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## 8 Multiple steps mediate ventricular layer attrition to form 10 the adult mouse spinal cord central canal

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28 ventral dissociation, floor plate, roof plate, cell cycle, FUCCI reporter, Shh signalling, BMP signalling.

28

30 **Abstract**

32 The ventricular layer of the spinal cord is remodelled during embryonic development and ultimately  
34 forms the adult central canal, which retains neural stem cell potential. This anatomical transformation  
36 involves the process of dorsal collapse, however, accompanying changes in tissue organization and  
38 cell behaviour as well as the origin of cells contributing to the adult central canal are not well understood.  
40 Here we describe sequential localised cell rearrangements which contribute to the gradual attrition of  
42 the spinal cord ventricular layer during development. This includes local breakdown of the  
44 pseudostratified organisation of the dorsal ventricular layer prefiguring dorsal collapse and evidence for  
46 a new phenomenon, ventral dissociation, during which the ventral-most floor plate cells separate from  
48 a subset that are retained in the central canal. Using cell proliferation markers and cell-cycle reporter  
mice, we further show that following dorsal collapse, ventricular layer attrition involves an overall  
reduction in cell proliferation, characterised by an intriguing increase in the percentage of cells in G1/S.  
In contrast, programmed cell death does not contribute to ventricular layer remodelling. By analysing  
transcript and protein expression patterns associated with key signalling pathways, we provide  
evidence for a gradual decline in ventral sonic hedgehog activity and an accompanying ventral  
expansion of initial dorsal bone morphogenetic protein signalling, which comes to dominate the forming  
central canal. This study identifies multiple steps that contribute to spinal cord ventricular layer attrition  
and adds to increasing evidence for the heterogenous origin of the adult spinal cord central canal, which  
includes cells from the floor plate and the roof plate as well as ventral progenitor domain.

## Introduction

50 The ependymal cells that form the central canal in the adult mammalian spinal cord constitute a largely  
52 quiescent stem cell niche (Adrian and Walker, 1962, Kraus-Ruppert et al., 1975, Alfaro-Cervello et al.,  
54 2012, Sabourin et al., 2009). These cells can be induced to re-enter the cell cycle in response to  
56 extrinsic stimuli, including mechanosensory stimulation (Shechter et al., 2011), physical exercise  
58 (Krytziarana et al., 2010), inflammation (Chi et al., 2006, Danilov et al., 2006) and injury (Adrian and  
60 Walker, 1962, Frisen et al., 1995, Barnabe-Heider et al., 2010, Johansson et al., 1999, Li et al., 2016,  
62 Li et al., 2018, Meletis et al., 2008). Moreover, most of these stimuli appear to promote the generation  
64 of new neurons and glial cells, consistent with *in vitro* studies of the differentiation potential of the spinal  
66 cord ependymal cell population (Weiss et al., 1996, Johansson et al., 1999, Li et al., 2016, Meletis et  
68 al., 2008, Sabourin et al., 2009). However, following spinal cord injury, central canal cells proliferate  
70 and migrate to the lesion site, but here differentiate into only glia (Barnabe-Heider et al., 2010, Li et al.,  
72 2016, Li et al., 2018, Meletis et al., 2008, Martens et al., 2002). These cells then contribute to scar  
74 tissue, many becoming astrocytes which reduce inflammation, but chronically inhibit axonal re-growth  
(Warren et al., 2018), while others differentiate into oligodendrocytes, which can promote survival of  
nearby neurons and help to maintain the integrity of the injured spinal cord (Sabelstrom et al., 2013).  
Together, these findings indicate that changes in environment determine the behaviour and  
differentiation of spinal cord ependymal cells. Importantly, this is a heterogenous cell population and  
the precise identity of cells with neural stem cell abilities has yet to be determined. This activity of spinal  
cord ependymal cells is also distinct from that of ependymal cells lining the brain ventricles, where  
instead the neural stem cells constitute a distinct sub-ependymal cell population (Mirzadeh et al., 2008,  
Shah et al., 2018, Lim and Alvarez-Buylla, 2016). In the healthy animal, adult spinal cord ependymal  
cells carry out specialised functions, including homeostatic regulation of cerebrospinal fluid (CSF)  
composition and acting as a barrier between CSF and the spinal cord parenchyma (reviewed in (Del  
Bigio, 1995, Bruni, 1998)). However, despite these significant roles in the injured and healthy spinal  
cord, little is known about how spinal cord ependymal cells arise and how the central canal is formed  
during development.

76 One aspect of central canal formation involves attrition of the progenitor cell population that  
78 constitutes the ventricular layer of the embryonic spinal cord (Fu et al., 2003, Shibata et al., 1997, Yu  
80 et al., 2013). This remodelling process includes a striking morphological phenomenon known as dorsal  
collapse, which mediates a pronounced reduction of the dorsal ventricular layer in a range of mammals  
(Barnes, 1883, Bohme, 1988, Elmonem et al., 2007, Sevc et al., 2009, Sturrock, 1981). However, the  
changes in cell behaviour that underlie this critical event are poorly understood. In contrast, the earlier  
82 dorso-ventral subdivision of the developing spinal cord has been well-characterised. This involves  
signals emanating from the roof plate located at the dorsal midline (including bone morphogenetic  
84 protein (BMP) and Wnt) and the floor plate at the ventral midline (Sonic hedgehog, Shh), which act in  
opposition to specify distinct neural progenitor cell populations along the dorso-ventral axis (Jessell,  
86 2000, Le Dreau and Marti, 2012, Ulloa and Briscoe, 2007). This involves regulation of homeodomain  
and other transcription factors, which act in combination to define neuronal subtype specific progenitors  
88 (Lee and Pfaff, 2001). Key transcription factors include *Pax6*, which is initially expressed broadly and

is involved in motor neuron progenitor specification, and *Nkx6-1*, which distinguishes V2 interneuron  
90 progenitors. Importantly, the continued expression of *Pax6* and *Nkx6-1* in the adult central canal has  
led to the notion that ependymal cells derive from this earlier population of ventral neural progenitors  
92 (Fu et al., 2003, Yu et al., 2013). It is apparent that this ventral region of the ventricular layer is also  
reduced over time and this may be associated with the switch from neurogenesis to gliogenesis  
94 between E11.5-12.5 and, ultimately, the migration of glial cells out of this layer (Deneen et al., 2006,  
Stolt et al., 2003) reviewed in (Laug et al., 2018). As the emerging central canal becomes separated  
96 from the most dorsal and ventral regions of the spinal cord, its formation may additionally involve the  
remodelling of these specialised cell populations. Indeed, dorsal collapse coincides with elongation of  
98 processes from nestin-expressing cells from the roof plate, which ultimately integrate into the adult  
central canal in mammals (Bohme, 1988, Sevc et al., 2009, Xing et al., 2018, Shinozuka et al., 2019,  
100 Ghazale et al., 2019) and fish (Kondrychyn et al., 2013). It is also possible that a similar ventral  
reorganisation takes place and that this may account for the apparent inclusion of some floor plate cells  
102 in the adult central canal (Khazanov et al., 2017).

Here, we describe sequential cell rearrangements associated with the attrition of the ventricular  
104 layer as the spinal cord matures during mid to late mouse embryogenesis. We determine whether  
changing patterns of cell proliferation and/or programmed cell death may contribute to this process. We  
106 further evaluate the contribution of floor plate cells to the forming central canal, assessing this in the  
context of changing expression patterns of genes and proteins associated with key dorsal and ventral  
108 signalling pathways.

## 110 **Methods**

### **Animals**

112 Mice were either wild type CD1 or C57BL/6J strains (Charles River). Embryos and spinal cords from  
Fucci2a transgenic mice (Mort et al., 2014) were provided by Professor Andrew Jackson (MRC HGU,  
114 University of Edinburgh) and embryos and spinal cords from Shh-GFP (Chamberlain et al., 2008) and  
GBS-GFP (Balaskas et al., 2012) transgenic mice were provided by Dr James Briscoe (Francis Crick  
116 Institute). For timed matings, the morning of the plug was considered E0.5. All animal procedures were  
approved by the UK Government Home Office and in accordance with European Community Guidelines  
118 (directive 86/609/EEC) under project licence numbers 6004454.

