in *hop* mice was less stable than in control mice, and in 5 of the 20 animals
 391 that displayed a synchronous segmental left-right and ipsilateral alternating rhythm, it was
 392 sustained for around 5 minutes, which was notably different from control mice, where the
 393 standard alternating rhythm was sustained for up to 60 min. There were no significant
 394 differences in cycle periods between *hop* mutants and controls (L₂: 5.19 ± 0.39 s, L₅: $5.53 \pm$
 395 0.37 s (mutants); L₂: 5.83 ± 0.98 s, L₅: 5.33 ± 0.80 s (controls); $n = 8$; $P > 0.5$). The
 396 synchronous fictive locomotion we observed in 20/28 *hop* mice was in accordance with the *in*
 397 *vivo* gait analyses as well as the EMG recordings.

398 In the 20 animals that generated synchronous segmental patterns and ipsilateral
 399 alternation, we found that the ipsilateral pattern of L_{2/3}-L₅ alternation was maintained
 400 following hemisection, suggesting that the circuitry coordinating flexor-extensor alternation
 401 was intact in these *hop* mutants (Fig. 1-1). Following hemisection, it was usually necessary to
 402 increase the concentration of the drug cocktail (DA to 75 μ M, 5-HT to 15 μ M) to maintain
 403 rhythmic activity [L_{2/3}-L₅: 1.18 ± 0.15 (PTCC), 5.53 ± 3.03 s (cycle period), 0.51 ± 0.04
 404 (phase lag), $n=2$]. The increase in concentration is typical of previous reports (Whelan et al.,
 405 2000).

406 The asymmetry between flexor and extensor bursts was not present in the hemisected cords
 407 (Fig. 1-1B), and was qualitatively similar to reports using normal mice (Whelan et al., 2000).

408 To further establish whether synchronous left-right rhythmic activity was the
 409 dominant pattern, we used electrical stimulation of the *cauda equina* or dorsal roots to elicit a
 410 rhythm (Gordon and Whelan, 2006; Whelan et al., 2000). We found that in *hop* mice,
 411 electrical stimulation did evoke a synchronous rhythm similar to that observed using
 412 pharmacological stimulation (Fig. 1-1C). In addition, in several mice, we were able to observe
 413 spontaneous bouts of synchronous left-right and alternating L₂-L₅ rhythm that would last a
 414 few seconds. Collectively, these data support the idea that the *hop* deficit is at least partly due
 415 to alterations in the lumbar network.

416 Previous studies have shown that neurotransmitter balance over the midline is critical
 417 for coordinated left-right output of the CPG, and during fictive locomotion, the balance
 418 between excitation and inhibition can be shifted using pharmacological strengthening
 419 (Hinckley et al., 2005; Juvin et al., 2005; Kullander et al., 2003; Wu et al., 2018). Following
 420 administration of nipecotic acid or NO-711 (data pooled since no difference was observed
 421 between the two drugs), we found that, although the left-right pattern did not switch to an
 422 alternating pattern, the rhythm became less stable (Fig. 1I, middle panel; [control: L₂-L₂: 1.11
 423 $\pm$ 0.06 (PTCC), 6.34 $\pm$ 1.56 s (cycle period), 0.03 $\pm$ 0.01 (phase lag); L₂-L₅: -1.10 $\pm$ 0.04
 424 (PTCC), 0.49 $\pm$ 0.04 (phase lag); with nipecotic acid or NO-711 (pooled data): L₂-L₂: 0.75 $\pm$
 425 0.33 (PTCC), 4.88 $\pm$ 0.55 s (cycle period), 0.11 $\pm$ 0.06 (phase lag); L₂-L₅: -1.01 $\pm$ 0.10
 426 (PTCC), 0.73 $\pm$ 0.13 (phase lag); n = 7]. We also tested both sarcosine and nipecotic acid bath
 427 applied simultaneously in a separate series of experiments. We found that this manipulation
 428 consistently altered network dynamics by changing the synchronous pattern to a less stable
 429 pattern, which at times appeared to be alternating (Fig. 1I right panel; [control: L₂-L₂: 1.29 $\pm$
 430 0.13 (PTCC), 3.73 $\pm$ 0.35 s (cycle period), 0.06 $\pm$ 0.003 (phase lag); L₂-L₅: -1.17 $\pm$ 0.07
 431 (PTCC), 0.58 $\pm$ 0.03 (phase lag); with sarcosine and nipecotic acid: L₂-L₂: 0.27 $\pm$ 0.44
 432 (PTCC), 4.54 $\pm$ 0.23 s (cycle period), 0.24 $\pm$ 0.15 (phase lag); L₂-L₅: -1.05 $\pm$ 0.10 (PTCC),
 433 0.55 $\pm$ 0.04 (phase lag); n = 3]).

434 Taken together, our locomotor analysis of *hop* mice confirmed the synchronous
 435 pattern previously reported and that defects in the lumbar spinal cord circuitry are sufficient to
 436 explain the altered hindlimb coordination. Further, whereas changing the balance of
 437 excitatory and inhibitory connections over the midline in *hop* mice caused altered network
 438 activity, the synchronous left-right output was not consistently switched back to alternation.

439 440 **Altered spinal cord morphology in *hop* mice**

441 Our electrophysiological experiments suggested that the aberrant locomotor phenotype arose
 442 from local spinal neuronal circuitry output, therefore we examined the morphology of the
 443 spinal cord. The gross morphology of wild type postnatal spinal cords is characterized by a
 444 cervical and lumbar enlargement, which contain, among others, the neurons that coordinate
 445 fore- and hindlimb movement, respectively. The cervical enlargement extends from C5 to T1,
 446 and the lumbar enlargement extends from L2 to L6. The spinal cord from early postnatal
 447 control mice was found to measure 14 ± 3 mm. In *hop* mice, the length of the spinal cord, 14
 448 ± 5 mm, and the size of the cervical enlargement were comparable to controls, whereas the
 449 lumbar enlargement was hardly detectable (Fig. 2A-B). Further, wild type spinal cords had a
 450 visible ventral spinal artery fed by a radicular artery termed the artery of Adamkiewicz, which
 451 in wild type mice usually originates at the level of L₁₋₂ (Fig. 2A, arrow). In the lumbar region
 452 of *hop* spinal cords, the blood supply was abnormal, including the absence of a ventral spinal
 453 artery together with an undefined artery of Adamkiewicz (Fig. 2A, star).

454 Next, we used the spinal cord open-book preparation, in which the spinal cord is cut
 455 dorsally along the rostrocaudal axis, and the left and right halves of the spinal cord are
 456 dissected to reveal the inside of the two halves and the remaining ventral part. In this
 457 preparation, using NeuN as a marker for neurons, we found that the morphology of the
 458 lumbar part in *hop* mice was severely affected showing a diffuse border between the NeuN

positive neurons and the ventral area, whereas the more rostral and caudal parts were unaffected (Fig. 2C,D). Transverse sections of lumbar spinal cords stained with NeuN (grey matter) and myelin basic protein (MBP) as a marker for myelinated axons (white matter), revealed less myelinated structures and misplaced neurons in *hop* mutants (Fig. 2E,F). Distribution plots obtained from these stainings further illustrate the misplacement of neurons along the medio-lateral axis (magenta) and a dorsally shifted central canal (blue) in *hop* mice (Fig. 2G,H).

Similarly, adult mice transverse sections of the cervical spinal cord from *hop* mice showed no morphological differences compared to controls (Fig. 2I). However, sections of lumbar spinal cords from *hop* mice showed an altered morphology in the ventral spinal cord, including an undefined border between white and grey matter, the absence of a ventral funiculus and a dorsally shifted, poorly delineated, and smaller central canal (Fig. 2I-L). Immunohistochemistry, using NeuN and MBP antibodies, demonstrated misplaced neurons within the white matter also in adult *hop* mutants (Fig. 2M-O). Other mouse mutants with locomotor phenotypes have been reported to display severe defects in several brain regions, including the corpus callosum, hippocampal-, anterior-, habenular- and posterior commissure as well as the pontine nucleus and cerebellar structures (Ackerman et al., 1997; Chiang et al., 1996; Fazeli et al., 1997; Kullander et al., 2001; Páez et al., 2007; Serafini et al., 1996). In contrast, morphological analysis of brains from adult *hop* mice, which display enlarged ventricles likely connected to the hydrocephalic phenotype, did not show any further brain abnormalities (Fig. 2-1).

Together, these findings suggest that the synchronous gait in *hop* mice is the result of ventral spinal cord alterations limited to the lumbar region responsible for controlling the hindlimbs. The rostro-caudal specificity is further supported by the observed forelimb alternating locomotion and normal cervical spinal cord morphology.

484

485 **Aberrant commissural fibres and neurotransmitter phenotype in *hop* mice**

486 Cross-inhibitory and excitatory actions from CINs between the two sides of the locomotor
 487 CPG ensure coordinated left-right movements (Cowley and Schmidt, 1995; Kjaerulff and
 488 Kiehn, 1996). In an effort to further explain the aberrant locomotor phenotype, we examined
 489 the neurotransmitter profile of the neurons interspersed in the lumbar ventral white matter of
 490 *hop* mice using *in situ* hybridization. GABA/glycinergic, glutamatergic and cholinergic
 491 neurons were identified by detecting mRNA encoding the vesicular inhibitory amino acid
 492 transporter (VIAAT), the vesicular glutamate transport (Vglut2), or the vesicular
 493 acetylcholine transporter (VACHT). We found both VIAAT and Vglut2 positive neurons in
 494 the ventral funiculus of *hop* mice demonstrating that misplaced neurons can be either
 495 excitatory or inhibitory (Fig. 3A,B,E,F,I,J). In addition, we found that central canal
 496 cholinergic cells were ventrally and medially displaced in *hop* mice compared to controls
 497 (Fig. 3C,G), whereas no apparent differences were found regarding position and number of
 498 motor neurons (Fig. 3D,H,K).