### 120 **Immunofluorescence**

From embryos, the inter-limb (thoracic) spinal cord tissue was dissected in ice-cold PBS, fixed for 2  
122 hours at 4°C in 4% paraformaldehyde (PFA), rinsed several times in PBS and cryopreserved in 30%  
sucrose/PBS at 4°C overnight. This tissue was embedded in 1.5% LB agar/5% sucrose, again  
124 cryopreserved in 30% sucrose/PBS overnight, frozen on dry ice, and either cryosectioned at 20 µm  
sections or stored at -20 °C for later use. To obtain adult spinal cords, 10 to 24-week old mice were  
126 deeply anaesthetised with an overdose of pentobarbital (Euthatal) or isoflurane and then transcardially  
perfused with ice-cold PBS followed by 4% PFA. Spinal cords were quickly dissected out and post-fixed  
128 in 4% PFA for 2 hours at 4°C and processed as above. Standard procedures were used for immune  
fluorescence, with the following exceptions. To detect phosphorylated SMAD1/5, all solutions were  
130 supplemented with PhospoStop phosphatase inhibitor (Roche, Cat. # 4906837001) and sections were  
dehydrated with methanol to improve permeability; to detect pH3 sections were exposed to citrate buffer  
132 (pH 6) at 95°C for 20 min. In all cases, sections were placed in blocking buffer (2-10% heat-inactivated  
donkey or goat serum and 0.3-1% Triton X-100 in PBS) for 30 minutes at room temperature and  
134 incubated at 4°C overnight with primary antibodies diluted in blocking buffer. Lists of primary and  
secondary antibodies used are provided in Table S1. Nuclear counterstaining was achieved with DAPI  
136 diluted in PBS (1:1000) and incubated for 5 minutes at RT. Slides were mounted with ProLong® Gold  
Antifade Mountant (Thermo Fisher Scientific, P36930) and images were captured on a DeltaVision or  
138 Leica TCS SP8 confocal microscope system.

### 140 **RNA *in situ* hybridisation**

Inter-limb spinal cord tissue was dissected, fixed and processed as described above, but cryosectioned  
142 at 50 µm. Adult mouse spinal cords (24 to 40-weeks old) were also prepared as above but post-fixed  
overnight in 4% PFA at 4°C. Plasmids, restriction endonucleases and RNA polymerases used for the  
144 generation of digoxigenin-labelled probes (Roche Applied Science) are provided in Table S2. Probes  
were diluted 1:100 in hybridisation buffer (50% deionised formamide, 5X SSC, 0.08% Tween 20, 50  
146 µg/mL heparin, 1 mg/mL t-RNA, 5 mM EDTA, 0.1% CHAPS, 0.02 g/mL Boehringer blocking reagent)  
and slides incubated at 65°C in a chamber humidified overnight. Sections were washed in post-

148 hybridisation buffer (1X SSC, 50% formamide) at 65°C for 15 minutes three times, followed by a 1:1  
149 post-hybridisation buffer/TBS-0.1% Triton X-100 (post-hyb) wash at 65°C for 20 minutes and blocked  
150 in 2% Boehringer blocking reagent, 20% heat-inactivated sheep serum in post-hyb for 1h. Probe was  
151 detected using alkaline phosphatase-labelled anti-digoxigenin antibody (1:1000, Promega,  
152 11093274910) in blocking buffer at 4°C overnight. Sections were then washed with in post-hyb and  
153 equilibrated for 10 min in NTMT buffer (100 mM NaCl, 100 mM Tris HCl pH 9.5, 50 mM MgCl<sub>2</sub>, 1%  
154 Tween 20). Finally, sections were incubated with alkaline phosphatase substrates nitro blue tetrazolium  
155 (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in NTMT buffer to reveal signal, washed in  
156 PBS-0.1% Triton X-100 and post-fixed in 4% PFA at 4°C and washed again in PBS-0.1% Triton X-100  
157 and slides mounted with ProLong® Gold Antifade Mountant. Images were acquired on a Leica DMRB  
158 microscope fitted with a Nikon D7100 camera.

#### 160 **TdT-UTP nick end labelling (TUNEL) assay**

161 The inter-limb spinal cord tissue from CD1 mouse embryos was dissected and fixed at 4°C for 2 hours  
162 or overnight in 4% PFA. TUNEL assay was performed on 20 µm thick cryosections using ApopTag®  
163 Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Cat. # S7100) according to manufacturer's  
164 instructions.

#### 166 **Tissue measurements and cell counts**

167 Images were processed using Fiji and assembled using Adobe Photoshop and Illustrator software.  
168 Measurements of the dorsal-ventral length and apical-basal width of the ventricular layer were  
169 determined using Deltavision SoftWorx software. The widest point was taken as the measure for width.  
170 The Cell Counter plugin in Fiji was used to count SOX2 expressing cells labelled with pH3 or PCNA  
171 and the same approach was used to count fluorescent cells in tissues from the Fucci mouse line.

172

#### **Statistical analysis**

173 All the results were summarised as mean ± standard error of the mean (SEM). A two-sided, unpaired  
174 Student's t-test with pooled/equal variance was used to test whether the differences in the means of  
175 two groups were statistically significant. A p-value below 0.05 was considered to be statistically  
176 significant. The number of samples studied in each experiment is mentioned in their respective figure  
177 legends and see Tables S3-S6. No statistical method was used to determine the sample size.  
178

## Results

### 180 **Changes in tissue dimensions and local cell rearrangements are associated with ventricular** 181 **layer attrition during formation of mouse spinal cord central canal**

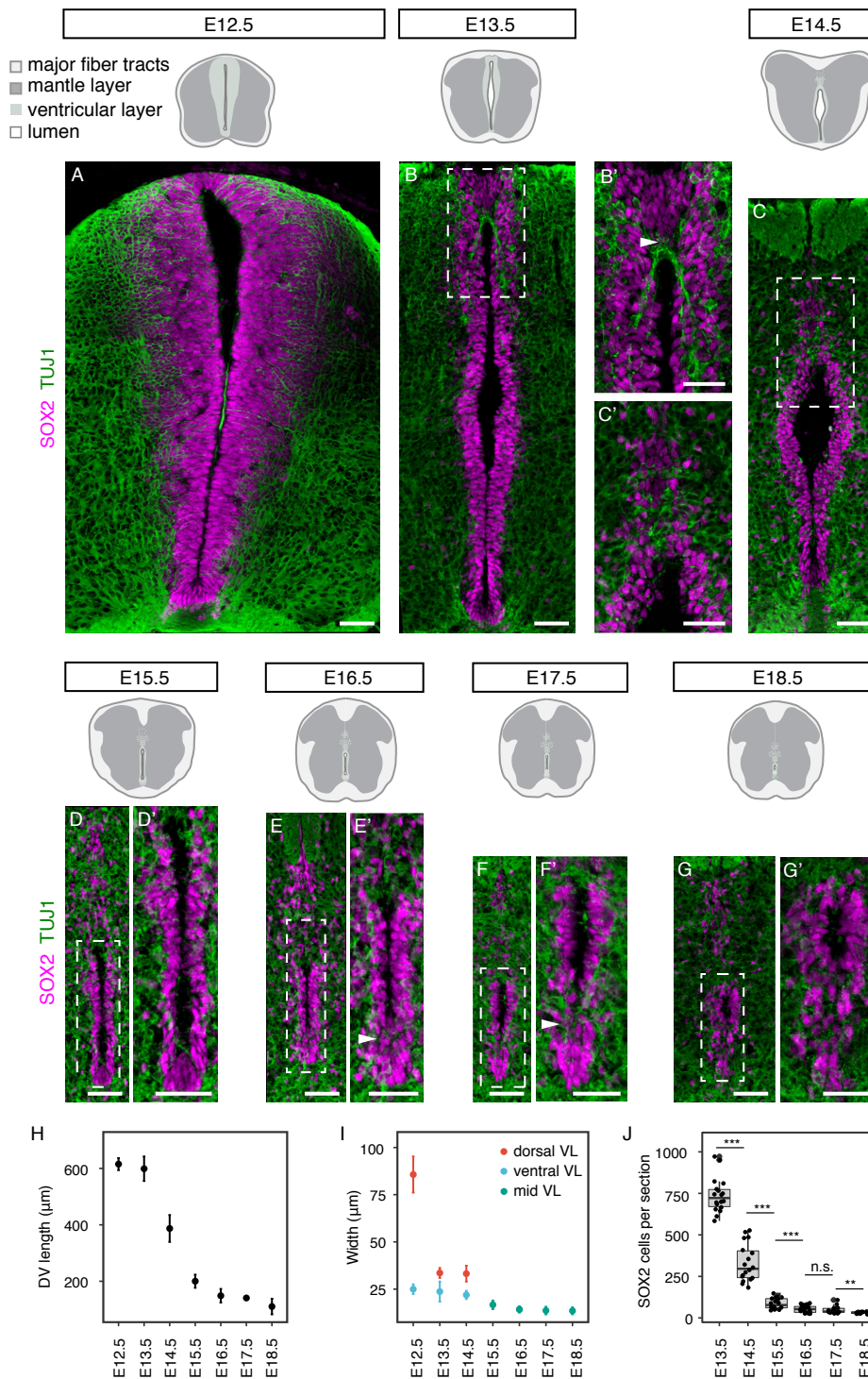
182 To characterise the changing dimensions and organisation of the ventricular layer in the developing  
183 mouse spinal cord, we delimited this cell population using immunofluorescence to detect SOX2-positive  
184 neural progenitors and surrounding TUJ1-expressing neurons. Measurements were made from  
embryonic day (E)12.5 to E18.5 in the thoracic spinal cord (Figs 1A-G' and Table S3).

186 At E12.5, the length of the ventricular layer spans the height of the transverse section of the  
spinal cord ( $615.2 \pm 7.1 \mu\text{m}$ ) and it is widest dorsally, tapering ventrally (Figs 1A, H, I). At this stage, the  
188 apical surface of the neuroepithelium often meets at the midline, obscuring the central lumen in all but  
the dorsal region (Fig 1A). The length of the ventricular layer is unchanged at E13.5; however, its dorsal  
190 width is now substantially reduced (from  $85.6$  to  $33.6 \mu\text{m}$  or from 10 to 4 cell diameters, Figs 1B, H, I).  
At this stage, the ventricular layer has an elongated rhomboid shape with the lumen open centrally (Fig  
192 1B). Here, in a nexus about five cell diameters below the roof plate, TUJ1-positive neuronal processes  
are now visible at the dorsal midline and interdigitate between SOX2-positive cells, which have lost their  
194 contiguous pseudostratified arrangement (Figs 1B'). This local remodelling of the ventricular layer  
appears to be the first indication of the process of dorsal collapse.

196 By E14.5, the length of the ventricular layer is drastically reduced (to  $387.1 \pm 15.9 \mu\text{m}$ ) due to  
dorsal collapse (Figs 1C, H). Almost all of the dorsal region now contains interdigitated neuronal  
198 processes and more sparsely distributed SOX2-positive cells (Fig 1C'). Some of these more dorsal  
SOX2 expressing cells have now come to abut the open central lumen, which has acquired an inverted  
200 tear-drop shape (Fig 1C'). This morphology, however, is quickly lost as dorsal collapse proceeds.  
Indeed, by E15.5 the ventricular layer is further reduced in length ( $200.1 \pm 7.3 \mu\text{m}$ ; or 15 to 20 cell  
202 diameters) and width, which becomes more uniform along the dorsal-ventral axis ( $16.7 \pm 2.2 \mu\text{m}$ ; or 2  
cell diameters), and only a narrow slit-like lumen is apparent (Figs 1D, D', H, I). At E16.5, the length  
204 and width of the ventricular layer continues to diminish (Figs 1E, E', H, I) and some of the ventral-most  
SOX2-positive cells appear to dissociate from the ventricular layer (arrowheads in Figs 1E'). Between  
206 E17.5 and E18.5, the ventricular layer is reduced in length just a few more cell diameters, as the more  
loosely arranged SOX2-positive cells both dorsally and ventrally are further dispersed ( $110.4 \pm 8.7 \mu\text{m}$   
208 in length) (Figs 1F-G', H). The remaining contiguous SOX2-expressing cell population that constitutes  
the ventricle appears now to have reached the final, oval-shaped arrangement of what will be the future  
210 spinal cord central canal (Figs 1G, G').

These data identify large scale morphological changes and local cell re-arrangements  
212 associated with ventricular layer attrition. These include, first, a reduction in dorsal width at E13.5  
accompanied by a local loss of pseudostratified epithelial organisation, followed by dramatic reduction  
214 in length associated with widespread loss of epithelial organisation in the dorsal region and so dorsal  
collapse at E14.5. This is reflected in a significant reduction in the number of SOX2-positive cells in the  
216 ventricular layer between E13.5 and E14.5 and between E14.5 and E15.5 (Fig 1J). In addition, a later

218 further attrition step, which we name here ventral dissociation, separates most ventral SOX2-positive cells from the future central canal.



220

222 **Figure 1 Changing ventricular layer dimensions during mouse spinal cord development (A-G')**  
 223 Immunofluorescence for SOX2 (magenta) and TUJ1 (green) reveals the changes in the organisation of the  
 224 ventricular layer and the surrounding mantle layer (black dashed boxes) at the indicated stages. Higher  
 225 magnification images of white dashed boxes from E13.5 to E18.5 are shown in B', C', D', E', F' and G', respectively.  
 226 Note that some of the ventral-most SOX2-expressing cells appear to dissociate from the ventricular layer at later  
 stages (arrowheads in E' and F'). (H-I) Ventricular layer length (H) and width (I) at indicated stages (at least 9  
 sections, n = 3 embryos for each stage). Widest horizontal distance across the dorsal region of the ventricular layer



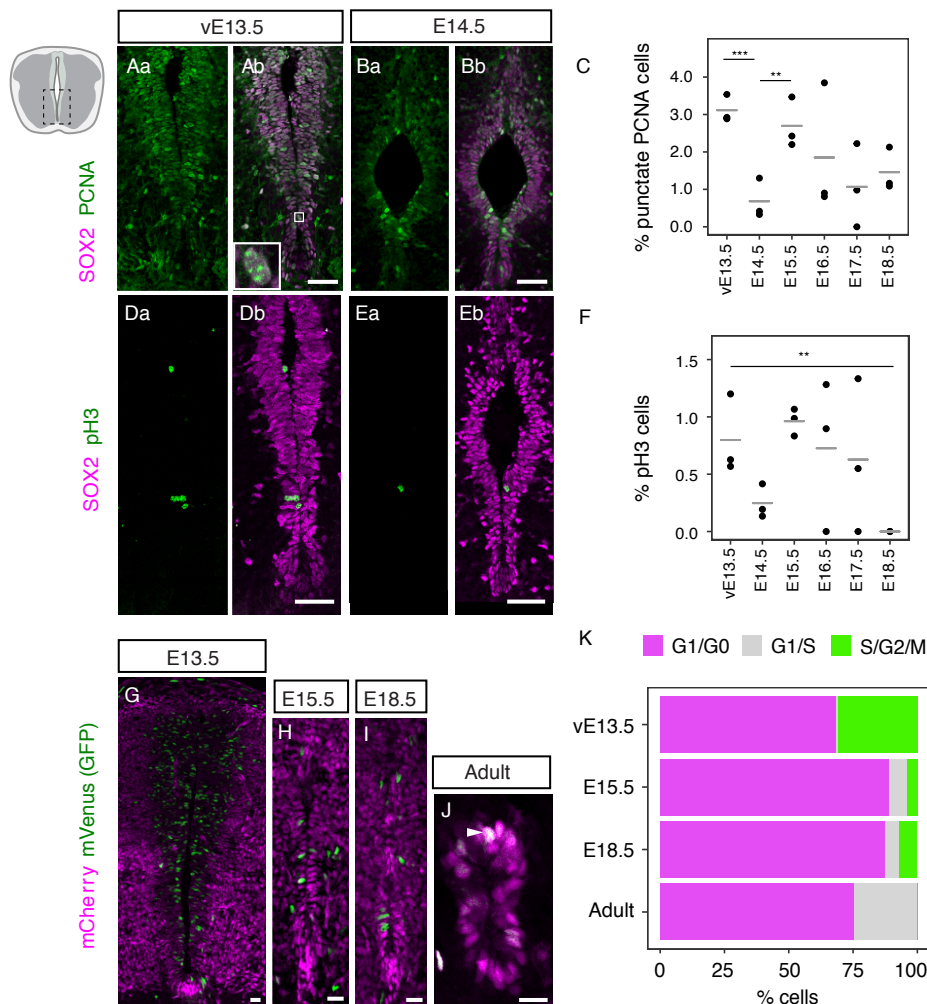
228 (dorsal VL) and adjacent to the floor plate (ventral VL) was measured. From E15.5 the width of the ventricular layer  
230 becomes fairly uniform along the dorsal-ventral axis, so measurement was at the mid ventricular layer (mid VL) at  
232 these stages; (J) SOX2 positive cells per section were quantified at each stage (see Table S3). Error bars represent  
234 mean values  $\pm$  standard error of the mean (SEM). A two-sided unpaired Student's t-test was used to determine  
whether differences in the mean values were statistically significant, \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*  $p < 0.05$ . Scale  
bars: 40  $\mu$ m.