499 Ventromedial CINs with axons crossing in the ventral commissure are necessary and
 500 sufficient for left-right coordination (Kjaerulff and Kiehn, 1996), which prompted us to
 501 investigate the CINs in *hop* mice. Short- and long-range projecting CINs were examined by
 502 tracer applications of fluorescein dextran amine (FDA) and rhodamine dextran amine (RDA)
 503 in early postnatal mice (Fig. 4A). Midline retrograde tracing, using FDA to identify locally
 504 CINs, revealed that CINs at the lumbar level 2 (L2, Fig. 4C,I) cluster together on the ventral
 505 midline of *hop* mice compared to the two separate clusters of CINs seen in control mice (Fig.
 506 4B,H), whereas similar numbers of local CINs were found (Fig. 4N). FDA application on one
 507 side of the L2 spinal cord illustrate a similar clustering of short projecting CINs on the ventral
 508 midline as well as aberrant fiber crossing (4D,E). Despite altered positioning (Fig. 4J,K), the

number of short-range projecting CINs in *hop* were similar compared to controls (Fig. 4O). To study the long-range projecting CINs, we examined three different subpopulations; the intersegmental ascending (RDA), descending (FDA), and bifurcating (RDA/FDA) CINs (Fig 4F-P). In the cervical spinal cord (Fig. 4-1), and in control mice also at the lumbar level, our tracings experiments showed that long-range projecting CIN populations clustered in the ventromedial area. At the lumbar level in *hop* mice, however, CINs were diffusely dispersed and partly located on the midline (Fig. 4L,M). In addition, lumbar long-range projection CINs were found to be reduced in *hop* compared to control mice (Fig. 4P).

Taken together, these results show that, in *hop* mice, the ventromedial inhibitory and excitatory CINs responsible for normal left-right coordination cluster together on the ventral midline, and that intersegmental CINs are partly lost and misplaced.

An early developmental defect underlies the altered functionality in *hop* mice

CPG activity in rodents develops between embryonic day (E)12 and birth. Correct assembly and maturation results in an established left-right coordination circuitry around birth (Branchereau et al., 2000; Nakayama et al., 2002; Nishimaru and Kudo, 2000). The prominent ventral spinal cord phenotype observed in postnatal *hop* mice prompted us to investigate the onset of the morphological phenotype. Immunohistochemistry on E15.5 *hop* spinal cord tissue using microtubule associated protein 2 (Map2), to visualize fibers, and NeuN, to visualize neurons, revealed a similar fused spinal cord phenotype as seen in adult mice (Fig. 5A-D). In addition, using RDA tracing at E15.5 and E12.5, we found that the traceable CIN population in *hop* embryos was misplaced towards the midline and at E15.5 was more dispersed throughout the ventromedial spinal cord (Fig. 5E-H). These data indicate deviant ventral spinal cord formation presumably due to aberrant developmental processes, such as midline axon guidance.

Several synchronous locomotor phenotypes in mice have been associated with mutations involving midline axon guidance molecules, including Netrin1 and ephrinB3. The absence of the midline chemo-attractant Netrin1 leads to a complete switch from alternating to synchronous fictive locomotor activity (Rabe et al., 2009). Further, mutations in either the ligand ephrinB3 or the receptor EphA4 cause abnormal midline crossing of corticospinal axons and interneuron axons in the CPG, resulting in mice with a characteristic hopping gait phenotype (Kullander et al., 2003). To assess the expression of axon guidance molecules in the midline of *hop* mice, we performed *in-situ* hybridization experiments on E15.5 and E12.5 spinal cord tissue with *Netrin1* and *EphrinB3* mRNA probes. The results showed that *Netrin1* expression levels were severely reduced in the lumbar ventral spinal cord of *hop* mice, with a clear loss in the floorplate itself whereas remaining expression could be observed in the ventricular area. *EphrinB3* expression levels were also severely reduced in the ventral, but not dorsal spinal cord (Fig. 5I-R). These data suggest an early axon guidance defect in the ventral part of the spinal cord, which could at least partly explain the physiological phenotype in *hop* mice. However, guidance of commissural axons is not entirely dependent of floor-plate-derived Netrin1 (Moreno-Bravo et al., 2019), so the loss of Netrin1 in *hop* mice may relate more to earlier morphogenic events.

Abnormal Shh and lumbar spinal cord patterning in *hop* mice

Next, we explored the expression of Shh, a key regulator of early development. Immunohistochemistry on E12.5 tissue with a Shh antibody revealed that the distance between the notochord and ventral spinal cord border was clearly decreased (Fig. 6A,B,G), and that Shh levels were reduced in both the notochord and spinal cord of *hop* mice (Fig. 6H). Strikingly, in the ventral spinal cord of *hop* mice, Shh expression was almost absent, likely as a result of a missing floorplate (Fig. 6A'',B''). Moreover, notochord morphology was

559 abnormal displaying disorganised cells and low Shh expression (Fig. 6A',B'). Furthermore,
 560 Shh levels were also reduced in *hop* mutant embryos at E10.5 (Fig. 6C,D).

561 A point mutation in the *Ttc26* gene was found in a previous study of *hop* mice
 562 (Swiderski et al., 2014). *Ttc26* has been classified as an intraflagellar transport (IFT) complex
 563 B protein (Follit et al., 2009). Studies conducted on other IFT mutants have revealed
 564 phenotypic defects similar to those in the *hop* mutant including ventral spinal cord patterning
 565 defects, loss of ventral cell types and polydactyly (Haycraft et al., 2007; Huangfu et al., 2003;
 566 Liu et al., 2005). Therefore, we evaluated protein expression of TTC26 using
 567 immunohistochemical analysis and antibodies against TTC26 in the early developing spinal
 568 cord (Fig. 6E,F). Despite the apparent improper shape and positioning of the notochord at
 569 E12.5 we found, that the truncated TTC26 protein was still detectable in *hop* mice (Fig. 6F,I).

570 Loss of Shh in mice results in degeneration of the notochord, absence of a
 571 morphologically distinct floorplate and abnormal dorsoventral patterning (Chiang et al.,
 572 1996). Consequently, we analyzed embryonic spinal cords of *hop* mice using antibodies
 573 against transcription factors as markers for specific developmental subpopulations to assess
 574 patterning defects (Fig. 6J-V, Fig. 6-1 (Gross et al., 2002; Moran-Rivard et al., 2001)). Most
 575 striking, this analysis revealed that the ventral most V3 progenitor domain labeled by Nkx2.2,
 576 although present in controls was undetectable in *hop* mice (Fig. 6 J,K). The complete loss of
 577 Nkx2.2 positive cells in *hop* mice was restricted to the lumbar level and was found to be less
 578 apparent with increasing distance in both rostral and caudal directions and again, these defects
 579 were not found in the thoracic region (Fig. 6V). Further, we used Brn3a to examine the dorsal
 580 subpopulations dI1– dI3 and dI5 interneurons, which were largely unaffected, except in the
 581 most ventral regions (Fig. 6L,M; S4A,F). Similarly, the dI4 – dI6 subpopulations labelled by
 582 Lbx1 (Fig. 6N,O; S4B,G) and the dI4, dI6, p0 and p4 subpopulations labelled by Pax2 (Fig.
 583 6P,Q; S4C,H) were present and appeared normal, except for the ventral-most labelled cells,

584 which were found in close vicinity to the midline in *hop* mice. Markers for ventral V0v cells
 585 labelled by *Evx1* and for dI3 and motor neurons labelled with *Isl1/2* identified cells residing
 586 on and close to the ventral midline in *hop* mice (Fig. 6S,U; S4I,J), which was not seen in
 587 controls (Fig. 6R,T; S4D,E). Thus, our results show that there is an early patterning defect
 588 specific to the lumbar spinal cord of *hop* mice, likely originating from a defect in the
 589 notochord early organizer.

591 **Validation of the *hop* mutation**

592 In the *hop* mouse, defects that are associated with early developmental patterning and *Shh*
 593 signalling are apparent, supporting the hypothesis that the identified point mutation in *Ttc26* is
 594 the causative mutation, and in line with a previous study (Swiderski et al., 2014). Our whole
 595 exome sequencing of a *hop* mouse and of *Ttc26* exon 15 in several heterozygous and
 596 homozygous individuals confirmed a C to A transversion at position 38,412,065 bp causing a
 597 premature stop codon and a truncated form of the TTC26 protein (Fig. 7-1). However, TTC26
 598 truncated protein remains expressed and it is possible that other mutations are present in
 599 neighboring genes, which may cause the observed phenotype. In the *hop* mutation area and in
 600 the close vicinity to the *Ttc26* gene, genes that have been associated with gait defects can be
 601 found. For example, *Hipk2* has been associated to TGF-beta signaling, another influential
 602 spinal cord morphant that plays an essential role in the regulation of survival in the nervous
 603 system (Doxakis et al., 2004; Isono et al., 2006; Zhang et al., 2007). Ablation of *Hipk2*
 604 generated mice with an ataxic-like phenotype implying that the *Hipk2* gene may contribute to
 605 the *hop* phenotype (Anzilotti et al., 2015).

606 We sought to exclude such additional possible causative mutations and determine
 607 whether the underlying genetic cause in the *hop* mouse is solely due to the identified mutation
 608 in *Ttc26*. Using clustered regularly interspaced short palindromic repeat/CRISPR-associated 9
 26

(CRISPR/Cas9) technology, we introduced a point mutation within *Ttc26* exon 15 at position 38,412,065 bp in wild type mice (Fig 7A). The generated CRISPR *Ttc26*^{Y430X} mutant mice displayed polydactyly of all feet (Fig. 7B,C), highly reminiscent of *hop* mice. However, whereas previous reports found a partial lethality between E10.5 and birth for *hop* mutant mice, we failed to receive live born homozygous *Ttc26*^{Y430X} pups (Bryan et al., 1977). Among 67 delivered pups from 9 heterozygous breedings, we received one live-born homozygous pup that died early. We therefore performed our phenotypic analysis of embryonic stages.

CRISPR *Ttc26*^{Y430X} mutant embryos showed a spinal cord phenotype similar to the *hop* mice (Fig. 7B-H). Ventral spinal cord morphology matched the *hop* mice, with a poorly delineated and dorsally shifted central canal (Fig. 7E,F). Furthermore, like in *hop* mutants, defects were regionalized to the lumbar spinal cord with the cervical region unaffected (Fig. 7D). NeuN immunolabeling to identify neurons corroborated that the border between the white and grey matter was poorly defined, as was found in *hop* mutants (Fig. 7G, H). There were also less myelinated structures and misplaced neurons within the white matter, resulting in an absent ventral funiculus (Fig. 7G). These findings demonstrate that the spinal cord phenotype found in *hop* mice was mirrored in *Ttc26*^{Y430X} mice, at least for embryonic stages. Thus, the C-to-A point mutation in the *Ttc26*-gene, exon 15 is likely the sole cause for the observed spinal cord defects in *hop* mice.