### Reduced cell proliferation, but not apoptosis, accompanies ventricular layer attrition

236 The reduction of SOX2-positive cells in the ventricular layer as development proceeds is at least in part  
accounted for by the delamination of cells from this proliferative zone to form neurons and, after E12.5,  
238 glial cells (Deneen et al., 2006, Stolt et al., 2003). In addition, it is also possible that progenitor cells  
remaining in the ventricular layer reduce their proliferation rate and so cell replacement slows,  
240 accelerating the reduction of this progenitor cell population. To test the latter possibility, we set out to  
investigate cell proliferation parameters in the ventricular layer from E13.5 to E18.5. As it is largely the  
242 ventral half of the ventricular layer at E13.5 that is retained following dorsal collapse, we focused on  
this cell population and on the whole contiguous SOX2-positive cell population from E14.5 onward (Figs  
244 2Aa-F). We first used the punctate pattern of proliferating cell nuclear antigen (PCNA), which indicates  
sites of DNA synthesis (Bravo and Macdonald-Bravo, 1985), to determine the percentage of SOX2-  
246 positive cells in S phase at each stage. This analysis revealed a reduction in the proportion of cells in  
S phase between E13.5 to E14.5 (Figs 2Aa-C) and that thereafter this remains low and approximately  
248 constant to E18.5 (Fig 2C). Next, the mitotic index was calculated by determining the percentage of  
phospho-Histone H3 (pH3)-positive SOX2 cells at each stage. This revealed a similar pattern, in that  
250 the percentage of mitotic cells is low at E13.5 and shows a tendency to decrease further at later stages  
(the mean differences between each two consecutive stages is not statistically significant, but the  
252 mitotic index at E18.5 is significantly lower than that at E13.5,  $p = 0.017$ ) (Figs 2Da-F).

A limitation of the above approaches is the small number of cells observed with a punctate  
254 PCNA pattern or undergoing mitosis (Table S3). To extend this cell proliferation analysis, we therefore  
next assessed cell cycle phase distribution in ventricular layer cells using Fucci2a transgenic mice (Mort  
256 et al., 2014) at key stages: before (ventral E13.5) and after (E15.5) dorsal collapse, after ventral  
dissociation (E18.5), and in the adult central canal (Figs 2G-J). These transgenic mice express two cell  
258 cycle-regulated proteins fused to fluorescent proteins, one to mCherry (mCherry-hCdt1) and the other  
to mVenus (mVenus-hGeminin). This results in the accumulation of mCherry-hCdt1 during the G1 and  
260 G0 phases of the cell cycle, making cells in G1 and G0 fluoresce red (shown here in magenta).  
mCherry-hCdt1 is degraded at the G1/S transition following interaction with Geminin (Wohlschlegel et  
262 al., 2000, Tada et al., 2001) and mVenus-hGeminin begins to accumulate in S, so cells in the G1/S  
transition fluoresce both red and green and appear yellow (shown here in white). mVenus-hGeminin  
264 continues to be expressed during S/G2/M phases, making cells in these phases fluoresce bright green.  
mVenus-hGeminin is then rapidly degraded before cytokinesis (Mort et al., 2014, Sakaue-Sawano et  
266 al., 2008). In agreement with our previous measurements, we found that the percentage of cells in  
S/G2/M decreases after E13.5 (from  $30.8 \pm 4.7\%$  to  $4.2 \pm 0.5\%$  E15.5, mean  $\pm$  SEM,  $p = 0.027$ ) and  
268 remains relatively constant to E18.5 ( $6.9 \pm 1.6\%$ ,  $p = 0.239$ ) (Fig 2K). In the adult central canal, cells in  
S/G2/M are rarely detected ( $0.1 \pm 0.1\%$ ,  $p = 0.003$ ). In contrast, the percentage of cells in the G1/G0

270 state increase from E13.5 to E15.5, from  $68.7 \pm 4.8\%$  to  $89.0 \pm 1.9\%$  ( $p = 0.06$ ), although the difference  
 is not statistically significant. The percentage of cells in G1/G0 remains rather similar between E15.5  
 272 and E18.5 ( $87.4 \pm 2.2\%$ ,  $p = 0.39$ ). This may reflect the intriguing emergence a group of cells  
 transitioning from G1 to S, which becomes first evident at late developmental stages (from  $0.48 \pm 0.24\%$   
 274 at E13.5 to  $6.8 \pm 2.0\%$  at E15.5,  $p = 0.006$ ) and increases still further in adult central canal cells (from  
 276  $5.6 \pm 1.6\%$  at E18.5 to  $24.4 \pm 1.3\%$ ,  $p = 0.0004$ ) (Fig 2K). This suggests that while the majority of adult  
 central canal cells reside in G1/G0, the rest of this cell population now appear poised to progress  
 through the cell cycle as they are beginning to upregulate Geminin (early S-phase) and lose Cdt1 (Figs  
 278 2J, K) (see Discussion). These findings show that after E13.5 gradually fewer cells progress through  
 S/G2/M phases of the cell cycle and instead spend longer in G1. Our analysis suggests that this involves  
 280 either arrest as cells exit the cell cycle into G0 or G1 lengthening, with about a quarter of adult cells  
 being in a G1/S phase transition state. Together, these changes indicate a reduction in cell proliferation  
 282 in the spinal cord ventricular layer as it forms the central canal.



284

286 **Figure 2 Cell proliferation declines as the spinal cord central canal forms (Aa-Ab)** Immunofluorescence for  
 the neural progenitor marker SOX2 (magenta) and the cell proliferation marker PCNA (green) in the ventral half of  
 288 the ventricular layer at E13.5 and (Ba-Bb) at E14.5. Only cells with a punctate PCNA pattern were counted in the  
 analysis (white full box in Ab); (C) Percentage of SOX2-positive cells with a punctate PCNA pattern in the spinal

cord at E13.5 to E18.5. Each dot represents one embryo and the horizontal bars are the mean values of each stage; (Da-Db) Immunofluorescence for SOX2 (magenta) and the mitotic marker pH3 (green) in the ventral half of the ventricular layer at E13.5 and (Ea-Eb) E14.5; (F) Percentage of SOX2-positive cells labelled with mitotic marker pH3 in the spinal cord at E13.5 to E18.5. Each dot represents one embryo and the horizontal bars are the mean values of each stage; (G) mCherry-hCdt1 (magenta) and mVenus-hGeminin (green) in the ventricular layer at E13.5, (H) E15.5, (I) E18.5 and (J) the adult central canal; (K) Percentage of cells in G1/G0 (magenta), G1/S (grey), and S/G2/M (green) in the ventral half of the ventricular layer at E13.5, the whole ventricular layer at E15.5 and E18.5, and in the adult central canal of Fucci2a mice. Asterisks show significant results from two-sided, unpaired Student's t-tests between each two correlative stages. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \* $p < 0.05$ . Only statistically significant differences are indicated. The number of cells and sections analysed for each embryo and the exact p-values for all comparisons can be found in Table S4 (for C), Table S5 (for F), and Table S6 (for K). Scale bars Aa-Eb: 40  $\mu$ m, G-J: 20  $\mu$ m.

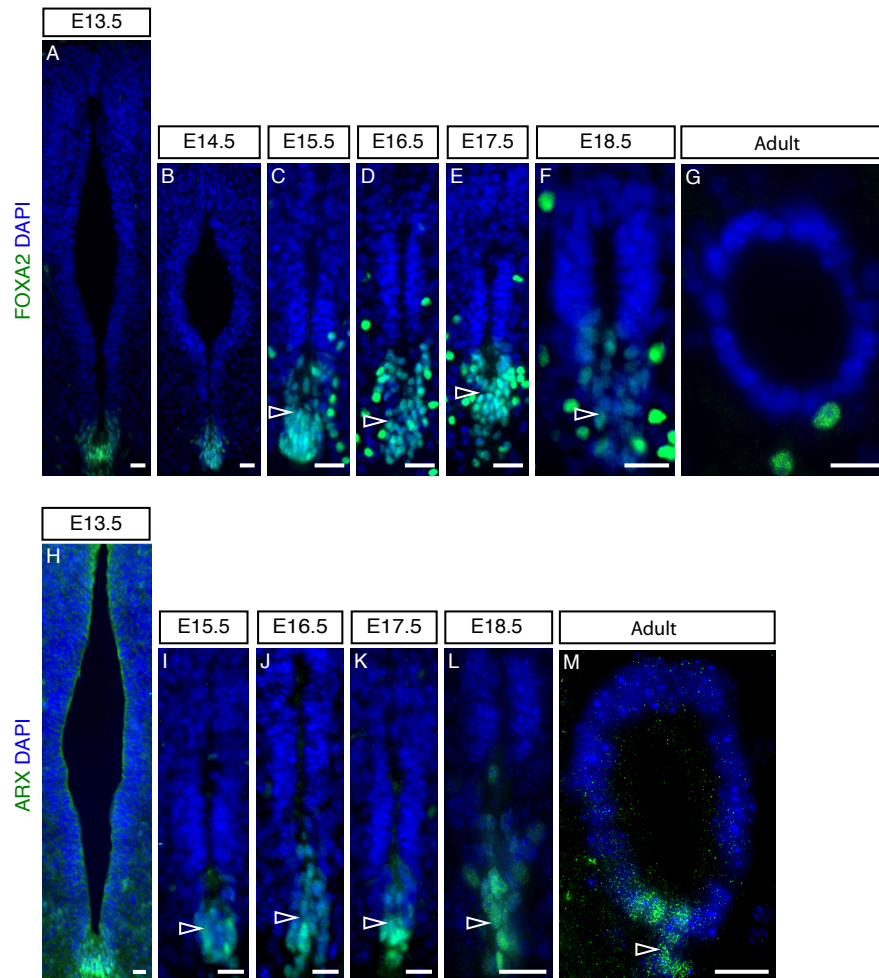
A further cellular process that could contribute to the attrition of the ventricular layer is localised programmed cell death (apoptosis). This might operate in the dorsal region as the collapse commences from E13.5 and/or during subsequent refinement of the ventricular layer cell population as the central canal forms. To investigate this possibility, we carried out a TdT-UTP nick end labelling (TUNEL) assay on transverse sections between E12.5 and E18.5 to detect DNA fragmentation, a hallmark of apoptosis. This revealed few apoptotic cells at any stage (Fig S1A-G) and this was confirmed by immunofluorescence for activated caspase-3 (Fig S1H-O). These findings indicate that apoptosis does not contribute to ventricular layer attrition.

310

### **Dissociation of a ventral-most cell population contributes to central canal formation**

Analysis of the ventral-most SOX2-positive cells during development revealed a spatial rearrangement from E15.5, which culminated in the dissociation of the majority of these cells from the forming central canal (Figs 1D-G'). To define further this ventral cell rearrangement and the cell populations involved, we analysed expression of marker proteins of the ventral-most cell population, the floor plate, as the central canal forms. FOXA2 is a transcription factor that is initially localised in the floor plate and the adjacent p3 interneuron domain (Fig 3A) (Cruz et al., 2010, Schafer et al., 2007). We found that this pattern of expression persists until E14.5 (Fig 3B) and from E15.5, while ventral ventricular layer cells remain FOXA2-positive, the ventral-most cells begin to dissociate into a distinct group (arrowheads in Figs 3C-F). In addition, a new group of cells that express FOXA2 highly, now also appears sub-ependymally (outside the ventricle) (Figs 3C-F). Moreover, while FOXA2 expression later is no longer detected in the adult central, these FOXA2-high sub-ependymal cells remain (Fig 3G) and may correspond to a subset of cerebrospinal fluid-contacting neurons (CSF-cN") (Petracca et al., 2016). *Arx*, is a further well-known floor plate marker from E9 to E12.5 (Miura et al., 1997, Cruz et al., 2010). A group of ARX-positive cells continued to define this cell population at E13.5 (Fig 3H). At later stages, ARX-positive cells form an elongated arrangement, again with some cells remaining in the ventral ventricular layer while others dissociate to form a distinct ventral group (arrowheads in Figs 3I-L). In contrast with FOXA2 expression, some ARX-positive cells then persist in the adult ventral central canal, as noted previously (Khazanov et al., 2017) (Fig 3M). These patterns of expression corroborate the dissociation of ventral-most floor plate cells observed by monitoring changing arrangement of SOX2-expressing cells and support the inclusion of some floor plate cells in the adult central canal.

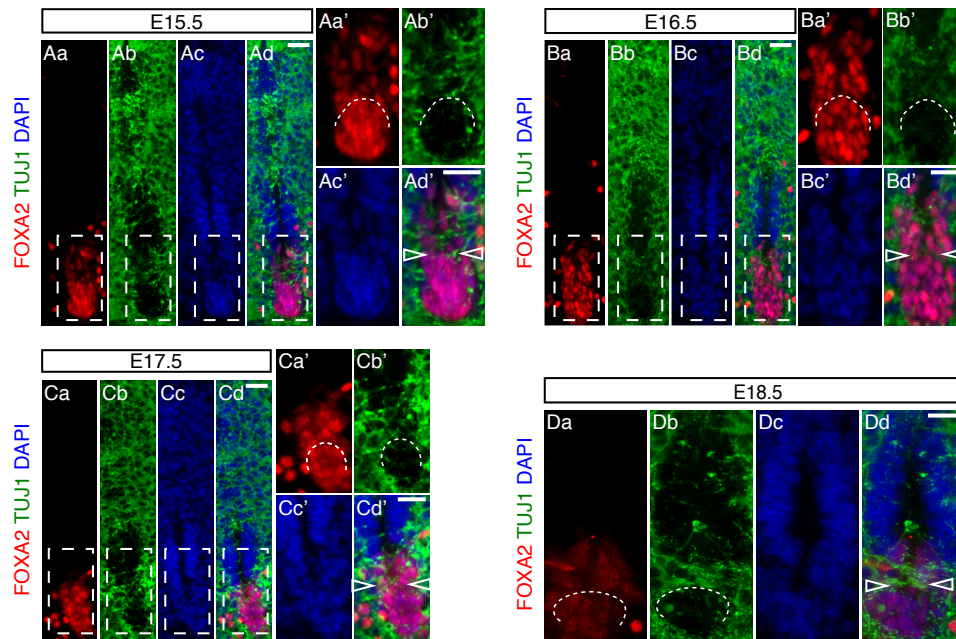
332



334 **Figure 3 FOXA2 and ARX expression in the developing and adult mouse spinal cord (A-G)**  
336 Immunofluorescence for FOXA2 (green) at the indicated stages (E13.5: 33 sections, n = 3 embryos; E14.5: 8  
338 sections, n = 2 embryos; E15.5: 18 sections, n = 5 embryos; E16.5: 22 sections, n = 5 embryos; E17.5: 16 sections,  
340 n = 4 embryos; E18.5: 26 sections, n = 4 embryos; adult (10 – 11 week old mice): 18 sections, n = 6 mice); (H-M)  
342 Immunofluorescence for ARX expression (green) at the indicated stages (E13.5: 12 sections, n = 1 embryo; E15.5:  
10 sections, n = 2 embryos; E16.5: 10 sections, n = 1 embryo; E17.5: 11 sections, n = 1 embryo; E18.5: 12 sections,  
n = 2 embryos; adult (10 and 24 week old mice): 5 sections, n = 2 mice each stage. Dorsal is top, ventral is bottom.  
Nuclei are stained with DAPI (blue). All scale bars: 20  $\mu$ m.