Finally, we sought to investigate how two populations of importance for locomotion were affected: motor neurons and a portion of the dI6 inhibitory interneurons defined by *Dmrt3* expression (Andersson et al., 2012; Perry et al., 2019). Immunohistochemistry using primary antibodies against vesicular acetylcholine transporter (VACHT) were used to label motor neurons in the lumbar part of *Ttc26*^{Y430X} and control spinal cords at E16.5. Quantification gave a mean value of 19.6 ± 9.4 motor neurons per hemi-section (51 hemisections counted) in the *Ttc26*^{Y430X} spinal cord and 20.6 ± 9.3 motor neurons per hemi-

634 section (57 hemisections counted) in the control, which was not significantly different
635 (independent-samples Student's t-test; $p=0.60$). Examination of the motor neuron distribution
636 in the lumbar region of the $Ttc26^{Y430X}$ spinal cord revealed two distinct populations on each
637 side of the midline, similar to controls (Fig 7I, J). This indicated that, despite the midline
638 fusion in the ventral spinal cord, the left and right populations of motor neurons remained
639 separate, as was also found in the adult *hop* mutants (Fig. 3). In contrast, central canal
640 cholinergic cells, that are normally separated by the midline, was distributed closer to and
641 even on the midline in the $Ttc26^{Y430X}$ spinal cord (Fig 7I',J'). We next explored the
642 distribution of dI6 interneurons using anti-Dmrt3 antibodies. As expected, in controls they
643 were located medioventrally in the spinal cord and formed two distinct populations on each
644 side of the midline. However, in $Ttc26^{Y430X}$ spinal cords, the Dmrt3 positive cells were
645 clustered on or close to the midline and positioned further ventrally (Fig. 7K,L,K',L').

646

647 **Discussion**

648 Here we analyzed *hop* mice, focusing on the underlying cause for their aberrant locomotor
649 phenotype. Behavioral tests and fictive locomotion experiments showed that *hop* mice move
650 using their hindlimbs in synchrony. Our developmental studies revealed an abnormal
651 notochord, a missing floorplate and the absence of a Nkx2.2 progenitor domain in *hop* mice
652 as well as a disorganized dorsoventral patterning with neurons residing on the ventral midline.
653 Further, we found reduced expression of the axon guidance molecules netrin-1 and ephrinB3.
654 By verifying the causative mutation in *Ttc26*, we revealed its important role for the formation
655 of a lumbar locomotor network containing two separate CPG half-centers.

657 **Lack of ventral spinal cord midline explains the synchronous gait**

658 According to the half-centre model of the spinal locomotor CPG proposed by Graham-Brown
659 (Graham-Brown T, 1911), the rhythmic pattern of alternating bursts of flexor and extensor
660 muscles is produced by neuronal populations in each half of the spinal cord. Commissural
661 projections by inhibitory and excitatory neurons ensure normal left-right coordination (Kiehn,
662 2006). After cutting the ventral commissure the normal left-right circuit disappears indicating
663 that ventromedially located CINs are indispensable for the bilateral coordination (Kjaerulff
664 and Kiehn, 1996; Restrepo et al., 2009). Our morphological studies of the spinal cord in *hop*
665 mice revealed defects in the ventral spinal cord, including an absent midline and a loosely
666 defined white/grey matter border. The disturbed expression of axon guidance molecules in
667 *hop* mice are likely to underlie the observed aberrant fibres of short-, long- and locally-
668 projecting INs. Furthermore, their cell bodies were spread on the ventral midline compared to
669 the two separate ventromedial clusters of commissural INs seen in control mice (Fig. 4). Also,

670 both excitatory and inhibitory neurons were found to reside on the ventral midline (Fig. 3).
671 Midline defects were discernable as early as E12.5, where we found that ventral neuronal
672 progenitors in the lumbar spinal cord were located where the midline should have been (Fig.
673 6). These results indicate that in *hop* mice, the neurons that normally form two separate CPG
674 half-centres, instead intermingle. This would cause a functionally fused CPG center,
675 explaining the observed synchronous gait.

676 Expression of pre-pattern genes along the dorsoventral axis of the spinal cord is
677 controlled by long range signals from the opposite poles of the neural tube. *Sonic Hedgehog*,
678 *Gli3* and *Smoothed* are among the most important molecules for ventral cell differentiation,
679 with the most ventral progenitor domain requiring the highest concentration of *Shh* to develop
680 normally (Briscoe and Ericson, 1999; Placzek, 1995). Already at E10.5, the most ventral
681 Nkx2.2 progenitor domain, which gives rise to V3 neurons, was absent in the *hop* mice. V3
682 neurons are one of the major classes of excitatory commissural interneurons in the mouse
683 spinal cord and are components of the locomotor network, with a role in establishing a stable
684 and balanced locomotor rhythm (Danner et al., 2019; Zhang et al., 2008).

685 Motor neuron populations, however, were not fused at the midline in *hop* mice.
686 Previous studies have shown that the upper edge of the Islet1 expressing motor column is
687 dorsally shifted in Netrin-1 mutant spinal cords at E10 (Kim et al., 2015). In *hop* mice, at
688 E12.5, cells expressing Islet1 were dispersed dorsally and clustered together on the ventral
689 midline (Fig. 6-1). Likewise, Robo mutant embryos (E9.5-E10.5) have a pack of misplaced
690 Islet1⁺ motor neurons within the floorplate. However, the ectopic Islet1⁺ cells of Robo
691 mutants are no longer visible in the floorplate by E12.5. Our study of the *Ttc26*^{Y430X} spinal
692 cords at E16.5 suggest that decreased levels of Netrin-1 in fact do affect the initial positioning
693 of motor neurons, but that compensatory mechanisms then correct the mis-placing before
694 birth. We did not find a significant difference between the number of motor neurons in
30

695 *Ttc26*^{Y430X} and control spinal cords, which indicates that the misplaced neurons most likely
 696 migrate back to their correct location, rather than undergo apoptosis. In addition to
 697 Semaphorin and Slit signalling governed by the transcription factors Islet1 and Islet2 (Lee et
 698 al., 2015; Mauti et al., 2007), motor neuron positioning is regulated by Reelin and Cadherin
 699 signalling causing a secondary reorganization of motor columns into sub-clusters consisting
 700 of motor pools (Demireva et al., 2011; Palmesino et al., 2010; Price et al., 2002). The
 701 migration and positioning of motor neurons seems to be more strictly regulated than the
 702 positioning of CINs, possibly explaining why motor neurons are not clustered on the ventral
 703 midline of *hop* and *Ttc26*^{Y430X} spinal cords. Further studies are needed to understand the exact
 704 underlying mechanisms for the observed differences between motor neuron and interneuron
 705 development in the ventral spinal cord.

706

707 **Rostrocaudal specificity of the midline fusion**

708 In addition to the previously reported characteristic hopping gait in *hop* mice (Johnson and
 709 Hunt, 1971; Wojnowski et al., 1998), our gait analysis showed that adult *hop* mice alternated
 710 their forelimbs while they moved their hind limbs in synchrony. Further, we found that the
 711 pattern of flexor-extensor alternation during fictive locomotion was maintained following
 712 hemisection, suggesting that the circuitry coordinating ipsilateral alternation is intact in *hop*
 713 mutants (Fig. 1-1). However, we did observe a tendency for asymmetry in flexor and extensor
 714 alternation (Fig. 1H,1-1A), suggesting that a bias toward extension exists, possibly due to a
 715 reduction in inhibitory drive from the flexor-related module of the CPG (Kiehn et al., 2008).
 716 This may be due to a reduced number of inhibitory descending CINs, or alternatively,
 717 ipsilateral flexor inhibitory projections onto extensor motoneurons. Even if we found a
 718 reduced number of traceable intersegmental CINs, both these scenarios remain possible and
 719 are not mutually exclusive. These findings suggest that the defect behind the characteristic

720 hopping phenotype is limited to the lumbar spinal cord controlling the left-right alternation of
 721 the hind limbs, and possibly descending INs that affect coordination. This is in accordance
 722 with the lumbar specific defects observed in *Ttc26* mutant mice early in development.

723 What might be the possible explanation for the rostro-caudal specificity of the defect?

724 Primary cilia and Shh signalling are critical for proper patterning of the neural tube along the
 725 dorsoventral axis (Dessaud et al., 2008; Tarchini et al., 2006), and TTC26 is required for
 726 accumulation of Gli at the ciliary tip (Swiderski et al., 2014). In Shh KO mice, motor neurons
 727 are not generated, however, in Shh and Gli3 double KO mice there is a rescue effect where
 728 motor neurons are generated predominantly in the lumbar region (Litingtung and Chiang,
 729 2000). This substantiates the involvement of the Shh signaling pathway in differential
 730 development along the rostral–caudal axis, possibly due to an unknown caudal factor such as
 731 retinoic acid (Litingtung and Chiang, 2000). Interestingly, missense mutations in the *ttc26*
 732 gene are more frequent in the rabbit compared to other mammals with alternating gaits (Fig.
 733 7-2A). To investigate if rabbits might have a rearranged lumbar region in their spinal cord
 734 explaining their synchronous hind limb gait, we analyzed rabbit tissue. There were, however,
 735 no signs of ventral fusion in the postnatal lumbar spinal cord (Fig. 7-2B) and spinal neuron
 736 populations were clearly separated (Fig. 7-2C-G), suggesting that mutations in *Ttc26* are not
 737 underlying the synchronous gait in rabbits.

738

739 **The mutation in *Ttc26* is causative for the spinal cord fusion**

740 Shh is crucial for induction of both floorplate and dorsoventral patterning of the spinal cord
 741 (Chiang et al., 1996; Patten and Placzek*, 2000; Placzek, 1995), and in particular, defective
 742 secretion of Shh can result in the spinal cord developing without floorplate cells and V3
 743 interneurons (Iulianella et al., 2018). *Shh* mutant mice have several defects in midline
 744 structures such as the notochord and cyclopia (Boije et al., 2012). A similar spinal cord

phenotype is present in HNF-3 β null mutant mice. HNF-3 β is a transcription factor expressed in the three midline organizing centers; the node, notochord and floorplate (Ang and Rossant, 1994). Similar to Swiderski et al., 2014, our positional cloning analysis localized the *hop* mutation to *Ttc26*. TTC26, a component of Intraflagellar Transport (IFT) complex B, is a structure necessary for cilia formation and protein transport, and is important for Shh signaling. In addition, Ttc26 (also named IFT56) regulates vertebrate developmental patterning by maintaining IFTB complex integrity and ciliary microtubule architecture (Xin et al., 2017). Moreover, cilia motility is important for embryonic left-right determination (Nonaka et al., 1998). Although it is likely that the found mutation is the causative one, we noticed some differences between our analysis and the Swiderski et al. (2014) study. The spinal cord ventralisation was found in both studies, but additionally, we found midline fusion defects as well as lack of the ventral midline and Nkx2.2 positive V3 progenitors in the lumbar spinal cord. These differences opened the possibility of other additional mutations in the *hop* mice. For example, *Gli1*^{-/-}; *Gli2*^{+/-} mice have a hopping gait (Park et al., 2000), and *Gli3*^{+/-} mice have preaxial polydactyly (Hui and Joyner, 1993; Park et al., 2000). In addition, a similar distortion of notochord, midline fusion (E14) and missing Nkx2.2 domain has been observed in *Gli1/2* double mutants. Also, in *Gli2*^{-/-} mouse embryos, the most ventral cells (the floor plate) are not specified (Park et al., 2000).