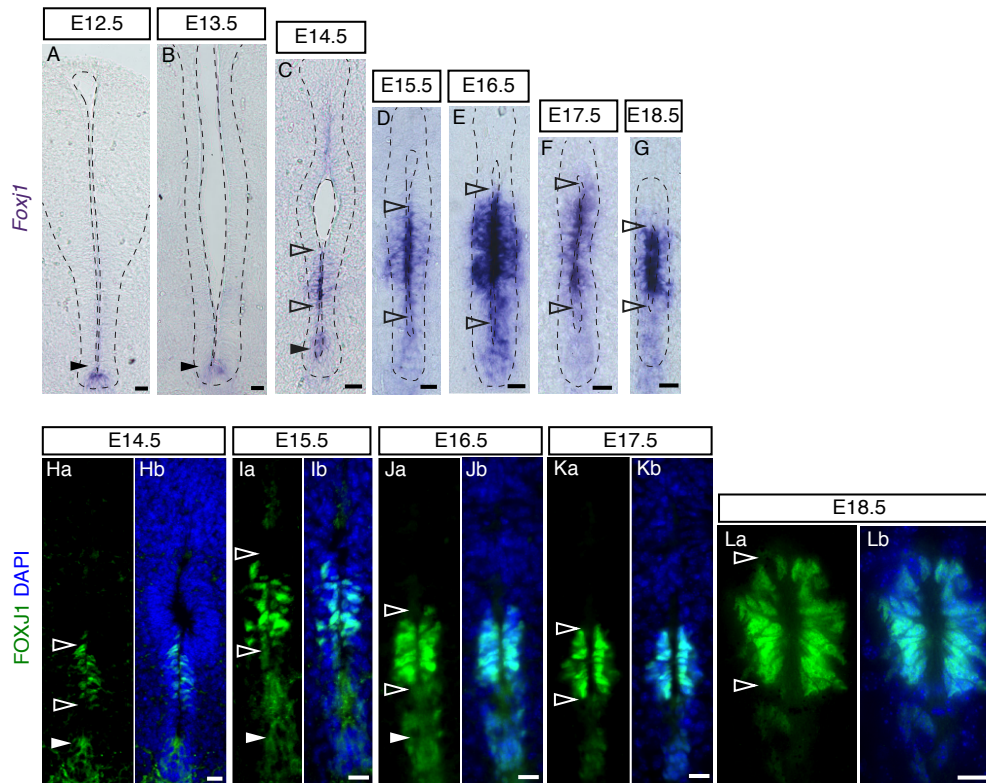
To elucidate further the remodelling of this ventral cell population, we next combined FOXA2  
344 and TUJ1 immunofluorescence, to visualise the relationship between FOXA2-positive cell groups and  
neuronal processes. This revealed TUJ1-positive cell processes intervening between the central canal  
346 and the dissociating ventral-most cell group from E15.5, with a pronounced ventral commissure  
apparent by E18.5 (Figs 4Aa-Dd).

348



350 **Figure 4 Ventral cell rearrangement during late mouse spinal cord development (Aa-Dd)**  
 352 Immunofluorescence for FOXA2 (red) and TUJ1 (green) in mouse spinal cord development at stages indicated  
 353 (E15.5: 11 sections, n = 3 embryos; E16.5: 10 sections, n = 3 embryos; E17.5: 9 sections, n = 3 embryos; E18.5:  
 354 9 sections, n = 2 embryos). Higher magnification of regions outlined in white dashed line boxes are shown in Aa'-  
 355 Ad', Ba'-Bd' and Ca'-Cd'. Partition of the ventral-most floor plate cell population by TUJ1-positive neuronal  
 356 processes, indicated by curved white dashed lines and by arrowheads in merged images. Nuclei are stained with  
 DAPI (blue). All scale bars: 20  $\mu$ m.

358 Intriguingly, this dissociation of the ventral-most cell population occurs concomitant with a  
 change in the expression pattern of *Foxj1*, the master regulator of the ciliogenesis programme (Choksi  
 360 et al., 2014) and a mediator of migratory cell behaviour of spinal cord ependymal cells in response to  
 injury (Li et al., 2018). *Foxj1* transcript and protein levels decline in the floor plate as ventral dissociation  
 362 begins at E15.5 (compare ventral region in Figs 5A-D and 5Ha-Ib), but is upregulated in ventral  
 progenitors from E14.5 (Figs 5C-5G and 5Ha-5Lb) and expressed in the majority of central canal cells,  
 364 although only at low-level in ventral-most cells at E18.5 (Li et al., 2018). Together, these observations  
 identify a distinct dissociation event which resolves the ventral ventricular cell population into two  
 366 anatomically distinct groups, the ventral central canal and the dissociated ventral-most floor plate.



368 **Figure 5 *Foxj1* mRNA and protein expression during mouse spinal cord development (A-G) *In situ***  
 370 **hybridisation showing the dynamic expression of *Foxj1* mRNA at the indicated stages (E12.5: 22 sections, n = 5**  
 372 **embryos; E13.5: 32 sections, n = 7 embryos; E14.5: 32 sections, n = 7 embryos; E15.5: 33 sections, n = 12**  
 374 **embryos; E16.5: 86 sections, n = 15 embryos; E17.5: 114 sections, n = 18 embryos; E18.5: 94 sections, n = 11**  
 376 **embryos). Note apical localisation of transcripts. Nuclei are stained with DAPI (blue); (Ha-Lb) Immunofluorescence**  
 378 **showing dynamic expression of the FOXJ1 protein (green) at indicated stages (E14.5: 66 sections, n = 4 embryos;**  
**E15.5: 33 sections, n = 3 embryos; we also confirm findings of Li et al 2018 at later stages E16.5: 12 sections, n =**  
**1 embryo; E17.5: 12 sections, n = 1 embryo; E18.5: 12 sections, n = 1 embryo). Nuclei are stained with DAPI**  
**(blue). Note that low levels/gaps in expression are found in the ventricular layer between the region closer to the**  
**dorsal lumen and the domain adjacent to the floor plate at E14.5 (arrowheads in C and Ha). Scale bars for A, B :**  
**40  $\mu$ m , for C-Lb :20  $\mu$ m.**

380

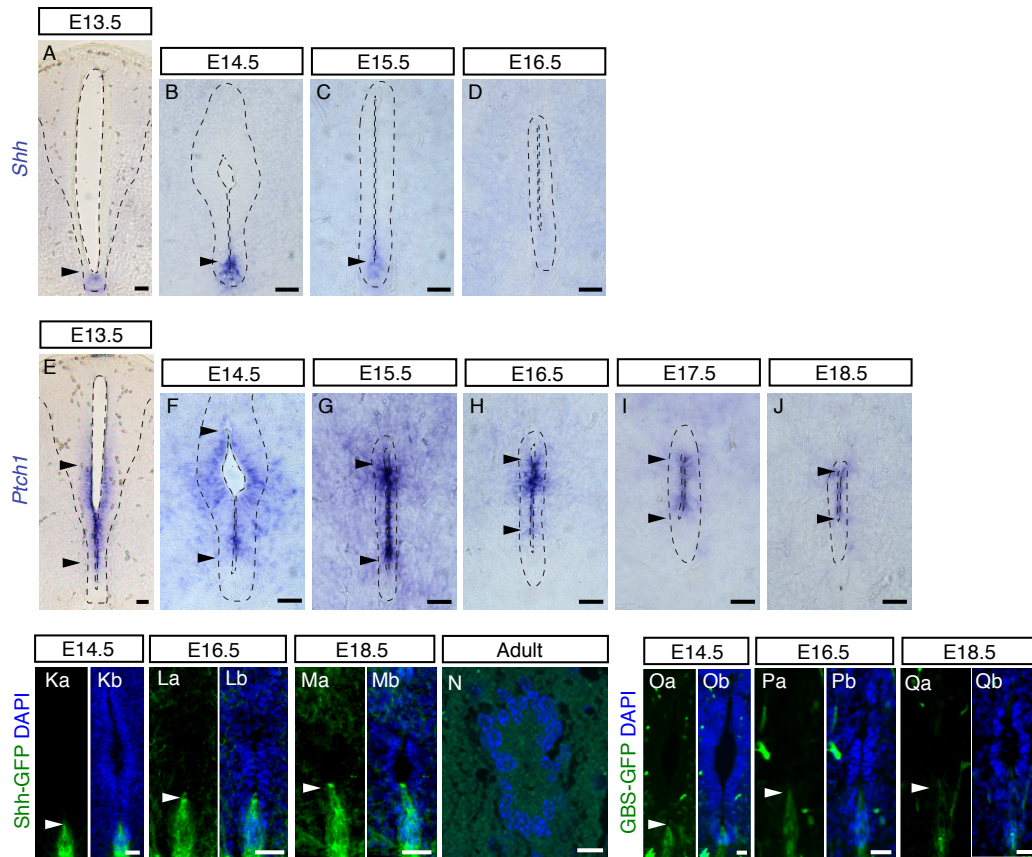
### 382 **A floor plate contribution to the spinal cord central canal is supported by analysis of *Shh* pathway components, which also reveals a gradual decline in *Shh* signalling**

384 The presence of ARX-positive cells in the ventral central canal supports the notion that some floor plate-  
 386 derived cells contribute to this structure. Indeed, lineage tracing of cells expressing the floor plate  
 388 marker *Nato3* (*Ferd3l*), using a *Nato3-LacZ* line, revealed some LacZ-positive cells in the spinal cord  
 390 central canal (Khazanov et al., 2017). Further, the regulatory relationships between *Foxa2*, *Nato3* and  
*Shh* signalling that underpin floor plate maturation (Cruz et al., 2010, Mansour et al., 2014, Mansour et  
 al., 2011), suggest that the *Shh* pathway is active as the central canal forms (Rowitch et al., 1999).  
 Moreover, as *Shh* acts as a mitogen as well as a morphogen in the early neural tube it is interesting to  
 assess whether cells retaining floor plate markers continue to act as a signalling centre during mid-late  
 development and postnatally.