The lower expression levels of Shh seen in *hop* mice could also be caused by mutations in upstream regulators of Shh, events that could take place earlier in development. As mentioned above, the HOX1 cluster, located on cytoband B3-C >25.40 cM in the vicinity of the *hop* region, could be affected in the *hop* mouse mutation. Mice with homozygous mutations in one or multiple members of the cluster display a range of phenotypes including developmental defects in the skeletal, reproductive and nervous systems (Raines et al., 2013).

Two other genes in the *hop* region are associated to gait defects or could explain a fused spinal cord midline. When the *Braf* gene is mutated it has been reported to cause developmental deficits such as craniofacial dysmorphism and extra digits (Moriya et al., 2015). Mice with a targeted mutation in the transcriptional cofactor homeodomain interacting protein kinase 2 (HIPK2) display abnormal gaits including shuffling, reduced width and short stride length (Zhang et al., 2007). For this reason, we introduced a point mutation in the *Ttc26* gene, which produced a premature stop codon mimicking the *hop* mouse mutation. Unfortunately, potentially due to different genetic backgrounds, unlike *hop* mutants, *Ttc26*^{Y430X} mutants displayed fully penetrant embryonic lethality why locomotor studies could not be performed. However, *Ttc26*^{Y430X} mutant mice displayed the same spinal cord ventral midline fusion as was found in *hop* mice, suggesting that this point mutation can sufficiently explain the observed mutant phenotype.

Conclusion

Our findings suggest that the aberrant spinal cord phenotype observed in *hop* mice is the result of abnormal developmental processes, including induction from the notochord and Shh signalling. Despite the loss of the ventral midline, functional neuronal networks develop and form connections able to produce coordinated, albeit synchronous, activity. Our data support the two half-centre hypothesis by demonstrating that midline separation is crucial for left-right alternation of locomotion. To our knowledge, the *hop* mutant is the first model with a specific ventral spinal cord fusion, in which the components of the CPG can be studied and understood.

793

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803 **References**

- 804 Ackerman, S.L., Kozak, L.P., Przyborski, S.A., Rund, L.A., Boyer, B.B., and Knowles,
805 B.B. (1997). The mouse rostral cerebellar malformation gene encodes an UNC-5-like
806 protein. *Nature* 386, 838–842.
- 807 Akay, T., McVea, D.A., Tachibana, A., and Pearson, K.G. (2006). Coordination of fore
808 and hind leg stepping in cats on a transversely-split treadmill. *Exp Brain Res* 175, 211–
809 222.
- 810 Andersson, L.S., Larhammar, M., Memic, F., Wootz, H., Schwochow, D., Rubin, C.-J.,
811 Patra, K., Arnason, T., Wellbring, L., Hjälml, G., et al. (2012). Mutations in *DMRT3*
812 affect locomotion in horses and spinal circuit function in mice. *Nature* 488, 642–646.
- 813 Ang, S.L., and Rossant, J. (1994). *HNF-3 beta* is essential for node and notochord
814 formation in mouse development. *Cell* 78, 561–574.
- 815 Anzilotti, S., Tornincasa, M., Gerlini, R., Conte, A., Brancaccio, P., Cuomo, O., Bianco,
816 G., Fusco, A., Annunziato, L., Pignataro, G., et al. (2015). Genetic ablation of
817 homeodomain-interacting protein kinase 2 selectively induces apoptosis of cerebellar
818 Purkinje cells during adulthood and generates an ataxic-like phenotype. *Cell Death Dis*
819 6, e2004.
- 820 Baddeley, A., and Turner, R. (2005). *spatstat* : An R Package for Analyzing Spatial Point
821 Patterns. *J. Stat. Soft.* 12.
- 822 Bannatyne, B.A., Edgley, S.A., Hammar, I., Jankowska, E., and Maxwell, D.J. (2003).
823 Networks of inhibitory and excitatory commissural interneurons mediating crossed
824 reticulospinal actions. *Eur. J. Neurosci.* 18, 2273–2284.
- 825 Boije, H., Harun-Or-Rashid, M., Lee, Y.-J., Imsland, F., Bruneau, N., Vieaud, A.,
826 Gourichon, D., Tixier-Boichard, M., Bed'hom, B., Andersson, L., et al. (2012). Sonic
827 Hedgehog-signalling patterns the developing chicken comb as revealed by exploration of
828 the pea-comb mutation. *PLoS ONE* 7, e50890.
- 829 Bracci, E., Ballerini, L., and Nistri, A. (1996). Localization of rhythmogenic networks
830 responsible for spontaneous bursts induced by strychnine and bicuculline in the rat
831 isolated spinal cord. *J. Neurosci.* 16, 7063–7076.
- 832 Branchereau, P., Morin, D., Bonnot, A., Ballion, B., Chapron, J., and Viala, D. (2000).
833 Development of lumbar rhythmic networks: from embryonic to neonate locomotor-like
834 patterns in the mouse. *Brain Res. Bull.* 53, 711–718.
- 835 Briscoe, J., and Ericson, J. (1999). The specification of neuronal identity by graded
836 Sonic Hedgehog signalling. *Semin. Cell Dev. Biol.* 10, 353–362.
- 837 Bryan, J.H.D., Hughes, R.L., and Bates, T.J. (1977). Brain development in
838 hydrocephalic-polydactyl, a recessive pleiotropic mutant in the mouse. *Virchows Arch. A*
839 *Path. Anat. and Histol.* 374, 205–214.

- 840 Butt, S.J.B., and Kiehn, O. (2003). Functional identification of interneurons responsible
841 for left-right coordination of hindlimbs in mammals. *Neuron* 38, 953–963.
- 842 Chiang, C., Litington, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., and Beachy,
843 P.A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog
844 gene function. *Nature* 383, 407–413.
- 845 Choi, M., Scholl, U.I., Ji, W., Liu, T., Tikhonova, I.R., Zumbo, P., Nayir, A., Bakkaloğlu,
846 A., Ozen, S., Sanjad, S., et al. (2009). Genetic diagnosis by whole exome capture and
847 massively parallel DNA sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19096–19101.
- 848 Cowley, K.C., and Schmidt, B.J. (1995). Effects of inhibitory amino acid antagonists on
849 reciprocal inhibitory interactions during rhythmic motor activity in the in vitro neonatal
850 rat spinal cord. *J. Neurophysiol.* 74, 1109–1117.
- 851 Crone, S.A., Quinlan, K.A., Zagoraïou, L., Droho, S., Restrepo, C.E., Lundfald, L., Endo,
852 T., Setlak, J., Jessell, T.M., Kiehn, O., et al. (2008). Genetic ablation of V2a ipsilateral
853 interneurons disrupts left-right locomotor coordination in mammalian spinal cord.
854 *Neuron* 60, 70–83.
- 855 Crone, S.A., Zhong, G., Harris-Warrick, R., and Sharma, K. (2009). In mice lacking V2a
856 interneurons, gait depends on speed of locomotion. *J. Neurosci.* 29, 7098–7109.
- 857 Danner, S.M., Zhang, H., Shevtsova, N.A., Borowska-Fielding, J., Deska-Gauthier, D.,
858 Rybak, I.A., and Zhang, Y. (2019). Spinal V3 Interneurons and Left-Right Coordination
859 in Mammalian Locomotion. *Front Cell Neurosci* 13, 516.
- 860 Demireva, E.Y., Shapiro, L.S., Jessell, T.M., and Zampieri, N. (2011). Motor neuron
861 position and topographic order imposed by β - and γ -catenin activities. *Cell* 147, 641–
862 652.
- 863 Dessaud, E., McMahon, A.P., and Briscoe, J. (2008). Pattern formation in the vertebrate
864 neural tube: a sonic hedgehog morphogen-regulated transcriptional network.
865 *Development* 135, 2489–2503.
- 866 Doxakis, E., Huang, E.J., and Davies, A.M. (2004). Homeodomain-interacting protein
867 kinase-2 regulates apoptosis in developing sensory and sympathetic neurons. *Curr. Biol.*
868 14, 1761–1765.
- 869 Drew, T., and Doucet, S. (1991). Application of circular statistics to the study of
870 neuronal discharge during locomotion. *J. Neurosci. Methods* 38, 171–181.
- 871 Eide, A.L., and Glover, J.C. (1995). Development of the longitudinal projection patterns
872 of lumbar primary sensory afferents in the chicken embryo. *J. Comp. Neurol.* 353, 247–
873 259.
- 874 Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G.,
875 Stoeckli, E.T., Keino-Masu, K., Masu, M., Rayburn, H., et al. (1997). Phenotype of mice
876 lacking functional Deleted in colorectal cancer (*Dcc*) gene. *Nature* 386, 796–804.

- 877 Follit, J.A., Xu, F., Keady, B.T., and Pazour, G.J. (2009). Characterization of mouse IFT
878 complex B. *Cell Motil. Cytoskeleton* 66, 457–468.
- 879 Gordon, I.T., and Whelan, P.J. (2006). Deciphering the organization and modulation of
880 spinal locomotor central pattern generators. *J. Exp. Biol.* 209, 2007–2014.
- 881 Graham-Brown T (1911). The intrinsic factors in the act of progression in the mammal.
882 *Proc. R. Soc. Lond. B* 84, 308–319.
- 883 Gross, M.K., Dottori, M., and Goulding, M. (2002). *Lbx1* specifies somatosensory
884 association interneurons in the dorsal spinal cord. *Neuron* 34, 535–549.
- 885 Hammar, I., Bannatyne, B.A., Maxwell, D.J., Edgley, S.A., and Jankowska, E. (2004).
886 The actions of monoamines and distribution of noradrenergic and serotonergic
887 contacts on different subpopulations of commissural interneurons in the cat spinal cord.
888 *Eur. J. Neurosci.* 19, 1305–1316.
- 889 Haycraft, C.J., Zhang, Q., Song, B., Jackson, W.S., Detloff, P.J., Serra, R., and Yoder,
890 B.K. (2007). Intraflagellar transport is essential for endochondral bone formation.
891 *Development* 134, 307–316.
- 892 Hinckley, C., Seebach, B., and Ziskind-Conhaim, L. (2005). Distinct roles of glycinergic
893 and GABAergic inhibition in coordinating locomotor-like rhythms in the neonatal mouse
894 spinal cord. *Neuroscience* 131, 745–758.
- 895 Hollander, W. (1976). Hydrocephalic-polydactyl, a recessive pleiotropic mutant in the
896 mouse and its location in chromosome 6. *Subject Strain Bibliography* 1976 13–23.
- 897 Huangfu, D., Liu, A., Rakeman, A.S., Murcia, N.S., Niswander, L., and Anderson, K.V.
898 (2003). Hedgehog signalling in the mouse requires intraflagellar transport proteins.
899 *Nature* 426, 83–87.
- 900 Hui, C.C., and Joyner, A.L. (1993). A mouse model of greig cephalopolysyndactyly
901 syndrome: the extra-toesJ mutation contains an intragenic deletion of the *Gli3* gene. *Nat.*
902 *Genet.* 3, 241–246.
- 903 Inui, M., Miyado, M., Igarashi, M., Tamano, M., Kubo, A., Yamashita, S., Asahara, H.,
904 Fukami, M., and Takada, S. (2015). Rapid generation of mouse models with defined point
905 mutations by the CRISPR/Cas9 system. *Sci Rep* 4, 5396.
- 906 Isono, K., Nemoto, K., Li, Y., Takada, Y., Suzuki, R., Katsuki, M., Nakagawara, A., and
907 Koseki, H. (2006). Overlapping roles for homeodomain-interacting protein kinases *hipk1*
908 and *hipk2* in the mediation of cell growth in response to morphogenetic and genotoxic
909 signals. *Mol. Cell. Biol.* 26, 2758–2771.
- 910 Iulianella, A., Sakai, D., Kurosaka, H., and Trainor, P.A. (2018). Ventral neural
911 patterning in the absence of a *Shh* activity gradient from the floorplate. *Dev. Dyn.* 247,
912 170–184.

- 913 Jankowska, E. (2008). Spinal interneuronal networks in the cat: elementary components.
914 *Brain Res Rev* 57, 46–55.
- 915 Johnson, D.R., and Hunt, D.M. (1971). Hop-sterile, a mutant gene affecting sperm tail
916 development in the mouse. *J Embryol Exp Morphol* 25, 223–236.
- 917 Juvin, L., Simmers, J., and Morin, D. (2005). Propriospinal circuitry underlying
918 interlimb coordination in mammalian quadrupedal locomotion. *J. Neurosci.* 25, 6025–
919 6035.
- 920 Kiehn, O. (2006). Locomotor circuits in the mammalian spinal cord. *Annu. Rev.*
921 *Neurosci.* 29, 279–306.
- 922 Kiehn, O., Quinlan, K.A., Restrepo, C.E., Lundfald, L., Borgius, L., Talpalar, A.E., and
923 Endo, T. (2008). Excitatory components of the mammalian locomotor CPG. *Brain Res*
924 *Rev* 57, 56–63.
- 925 Kim, M., Fontelonga, T., Roesener, A.P., Lee, H., Gurung, S., Mendonca, P.R.F., and
926 Mastick, G.S. (2015). Motor neuron cell bodies are actively positioned by Slit/Robo
927 repulsion and Netrin/DCC attraction. *Dev. Biol.* 399, 68–79.
- 928 Kjaerulff, O., and Kiehn, O. (1996). Distribution of networks generating and
929 coordinating locomotor activity in the neonatal rat spinal cord in vitro: a lesion study. *J.*
930 *Neurosci.* 16, 5777–5794.
- 931 Kong, Y. (2011). Btrim: a fast, lightweight adapter and quality trimming program for
932 next-generation sequencing technologies. *Genomics* 98, 152–153.
- 933 Kullander, K., Croll, S.D., Zimmer, M., Pan, L., McClain, J., Hughes, V., Zabski, S.,
934 DeChiara, T.M., Klein, R., Yancopoulos, G.D., et al. (2001). Ephrin-B3 is the midline
935 barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral
936 motor control. *Genes Dev.* 15, 877–888.
- 937 Kullander, K., Butt, S.J.B., Lebre, J.M., Lundfald, L., Restrepo, C.E., Rydström, A.,
938 Klein, R., and Kiehn, O. (2003). Role of EphA4 and EphrinB3 in local neuronal circuits
939 that control walking. *Science* 299, 1889–1892.
- 940 Lanuza, G.M., Gosgnach, S., Pierani, A., Jessell, T.M., and Goulding, M. (2004). Genetic
941 identification of spinal interneurons that coordinate left-right locomotor activity
942 necessary for walking movements. *Neuron* 42, 375–386.
- 943 Lee, H., Kim, M., Kim, N., Macfarlan, T., Pfaff, S.L., Mastick, G.S., and Song, M.-R.
944 (2015). Slit and Semaphorin signaling governed by Islet transcription factors positions
945 motor neuron somata within the neural tube. *Exp. Neurol.* 269, 17–27.
- 946 Lemieux, M., Laflamme, O.D., Thiry, L., Boulanger-Piette, A., Frenette, J., and Bretzner,
947 F. (2016). Motor hypertonia and lack of locomotor coordination in mutant mice lacking
948 DSCAM. *J. Neurophysiol.* 115, 1355–1371.

- 949 Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-
950 Wheeler transform. *Bioinformatics* 25, 1754–1760.
- 951 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G.,
952 Abecasis, G., Durbin, R., and 1000 Genome Project Data Processing Subgroup (2009).
953 The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079.
- 954 Litingtung, Y., and Chiang, C. (2000). Specification of ventral neuron types is mediated
955 by an antagonistic interaction between *Shh* and *Gli3*. *Nat. Neurosci.* 3, 979–985.
- 956 Liu, A., Wang, B., and Niswander, L.A. (2005). Mouse intraflagellar transport proteins
957 regulate both the activator and repressor functions of *Gli* transcription factors.
958 *Development* 132, 3103–3111.
- 959 Lundfald, L., Restrepo, C.E., Butt, S.J.B., Peng, C.-Y., Droho, S., Endo, T., Zeilhofer,
960 H.U., Sharma, K., and Kiehn, O. (2007). Phenotype of V2-derived interneurons and their
961 relationship to the axon guidance molecule *EphA4* in the developing mouse spinal cord.
962 *Eur. J. Neurosci.* 26, 2989–3002.
- 963 Lyon, M. (1973). Further data locating *hpy* in Linakge Group XI. *Mouse News Lett.* 49.
- 964 Madriaga, M.A., McPhee, L.C., Chersa, T., Christie, K.J., and Whelan, P.J. (2004).
965 Modulation of locomotor activity by multiple 5-HT and dopaminergic receptor subtypes
966 in the neonatal mouse spinal cord. *J. Neurophysiol.* 92, 1566–1576.
- 967 Mauti, O., Domanitskaya, E., Andermatt, I., Sadhu, R., and Stoeckli, E.T. (2007).
968 *Semaphorin6A* acts as a gate keeper between the central and the peripheral nervous
969 system. *Neural Dev* 2, 28.
- 970 Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M.K., Burrill, J., and Goulding,
971 M. (2001). *Evx1* is a postmitotic determinant of v0 interneuron identity in the spinal
972 cord. *Neuron* 29, 385–399.
- 973 Moreno-Bravo, J.A., Roig Puiggros, S., Mehlen, P., and Chédotal, A. (2019). Synergistic
974 Activity of Floor-Plate- and Ventricular-Zone-Derived *Netrin-1* in Spinal Cord
975 Commissural Axon Guidance. *Neuron* 101, 625-634.e3.
- 976 Moriya, M., Inoue, S.-I., Miyagawa-Tomita, S., Nakashima, Y., Oba, D., Niihori, T.,
977 Hashi, M., Ohnishi, H., Kure, S., Matsubara, Y., et al. (2015). Adult mice expressing a
978 *Braf* Q241R mutation on an ICR/CD-1 background exhibit a cardio-facio-cutaneous
979 syndrome phenotype. *Hum. Mol. Genet.* 24, 7349–7360.
- 980 Nakayama, K., Nishimaru, H., and Kudo, N. (2002). Basis of Changes in Left–Right
981 Coordination of Rhythmic Motor Activity during Development in the Rat Spinal Cord. *J.*
982 *Neurosci.* 22, 10388–10398.
- 983 Nishimaru, H., and Kudo, N. (2000). Formation of the central pattern generator for
984 locomotion in the rat and mouse. *Brain Res. Bull.* 53, 661–669.