392 Using *in situ* hybridization, we found that transcripts for *Shh* in the floor plate were attenuated  
at E15.5 and undetectable by E16.5 (Figs 6A-D). In contrast, transcripts for the Shh receptor and  
394 transcriptional target *Ptch1* (Goodrich et al., 1997, Marigo and Tabin, 1996, Pearse et al., 2001, Vokes  
et al., 2008) persisted for longer in the presumptive central canal, but were much reduced by E18.5  
396 (Figs 6E-J). These observations suggest that Shh signalling persists after *Shh* transcription ceases, but  
declines as the central canal forms. We investigated this further by looking at GFP expression in two  
398 Shh signalling reporter mouse lines: one expressing a tagged Shh-GFP transgene (Chamberlain et al.,  
2008) and another expressing Gli-binding site motifs linked to GFP (GBS-GFP), which provides a  
400 further readout of Shh signalling activity (Balaskas et al., 2012). Expression of Shh-GFP was restricted  
to the floor plate at all embryonic stages examined including E18.5 (Figs 6Ka-Mb). This persistence of  
402 Shh-GFP compared with that of *Shh* transcripts may be indicative of residual Shh protein (consistent  
with continued *Ptch1* expression), but could also reflect a longer half-life of the GFP tagged protein.  
404 Ependymal cells of the adult central canal, however, lacked any Shh-GFP (Fig 6N).

The presence of cytoplasmic GFP in these Shh-GFP embryos further allowed us to observe  
406 changing cell morphologies and confirmed the elongation of cells associated with the ventral  
dissociation event (Figs 6Ka-Lb). Moreover, some GFP-expressing cell processes remain integrated  
408 within the apical surface of the ventricle even at late stages (Fig 6Ma-Mb); this may reflect inclusion of  
floor plate-derived cells in the central canal or additional retained contact of the ventral-most dissociated  
410 cell group.

GBS-GFP was expressed more widely than Shh-GFP and was detected in the floor plate and  
412 adjacent ventricular layer cells at E14.5 (Fig 6Oa-Ob). However, it too also became restricted to the  
floor plate at subsequent stages, with a dwindling number of expressing cells continuing to contact the  
414 ventricular lumen at E18.5 (Figs 6Oa-Qb). These data indicate that Shh signalling persists locally for  
some days after *Shh* transcription ceases at E14.5, declining slowly as the central canal forms and  
416 appears lost in the adult. The continued contact of floor plate-derived cells with the apical surface of  
central canal lumen, however, further substantiates the contribution of some cells of floor plate origin to  
418 this structure.



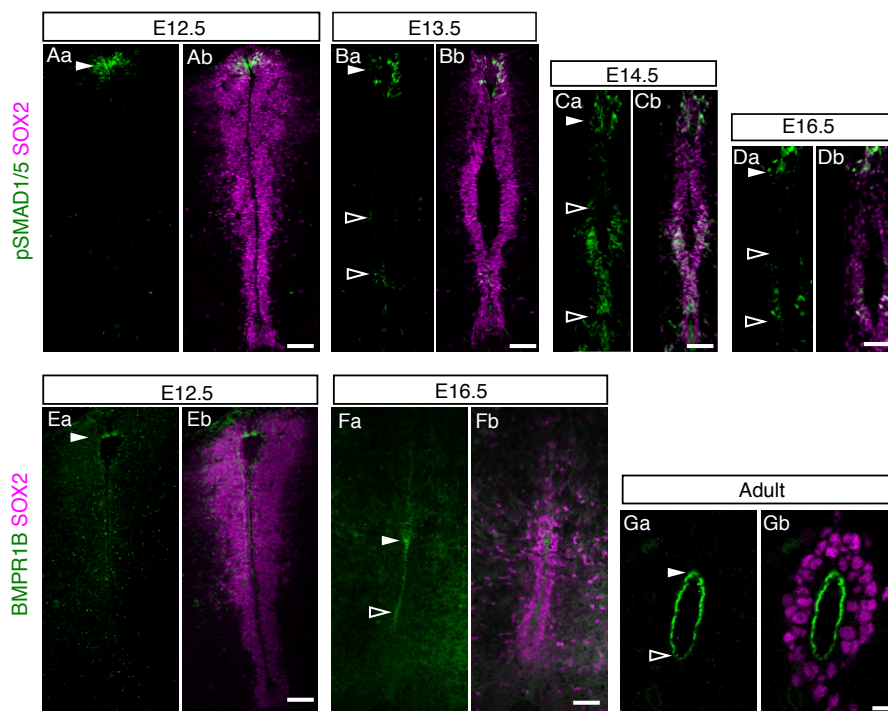
420 **Figure 6 Shh activity persists after decline in *Shh* transcripts during mouse spinal cord development (A-D)**  
 422 *In situ* hybridisation showing *Shh* mRNA expression in the floor plate (arrowheads) at the indicated stages (E13.5:  
 424 14 sections, n = 3 embryos; E14.5: 15 sections, n = 5 embryos; E15.5: 58 sections/explants, n = 9 embryos; E16.5:  
 426 38 sections/explants, n = 9 embryos). From E17.5 onwards, *Shh* mRNA expression was no longer detected in the  
 428 spinal cord (E17.5: 38 sections/explants, n = 8 embryos; E18.5: 18 sections, n = 3 embryos; 24- and 40-week-old:  
 430 17 sections, n = 3 adult mice; data not shown); (E-J) *Ptch1* mRNA expression at the indicated stages (E13.5: 27  
 432 sections, n = 5 embryos; E14.5: 26 sections/explants, n = 5 embryos; E15.5: 43 sections/explants, n = 6 embryos;  
 434 E16.5: 49 sections/explants, n = 8 embryos; E17.5: 40 sections/explants, n = 8 embryos; E18.5: 16 sections, n =  
 436 3 embryos). Note *Ptch1* is excluded from the floor plate and detected in ventral half of the ventricular layer (between  
 arrowheads) and persists in the forming central canal E18.5, but was no longer detected in adult (40-week-old: 24  
 sections, n = 3 mice; data not shown). (Ka-Mb) Immunofluorescence for GFP in Shh-GFP embryos was detected  
 in the floor plate during development (arrowheads) E14.5: 17 sections, n = 1 embryo; E16.5: 17 sections, n = 1  
 embryo; E18.5: 18 sections, n = 1 embryo, (N) but not in adult central canal (9-10 week adult (88 sections, n = 3  
 mice); (Oa-Qb) Immunofluorescence for GFP (arrowheads) in GBS-GFP embryos revealed positive cells in the  
 ventral region at indicated stages (E14.5: 47 sections, n = 3 embryos; E16.5: 43 sections, n = 3 embryos; E18.5:  
 33 sections, n = 3 embryo). Ka-Qb, nuclei are stained with DAPI (blue). All scale bars: 40  $\mu$ m, except N: 10  $\mu$ m.

#### 438 **Dorsal BMP signalling persists and extends ventrally during central canal formation**

The roof plate origin of dorsally located radial glia-like cells that remain attached to the central canal  
 440 during dorsal collapse was recently confirmed in mice (Xing et al., 2018, Shinozuka et al., 2019). During  
 development, roof plate cells express Wnt and BMP ligands, which act in opposition to Shh signalling  
 442 (Le Dreau and Marti, 2012). Moreover, BMP signalling has been implicated in the regulation of  
 quiescence and proliferation of adult brain neural stem cells (Llorens-Bobadilla et al., 2015, Mira et al.,  
 444 2010).