- 985 Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M., and
986 Hirokawa, N. (1998). Randomization of left-right asymmetry due to loss of nodal cilia
987 generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein.
988 *Cell* 95, 829–837.
- 989 Norreel, J.-C., Pflieger, J.-F., Pearlstein, E., Simeoni-Alias, J., Clarac, F., and Vinay, L.
990 (2003). Reversible Disorganization of the Locomotor Pattern after Neonatal Spinal Cord
991 Transection in the Rat. *J. Neurosci.* 23, 1924–1932.
- 992 Páez, P., Báltiz, L.-F., Roales-Buján, R., Rodríguez-Pérez, L.-M., Rodríguez, S., Jiménez,
993 A.J., Rodríguez, E.M., and Pérez-Fígares, J.M. (2007). Patterned neuropathologic events
994 occurring in hyh congenital hydrocephalic mutant mice. *J. Neuropathol. Exp. Neurol.* 66,
995 1082–1092.
- 996 Palmesino, E., Rousso, D.L., Kao, T.-J., Klar, A., Laufer, E., Uemura, O., Okamoto, H.,
997 Novitch, B.G., and Kania, A. (2010). *Foxp1* and *Lhx1* Coordinate Motor Neuron
998 Migration with Axon Trajectory Choice by Gating Reelin Signalling. *PLoS Biol* 8,
999 e1000446.
- 1000 Park, H.L., Bai, C., Platt, K.A., Matise, M.P., Beeghly, A., Hui, C.C., Nakashima, M.,
1001 and Joyner, A.L. (2000). Mouse *Gli1* mutants are viable but have defects in *SHH*
1002 signaling in combination with a *Gli2* mutation. *Development* 127, 1593–1605.
- 1003 Patten, I., and Placzek*, M. (2000). The role of Sonic hedgehog in neural tube
1004 patterning: CMLS, *Cell. Mol. Life Sci.* 57, 1695–1708.
- 1005 Perry, S., Larhammar, M., Vieillard, J., Nagaraja, C., Hilscher, M.M., Tafreshiha, A.,
1006 Rofo, F., Caixeta, F.V., and Kullander, K. (2019). Characterization of *Dmrt3*-Derived
1007 Neurons Suggest a Role within Locomotor Circuits. *J. Neurosci.* 39, 1771–1782.
- 1008 Placzek, M. (1995). The role of the notochord and floor plate in inductive interactions.
1009 *Curr. Opin. Genet. Dev.* 5, 499–506.
- 1010 Price, S.R., De Marco Garcia, N.V., Ranscht, B., and Jessell, T.M. (2002). Regulation of
1011 motor neuron pool sorting by differential expression of type II cadherins. *Cell* 109, 205–
1012 216.
- 1013 Pruitt, K.D., Tatusova, T., Klimke, W., and Maglott, D.R. (2009). NCBI Reference
1014 Sequences: current status, policy and new initiatives. *Nucleic Acids Res.* 37, D32–36.
- 1015 Quinlan, K.A., and Kiehn, O. (2007). Segmental, synaptic actions of commissural
1016 interneurons in the mouse spinal cord. *J. Neurosci.* 27, 6521–6530.
- 1017 R Core Team (2021). *R: A language and environment for statistical computing.*
- 1018 Rabe, N., Gezelius, H., Vallstedt, A., Memic, F., and Kullander, K. (2009). *Netrin-1*-
1019 *Dependent Spinal Interneuron Subtypes Are Required for the Formation of Left-Right*
1020 *Alternating Locomotor Circuitry.* *Journal of Neuroscience* 29, 15642–15649.

- 1021 Rabe Bernhardt, N., Memic, F., Gezelius, H., Thiebes, A.-L., Vallstedt, A., and
1022 Kullander, K. (2012). DCC mediated axon guidance of spinal interneurons is essential
1023 for normal locomotor central pattern generator function. *Dev. Biol.* 366, 279–289.
- 1024 Raines, A.M., Adam, M., Magella, B., Meyer, S.E., Grimes, H.L., Dey, S.K., and Potter,
1025 S.S. (2013). Recombineering-based dissection of flanking and paralogous Hox gene
1026 functions in mouse reproductive tracts. *Development* 140, 2942–2952.
- 1027 Restrepo, C.E., Lundfald, L., Szabó, G., Erdélyi, F., Zeilhofer, H.U., Glover, J.C., and
1028 Kiehn, O. (2009). Transmitter-phenotypes of commissural interneurons in the lumbar
1029 spinal cord of newborn mice. *J. Comp. Neurol.* 517, 177–192.
- 1030 Satoh, D., Pudenz, C., and Arber, S. (2016). Context-Dependent Gait Choice Elicited by
1031 EphA4 Mutation in Lbx1 Spinal Interneurons. *Neuron* 89, 1046–1058.
- 1032 Schaeren-Wiemers, N., and Gerfin-Moser, A. (1993). A single protocol to detect
1033 transcripts of various types and expression levels in neural tissue and cultured cells: in
1034 situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* 100, 431–
1035 440.
- 1036 Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Beddington, R., Skarnes, W.C.,
1037 and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in
1038 the developing vertebrate nervous system. *Cell* 87, 1001–1014.
- 1039 Swiderski, R.E., Nakano, Y., Mullins, R.F., Seo, S., and Bánfi, B. (2014). A mutation in
1040 the mouse *ttc26* gene leads to impaired hedgehog signaling. *PLoS Genet.* 10, e1004689.
- 1041 Talpalar, A.E., Bouvier, J., Borgius, L., Fortin, G., Pierani, A., and Kiehn, O. (2013).
1042 Dual-mode operation of neuronal networks involved in left-right alternation. *Nature* 500,
1043 85–88.
- 1044 Tarchini, B., Duboule, D., and Kmita, M. (2006). Regulatory constraints in the evolution
1045 of the tetrapod limb anterior-posterior polarity. *Nature* 443, 985–988.
- 1046 Thiry, L., Lemieux, M., and Bretzner, F. (2018). Age- and speed-dependent modulation of
1047 gaits in DSCAM2J mutant mice. *J. Neurophysiol.* 119, 723–737.
- 1048 Vallstedt, A., and Kullander, K. (2013). Dorsally derived spinal interneurons in
1049 locomotor circuits. *Ann. N. Y. Acad. Sci.* 1279, 32–42.
- 1050 Wallén-Mackenzie, A., Gezelius, H., Thoby-Brisson, M., Nygård, A., Enjin, A., Fujiyama,
1051 F., Fortin, G., and Kullander, K. (2006). Vesicular glutamate transporter 2 is required
1052 for central respiratory rhythm generation but not for locomotor central pattern
1053 generation. *J. Neurosci.* 26, 12294–12307.
- 1054 Wegmeyer, H., Egea, J., Rabe, N., Gezelius, H., Filosa, A., Enjin, A., Varoqueaux, F.,
1055 Deininger, K., Schnütgen, F., Brose, N., et al. (2007). EphA4-dependent axon guidance is
1056 mediated by the RacGAP alpha2-chimaerin. *Neuron* 55, 756–767.

- 1057 Whelan, P., Bonnot, A., and O'Donovan, M.J. (2000). *Properties of rhythmic activity*
1058 *generated by the isolated spinal cord of the neonatal mouse. J. Neurophysiol.* 84, 2821–
1059 2833.
- 1060 Wojnowski, L., Berna, R., Park, C.M., Handel, M.A., Hollander, W.F., and Zimmer, A.
1061 (1998). *Reduced activity of BRAF protein kinase in hop and hop(hpy) mouse mutants.*
1062 *Mamm. Genome* 9, 905–906.
- 1063 Wu, C.-Y., Gagnon, D.A., Sardin, J.S., Barot, U., Telenson, A., Arratia, P.E., and Kalb,
1064 R.G. (2018). *Enhancing GABAergic Transmission Improves Locomotion in a*
1065 *Caenorhabditis elegans Model of Spinal Muscular Atrophy. ENeuro* 5.
- 1066 Xin, D., Christopher, K.J., Zeng, L., Kong, Y., and Weatherbee, S.D. (2017). *IFT56*
1067 *regulates vertebrate developmental patterning by maintaining IFTB complex integrity*
1068 *and ciliary microtubule architecture. Development* 144, 1544–1553.
- 1069 Zagoraiou, L., Akay, T., Martin, J.F., Brownstone, R.M., Jessell, T.M., and Miles, G.B.
1070 (2009). *A cluster of cholinergic premotor interneurons modulates mouse locomotor*
1071 *activity. Neuron* 64, 645–662.
- 1072 Zhang, J., Pho, V., Bonasera, S.J., Holtzman, J., Tang, A.T., Hellmuth, J., Tang, S.,
1073 Janak, P.H., Tecott, L.H., and Huang, E.J. (2007). *Essential function of HIPK2 in*
1074 *TGFbeta-dependent survival of midbrain dopamine neurons. Nat. Neurosci.* 10, 77–86.
- 1075 Zhang, Y., Narayan, S., Geiman, E., Lanuza, G.M., Velasquez, T., Shanks, B., Akay, T.,
1076 Dyck, J., Pearson, K., Gosgnach, S., et al. (2008). *V3 spinal neurons establish a robust*
1077 *and balanced locomotor rhythm during walking. Neuron* 60, 84–96.

1078

1079 **Figure legends**

1080 **Figure 1: Abnormal gait in hind limbs of *hop* mice** (A) Gait analysis painting forelimbs (red) and
1081 hind limbs (blue) show that control mice move their fore- and hind limbs in an alternating pattern
1082 while *hop* mice move their hind limbs in a synchronous manner. Horizontal arrow L is the distance
1083 between the left and right paw and R the distance covered by the same right paw. (B) Graph showing
1084 quantification of walking pattern with the ratio L/R and significant genotype differences for hindlimb
1085 ($p = 0.021$, $r = -0.81$) but not forelimb ($p = 1$, $r = 0$) coordination (n=4 mice per group over a distance
1086 of 30 cm). (C-E) *hop* mice show a characteristic synchronous left-right air-stepping pattern *in vivo*. (C)
1087 Schematic drawing of the experimental setup for recording from neonatal mice. (E,G) Raw EMG
1088 recordings from the left and right tibialis muscles of *hop* (G) and control (E) mice following a tail
1089 pinch stimulus illustrating representative synchronous and alternating traces, respectively (n = 2 mice
1090 per group). The circular plots in (E,G) represent the phase of onset of the left and right tibialis anterior
1091 bursts with respect to each other. Each dot represents an individual burst, while the arrow indicates the
1092 tendency for the bursts to be coupled to each other. A phase value close to zero indicates synchrony in
1093 the case of the *hop* while a value close to 180° suggests an alternating pattern in the case of the control
1094 animals ($p < 0.01$). (D) Schematic illustration of ventral root recording setup measuring fictive
1095 locomotion. (F,H) The *hop* deficit can be reproduced *in vitro* using isolated spinal cord preparations.
1096 Control mice exhibit an alternating (F) and *hop* mice a synchronous (H) left-right pattern (n = 20 mice
1097 per group). Circular plots (F,H) indicate phase values across experiments. Each point equals one 60
1098 second section of data (5 per animal). The length of the arrow provides an index of the strength of the
1099 coupling between the rhythms and the direction equals phase. (I-K) Bath application of GABA and
1100 glycinergic reuptake blockers affect the synchronous rhythm's stability in the *hop* mutant mouse.
1101 Control rhythm in *hop* mice (I) evoked by bath application of NMDA, 5-HT and DA. 20 minutes after
1102 adding nipecotic acid (J), one L2 root would double burst occasionally and at random. After
1103 application of nipecotic acid and sarcosine, occasional alternating segmental activity could be

1104 observed (K), although the ipsilateral L2-L5 rhythm was disrupted. For burst pattern following
1105 hemisection please refer to Fig 1-1. *Significant effect of genotype $p < 0.05$.

1106

1107 **Figure 2: Altered spinal cord morphology in postnatal *hop* mice.** (A) Photomicrographs (ventral
1108 view) and (B) graph of measured sizes of early postnatal (p0-p5) cervical and lumbar spinal cords. At
1109 cervical levels from control and *hop* mice, no difference is apparent ($p = 1$, $r = 0$; n=4 animals per
1110 group). At lumbar levels, the lumbar enlargement of the spinal cord is less prominent in *hop* mice
1111 (black bar) compared to control mice (white bar, $p = 0.009$, $r = -0.83$; n=5 animals per group). The
1112 visible ventral spinal artery in control wild type mice (arrow) is absent from the lumbar part of the *hop*
1113 spinal cord (position noted with star). (C) Photomicrographs of immunohistochemistry staining with
1114 NeuN in red on spinal cord open-book preparations demonstrated lumbar specific defects in *hop* mice.
1115 White boxes indicate areas of higher magnification displayed in C'. (C') Arrows point to the border
1116 between white matter and NeuN positive cells, and stars denote areas where aberrant NeuN positive
1117 cells are found. (D) Fluorescence intensity analysis of NeuN signal within region of interest as
1118 indicated (dotted lines in C') reflects aberrant NeuN positioning in *hop* mice ($p = 0.009$, $r = -0.83$; n=8
1119 per group). (E-H) Immunohistochemistry staining with MBP in green and NeuN in red, coupled to
1120 density plots in (G,H), shows misplaced neurons in the ventral spinal cord and a dorsally shifted
1121 central canal in *hop* mice at P5 (F, H) compared to control (E, G). (I) Peroxidase staining on 60 μ m
1122 transverse sections of adult spinal cords showed abnormal morphology in the lumbar spinal cord of
1123 *hop* mice. The central canal is dislocated dorsally and is smaller in size (arrowhead), the ventral
1124 commissure is missing and the ventral white matter is disorganized or absent (black arrow). (J-L)
1125 Close up and quantification of central canal phenotype. (K) Dorsal shift ($p \leq 0.001$, $d = 3.88$) and (L)
1126 smaller size ($p \leq 0.001$, $d = 6.10$) of central canal in *hop* mice (n=12) compared to controls (n=10).
1127 (M, N) Immunohistochemistry staining with NeuN in red and MBP in green confirms misplaced
1128 neurons in the grey matter of ventral spinal cord in *hop* mice at adult age (N) compared to controls
1129 (M). (O) Quantification of number NeuN positive cells/section (n=10 per genotype) in the lumbar

1130 white matter in the *hop* adult mice compared to control ($p \leq 0.001$, $d = 7.21$). For brain morphology
1131 refer to Fig. 2-1. The dotted line in (E) indicates the midline, and in (M, N) the border between white
1132 and grey matter. Scale bars 300 μm (A), 150 μm (I), 100 μm (J) and 50 μm (C-E, M, N). Significant
1133 effect of genotype ** $p < 0.01$ *** $p \leq 0.001$.

1134

1135 **Figure 3: Neurotransmitter phenotype in the ventral spinal cord of postnatal *hop* mice.** (A-H) In
1136 situ hybridization on adult 60 μm floating sections using probes against *Viaat* (A,E), *Vglut2* (B,F) and
1137 *VACHT* (C,D,G,H). In control mice, neurons marked with either *Viaat* (A) or *Vglut2* (B) respect the
1138 border between white and grey matter, whereas in *hop* mice, *Viaat* (E) or *Vglut2* (F) mRNA
1139 expressing neurons intermingle with the white matter. Region of interest (ROI) as indicated with
1140 dotted black line. (I) Number of displaced *Viaat*⁺ cells ($p < 0.001$, $d = -2.31$) and (J) *Vglut2*⁺ ($p <$
1141 0.001 , $d = -3.16$) neurons in ROI are significant higher in lumbar but not cervical spinal cord ($p \geq$
1142 0.05). *VACHT* stained cholinergic cells are found around the central canal in control (C) but are
1143 ventrally displaced relative to the central canal in *hop* mice (G). (D,H,K) Motor neuron position
1144 (LMC-MMC) and number are similar (K) in *hop* mice (D) and controls (H). $n = 11$ -13 sections (L1 to
1145 L3) from $n = 3$ mice per genotype; Black box in (A) indicates area of enlargements. Neurons in the
1146 white matter are marked with black arrows. Arrowheads indicate central canal. Scale bars 50 μm (A-
1147 G), 150 μm (D-H). Significant effect of genotype *** $p \leq 0.001$.

1148

1149 **Figure 4: Aberrant commissural fibres and interneuron positioning in the ventral lumbar spinal**
1150 **cord of early postnatal (P0-P1) *hop* mice.** (A) Schematics of different strategies for tracer
1151 application to detect commissural interneurons. Midline tracing using FDA to show local crossing
1152 commissural interneurons (B-C), local tracing using FDA to label short projecting CINs (D-E') or
1153 intersegmental FDA and RDA tracing to show ascending (aCINs), descending (dCINs) and bifurcating
1154 commissural interneurons (adCINs) in (F-G'). (B, C) Transverse sections within the L2 level of spinal
1155 cords and density maps (H, I) show interneuron clustering on the midline in *hop* mice (C, I) as

1156 opposed to two ventromedial clusters in controls (B, H). N) Quantification of traced cells show similar
 1157 number of cells found ($p \geq 0.05$; $n=9-12$ sections from 3 mice per genotype). (D-E') Transverse
 1158 sections within the L2 level traced on L2 (D, E) shows that the fibres freely cross the midline in *hop*
 1159 (E') compared to control mice (D'). (J, K) Density maps illustrate diffuse and aberrant positioning of
 1160 CINs on the midline in *hop* mice compared to controls. O) Quantification of traced cells show similar
 1161 CIN numbers ($p \geq 0.05$; $n=6-9$ sections from 3 mice per genotype). (F-G') Aberrant fibres cross over
 1162 the midline in spinal cords of *hop* mice (G, G') ventral from the central canal and the amount of long-
 1163 range projecting CINs are reduced compared to controls. (L,M) Density plots of aCINs (red) and
 1164 dCINs (green) in control (L) and *hop* (M) spinal cords. (P) Quantification of traced cells show a
 1165 significant reduction of intersegmental projections over the midline (RDA: $p \leq 0.001$, $d = 2.92$, FDA:
 1166 $p \leq 0.001$, $d = 2.96$; aCIN, dCIN and adCIN: $p \leq 0.001$, $d = 4.46$; $n=8-11$ sections from 4 mice per
 1167 genotype). For intersegmental tracing on cervical spinal refer to Fig. 4-1. White box in (D, F) indicates
 1168 the area of close-up (D', G'). The outline of the spinal cord is indicated by a dashed line (B, C), central
 1169 canal and midline are indicated by a dotted line (F', G'). Scale bars (B-G) 100 μm and (D'-G') 200
 1170 μm . Significant effect of genotype *** $p \leq 0.001$.

1171
 1172 **Figure 5: The distinct spinal cord phenotype in *hop* mice is present at embryonic stages.** (A-B)
 1173 Photomicrographs of immunohistochemistry staining on E15.5 *hop* and control spinal cords with
 1174 NeuN (green) and Map2 (red) showing aberrant fibres and misplaced neurons disrespecting the ventral
 1175 midline. (C-D') White box in (A, B) indicates region of interest (ROI) for statistical analysis of
 1176 aberrant positioned NeuN+ cells and fiber crossing (C, NeuN+ cells: $p < 0.001$, $d = -5.46$; D', Map2
 1177 intensity as ratio to control: $p < 0.001$, $d = -2.81$; $n=5$ per genotype). White line in A' and B' indicates
 1178 the thickness of the ventral commissure, quantified length as ratio to controls in (D) ($p < 0.001$, $d = -$
 1179 5.71 ; $n=5$ per genotype). (E-H) RDA retrograde commissural interneuron (CIN) tracing on E15.5 (E-F)
 1180 and E12.5 (G-H) spinal cord. Dotted line indicates midline and outline of the spinal cord. (E'-H')
 1181 Density plots visualizing medial displacement of traced CIN in *hop* ($n=12$ sections from 3 embryos

per genotype). (I-P) In-situ hybridization on E15.5 (I, J, M, N) and E12.5 (K, L, O, P) lumbar spinal cords using probes detecting *Netrin1* and *EphrinB3*. Arrow heads point to regions of ventral *Netrin1* and *EphrinB3* mRNA expression. (Q, R) Staining intensity (background corrected ratio) is significantly reduced in *hop* compared to control spinal cords for both axon guidance molecules and time points (*Netrin*; E12.5 $p < 0.001$, $d = 5.86$, $n=7$ per group, E15.5: $p < 0.001$, $d = 3.47$, $n = 5$ per group; *EphrinB3*; E12.5 $p < 0.001$, $d = 6.46$, $n=7$ per group, E15.5: $p < 0.001$, $d = 5.19$, $n = 10$ per group). Scale bars 50 μm (A-H), 100 μm (I-P). Significant effect of genotype *** $p \leq 0.001$.

1189

Figure 6 Abnormal Shh and lumbar spinal cord patterning in *hop* mice

(A-D) Photomicrographs of Shh immunohistochemistry staining on transverse spinal cord sections at E12.5 (A, B) and E10.5 (C, D). Notochord and floorplate phenotype at E12.5 in low power (A, B) and close up from control (A', A'') and *hop* (B', B'') spinal cords. Dashed lines outline the spinal cord and notochord. The distance between the notochord and spinal cord is indicated by stars (A, B) and is reduced in *hop* spinal cords (G; $p < 0.001$, $d = 7.23$, $n=15$ per group). (A', B') A malformed notochord together with reduced amounts of Shh (H; $p = 0.002$, $d = 2.50$, $n=5$ per group). Lack of floorplate cells and severely reduced Shh expression in the spinal cord (A'', B'') of *hop* mice compared to controls (H), ($p = 0.016$, $d = 1.93$, $n=5$ per group). (C, D) Similarly, we found reduced amounts of Shh at E10.5 in *hop* spinal cords compared to controls (H), ($p < 0.001$, $d = 1.36$, $n=15$ per group). (E, F) Ttc26 immunostaining on E12.5 spinal cord transverse sections. For clarity, blue nuclear counterstain with DAPI has been omitted. (I) The expression of Ttc26 is found in the notochord at similar intensity levels both in control and *hop* spinal cords. (J-V) Immunostainings on E12.5 spinal cord sections reveal a missing Nkx2.2 progenitor domain and a migration defect of neurons in the lumbar spinal cord of *hop* mice. (J, K) The expression of the ventral most V3 progenitor marker Nkx2.2 is absent in lumbar sections from *hop* mice (K). White box indicates area of higher magnification (J', K'). (V) Nkx2.2 phenotype at different rostrocaudal levels exemplified with photomicrographs and visualized using density plots. The midline is indicated by lines ($n = 4$ sections per level). (L-V) Density plots

show misplaced cells and lack of a ventral midline for additional progenitor markers as specified below each panel (n=3), for representative photomicrographs refer to Fig. 6-1). (W) Schematic summary of defects found in the *hop* mice in the lumbar part of the spinal cord including weak Shh expression, misplaced and malformed notochord, a dorsally shifted central canal, absence of floorplate and p3 domains as well as absence of a ventral midline. pdINs - progenitor domains for dorsal interneurons, FP - floor plate. The outline of the spinal cord and midline are indicated by dashed lines. FP = floorplate, NC=notochord, scale bars: 100 μ m (A-B, J-K, M-V), 25 μ m (A',B') 30 μ m (A'', B'', J' K') and 50 μ m (C, D). Significant effect of genotype $*p < 0.05$ $**p < 0.01$ $***p \leq 0.001$.

Figure 7: Introduction of a point mutation in *Ttc26* and analysis *Ttc26*^{Y430X} mutants. For initial validation of the *hop* mouse mutation refer to Fig. 7-1. (A) We then applied the CRISPR/Cas9 system to generate mice with a point mutation in exon 15 of the *Ttc26* gene, generating a substitution of tyrosine to a premature stop codon. By simultaneous microinjection of guideRNA, hCas9 mRNA and a single-stranded donor oligonucleotide (ssDON) into mouse oocytes, mouse chromosome 6 harbouring the *Ttc26* gene was modified (left). The exon (black) and intron (red) organization is indicated together with Sanger sequencing traces of wild type (bottom) and the mutated allele (top) highlighting the premature stop mutation. The introduction of a PvuII site for screening purposes (blue) and the location of the guideRNA are indicated. (B,C) Images of E12.5 (B) and E16.5 (C) *Ttc26*^{Y430X} mice showing preaxial polydactyly (arrowheads, star). (D-F) Photomicrographs of Hematoxylin and Eosin staining on E16.5 transverse spinal cord sections. (D) Lumbar spinal cord of control mice showing a well delineated central canal (upper black arrow), a present ventral funiculus with a border between white and grey matter (dashed line) and a present ventral spinal artery (lower arrow). (E) Lumbar spinal cord of *Ttc26*^{Y430X} mice showing a poorly delineated dorsally shifted central canal (upper arrow), an absent ventral funiculus (upper star) and an absent ventral spinal artery (lower arrow). (F) Cervical spinal cord of *Ttc26*^{Y430X} mice showing a well delineated central canal (upper arrow) and a present ventral funiculus with a clear midline border (lower arrow). (G-L)

1234 Photomicrographs of immunohistochemistry experiments on E16.5 transverse lumbar spinal cord
 1235 sections of control and *Ttc26*^{Y430X} mice. (G) NeuN staining showing a poorly defined ventral midline
 1236 in *Ttc26*^{Y430X} mice, with misplaced neurons within the white matter of the ventral funiculus.
 1237 Enlargements on the right show cells positioned at the location of the missing midline in mutant but
 1238 not control spinal cord sections (G-H). (I-J) Cholinergic neurons as identified by their VACHT
 1239 expression (red) were found in the medial and lateral motor neuron cluster, as well as in neurons
 1240 around the central canal. Dmrt3 expressing interneurons were found medioventrally from the central
 1241 canal. In mutant *Ttc26*^{Y430} spinal cords, medial and lateral motor neuron nuclei were located at their
 1242 expected positions. In contrast, central canal cholinergic neurons and Dmrt3 expressing interneurons
 1243 were found ventral of the central canal and positioned in close proximity or even on top of the
 1244 presumed position of the midline. (I, J) VACHT immunostaining showing no apparent differences
 1245 regarding position and size of motor neurons between *Ttc26*^{Y430} and control mice. (I',J') Central canal
 1246 VACHT expressing cells, as well as (K-L) Dmrt3 expressing cells, were ventrally and medially
 1247 displaced in *Ttc26*^{Y430} spinal cords compared to controls. (J',L') Enlargement of the ventromedial area
 1248 of the spinal cord demonstrating misplaced cells in *Ttc26*^{Y430} spinal cords (J', arrows). Scale bars (D-
 1249 H) 100 μ m, (I-L) 50 μ m, (I'-L') 100 μ m. For comparative analysis in the rabbit refer to Fig. 7-2.

1250

1251 **Extended data**

1252

1253 **Figure 1-1 (related to figure 1): Mid-sagittal hemisection does not disrupt the ipsilateral**

1254 **alternating burst pattern.** (A) Neurograms illustrating a typical pattern recorded from a *hop* mouse.

1255 (B) Pattern recorded from the preparation following a midsagittal hemisection. Note the preservation

1256 of the L3-L5 pattern suggesting that flexor-extensor coordination is maintained. (C) Schematic

1257 illustrating stimulation and recording arrangement for cauda equina stimulation. Cauda equina evoked

1258 rhythmic pattern (4 Hz, 10 s train) showed segmental synchronous pattern and ipsilateral alternating

1259 pattern.

1260

1261 **Figure 2-1 (related to figure 2): Brain commissure morphology appears normal in *hop* mice.**

1262 Photomicrographs of horizontal sections from adult control (A,C,E,G,I,K) and *hop* (B,D,F,H,J,L)

1263 tissue. (A,B) The corpus callosum (CC) and hippocampal commissure (HC) showed no gross defects.

1264 (C,D,G,H) The anterior commissure (AC) also appeared normal. (E,F) Sections showing the habenular

1265 commissure (HaC), the posterior commissure (PC) and the subcommissural organ (sc), all three

1266 present and with normal appearance in both control and *hop* mice. (I,J) The pontine nuclei (pn) is also

1267 present in both genotypes. (K,L) No aberrant commissure was found between the roof of the fourth

1268 ventricle (FV), and cerebellum (ce) at the junction of midbrain and hindbrain, in control and *hop* mice.

1269 Scale bars (A,B,C,D,G,H) 1000 μ m, (E,F,I,J,K,L) 500 μ m

1270

1271 **Figure 4-1 (related to figure 4): Normal commissural interneurons and axon midline crossing in**

1272 **the cervical spinal cord of early postnatal (P0-P1) *hop* mice.** (A-F) Intersegmental tracing using

1273 FDA/RDA to locate ascending (aCINs), descending (dCINs) and bifurcating commissural

1274 interneurons (adCINs). No apparent defects in *hop* mice (D-F') compared to controls (A-C') on

1275 cervical sections were found.

1276

1277 **Figure 6-1 (related to figure 6): Embryonic Patterning defects in *hop* spinal cords.**

1278 Photomicrographs of immunohistochemistry experiments on E12.5 spinal cord transverse sections
1279 using antibodies against transcription factors as indicated at the top. White boxes in (A-E) indicate
1280 areas of higher magnification in the respective panel below (A'-E'). The stainings show misplaced
1281 cells and lack of ventral midline for all tested progenitor markers. The outline of the spinal cord,
1282 central canal and midline are indicated by dashed lines. Scale bars (A-J) 100 μ m, (A',F') 50 μ m and
1283 (B', G'-J') 30 μ m.

1284

1285 **Figure 7-1 (related to figure 7): Validation of the *hop* mouse mutation.** The exon/intron
1286 organization of *Ttc26* is indicated together with Sanger sequencing traces of wild type and *hop*
1287 highlighting the premature stop mutation in exon 15 (out of 18) of *Ttc26*. Genomic DNA from a *hop*
1288 homozygote (stock 002718) and BALB/cByJ control (stock 001026) was obtained from the Jackson
1289 Laboratory's DNA Resource (n=3 per genotype). Exomes were captured at the Yale Center for
1290 Genome Analysis, using the NimbleGen SeqCap EZ Mouse Exome and following NimbleGen
1291 protocols. Captured pools were sequenced (75bp, paired-end) on an Illumina HiSeq 2000 using
1292 previously described methods (Choi et al., 2009). We obtained ~91 million (BALB/cByJ) to ~113
1293 million (*hop*) high-quality reads. Illumina reads were first trimmed based on their quality scores to
1294 remove low-quality regions using the program Btrim (Kong, 2011). A cutoff of 20 for average quality
1295 scores within a moving window of size 5-bp was used. Minimum acceptable read length was 25-bp.
1296 Other parameters of Btrim were set to defaults. The pre-processed reads were then aligned to the
1297 mouse genome reference sequence (mm9) using the BWA mapping program (Li and Durbin, 2009).
1298 The mapping results were converted into SAMtools pileup format using SAMtools programs (Li et al.,
1299 2009). PCR duplicates were removed using the rmdup command from SAMtools, resulting in ~84x
1300 (BALB/cByJ) or ~100x (*hop*) coverage across the exome. >97% of all bases included in the exome
1301 showed at least 8x coverage and >90% of the bases showed at least 20x coverage. Single nucleotide
1302 variations (SNVs) were called using SAMtools' pileup command. Further filtering was performed
1303 using in-house scripts to exclude those SNV calls that had less than 3 reads or a SNP score less than
52

1304 20. Annotation was added based on the UCSC RefSeq gene model (<http://genome.ucsc.edu/> (Pruitt et
1305 al., 2009)). Based on SNV homozygosity mapping, the interval was narrowed to ~700 Kb on
1306 Chromosome 6, (37.9-38.6 Mb) which fell within the previously mapped *hop* genomic region. After
1307 filtering for known SNPs and repeat regions, we identified 17 possible point mutations, 16 of which
1308 were located in introns or 3' untranslated regions.

1309

1310 **Figure 7-2 (related to figure 7): Relatively high number of missense mutations in the rabbit**

1311 ***Ttc26* gene.** (A) Amino acid alignment of TTC26 sequences from eight mammals made using
1312 MUSCLE with missense mutations in rabbit marked (pink boxes). (B) Photomicrograph of HE stained
1313 lumbar spinal cord section from wild type adult rabbit (box shown magnified region). The morphology
1314 is similar to the wild type mouse spinal cord, with a well delineated central canal (upper arrow), a
1315 ventral funiculus with a clear border between white and grey matter and a ventral spinal artery (lower
1316 arrow). (C-G) Immunohistochemistry performed on postnatal rabbits towards NeuN (C), Chat (D),
1317 Lbx1 (E), Parvalbumin (F) and Pax2 (G) show a clear bilateral separation of the spinal neuronal
1318 populations. Scale bars (C-G) 200 μ m.

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7