To investigate how BMP signalling changes as the central canal forms, we carried out  
446 immunofluorescence against phosphorylated SMAD1/5 (pSMAD1/5), a readout of BMP signalling, and  
the BMP receptor BMPR1B. Analysis of patterns of pSMAD1/5 detection revealed that BMP activity is  
448 restricted to roof plate cells at E12.5 and persists in SOX2-positive cells located dorsally until at least  
E16.5 (Figs 7Aa-Db). However, from E13.5 pSMAD1/5 was additionally detected in a ventral domain  
450 (Fig 7Ba-Bb) which became more widespread at E14.5 (Fig 7Ca-Cb) and continued to be detected  
dorsally and ventrally at E16.5 (Fig 7Da-Db). Analysis of BMPR1B expression revealed that this was  
452 present in the roof plate at E12.5 (Fig 7Ea-Eb) and that this persisted and extended ventrally at E16.5,  
where the protein was enriched apically (Fig 7Fa-Fb). This pattern of expression continued in the adult  
454 central canal, with low level protein detected ventrally (Fig 7Ga-Gb). These findings indicate that BMP  
signalling is dynamically regulated in the developing spinal cord: initial dorsally-restricted activity  
456 spreads to ventral regions and this is mirrored in the changing expression pattern of BMPR1B.



458

**Figure 7 Expanding BMP signalling and BMPR1B expression during mouse spinal cord development** (Aa-  
460 Bb) Immunofluorescence for phospho-SMAD1/5 (green) detected in a dorsal cell population at E12.5 (16 sections,  
n = 2 embryos) and E13.5 (16 sections, n = 3 embryos) (full arrowheads), phospho-SMAD1/5 was also detected  
462 in the ventral ventricular layer at E13.5 (outlined arrowhead); (Ca-Db) At E14.5 (36 sections, n = 3 embryos) and  
E16.5 (14 sections, n = 2 embryos), phospho-SMAD1/5 was detected in dorsal (full arrowheads) and ventral  
464 regions of the ventricular layer (outlined arrowheads). (Ea-Fb) BMPR1B (green) was enriched dorsally at E12.5  
(10 sections, n = 1 embryo) (full arrowhead) and was detected dorsally and apically in ventricle abutting cells  
466 (between arrowheads) at E16.5 (23 sections, n = 3 embryos); (Ga, Gb) Pronounced apical localisation of BMPR1B  
468 in adult central canal (10 – 11 week old mice: 22 sections, n = 2 mice), which was more weakly detected ventrally  
(outline arrowhead). Progenitor cells within the ventricular layer are labelled with SOX2 (magenta). All scale bars:  
470 50  $\mu$ m, except Adult: 10  $\mu$ m.

## 472 Discussion

473 This study provides evidence for multiple steps that contribute to the reduction of the spinal cord  
474 ventricular layer during development and lead ultimately to the formation of the adult spinal cord central  
canal. These include initial local loss of ventricular layer organisation below the roof plate, which  
476 spreads as dorsal collapse proceeds; a reduction in cell proliferation, involving an increase in the  
percentage of cells in the G1/S phase of the cell cycle; and cell rearrangement in the floor plate,  
478 involving dissociation of the ventral-most cell population. Despite this ventral remodelling, we provide  
further evidence that some floor plate cells are incorporated into the forming central canal. Overall,  
480 these data support a heterogeneous origin of the ependymal cell population that constitutes the adult  
spinal cord central canal, including roof plate and floor plate cells as well as ventral progenitor cells  
482 derived from *Pax6* and *Nkx6-1* expression domains.

We show that ventricular layer attrition begins not long after the switch from neurogenesis to  
484 gliogenesis in the developing spinal cord, and is first manifest by the narrowing and local disorganisation  
of the dorsal ventricular layer at E13.5. The disruption of the close alignment of SOX2-expressing  
486 progenitor cells below the roof plate appears to be the initiation site for dorsal collapse, which  
progresses rapidly in subsequent days. The appearance of neuronal cell processes between these  
488 progenitor cells and across the midline, may equate to the initiation of the dorsal commissure, which is  
more apparent at later stages in this position (Comer et al., 2015). It is interesting that the novel cell  
490 rearrangement we observe later in ventral-most regions from E16.5, named here as ventral  
dissociation, is also associated with the appearance of neuronal cell processes that traverse the  
492 midline, here to form the ventral commissure (Comer et al., 2015). In this case, these axons spatially  
separate most floor plate cells from cells that will form the adult central canal. This coincidence raises  
494 the possibility that commissure establishment is one of the mechanisms by which the ventricular layer  
is restricted in ventral as well as dorsal regions (Fig 8).

496 Studies at earlier stages in the mouse embryo (between E9.5 and E11.5) have shown that the  
cell proliferation rate in the ventricular layer declines during neurogenesis, with little difference between  
498 dorso-ventrally distinct progenitor cell populations (Kicheva et al., 2014). Our finding of a reduction in  
the proportion of cells in S and M phases of the cell cycle at later stages suggests that this decline in  
proliferation continues and contributes to ventricular layer attrition after E13.5. Intriguingly, our analysis  
500 of the cell cycle using Fucci2a reporter mice uncovered a progressive increase in cells in G1/S from  
E15.5, which ultimately accounted for almost a quarter of the cells in the adult central canal. As cells  
502 are asynchronously distributed across the cell cycle, this could be attributed to G1 lengthening: with  
more cells in G1 resulting in an increase in cells transitioning from G1 to S phase at a given time.  
504 Another intriguing possibility is that ventricular cells progressively switch to a cell cycle variant known  
as endocycle as they differentiate into ependymal cells. Endocycling cells go through G1 and S but do  
506 not undergo mitosis, becoming polyploid (Edgar et al., 2014). This would be consistent with the  
progressive increase in the percentage of G1/S cells and the decrease in S/G2/M cells we detect in  
508 Fucci2a embryos and mice. Indeed, in other studies using Fucci reporters, endoreplicating cells seem  
to co-express Geminin and Cdt1 (and thus appear yellow) for longer (Cao et al., 2017, Lazzeri et al.,  
510

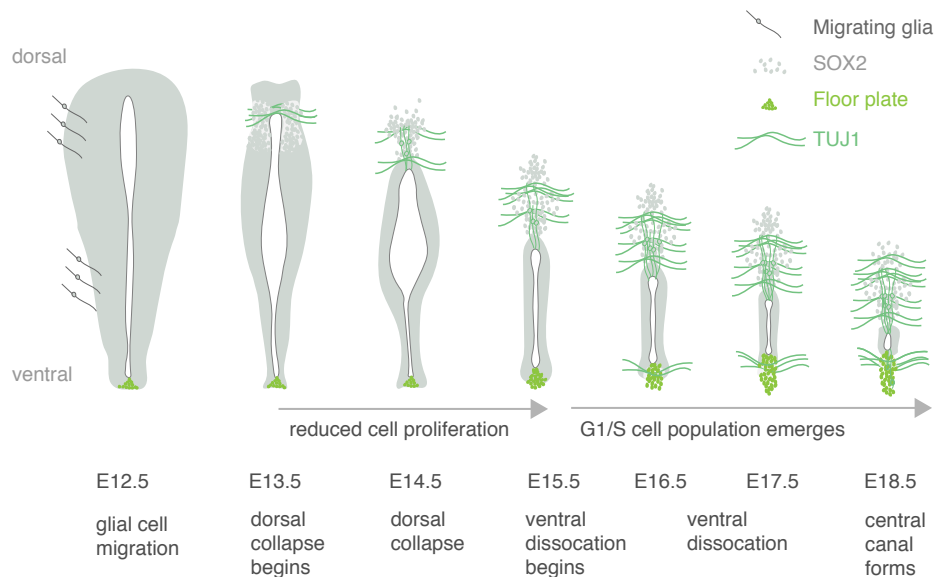
2018). A switch to endocycling would potentially lead to higher genomic output, which might then  
512 enhance the secretory function of these cells. A further possibility is that the overall decline in cell  
proliferation is due to an increase in the cells that enter the quiescent state (G0) in G1. However, it is  
514 difficult to distinguish cells in G1 or G0 using the Fucci2a system as they both appear red. Whether the  
movement of cells into a reversible G0 state explains why adult central canal cells are mostly quiescent  
516 but can readily re-enter the cell cycle in response to multiple extrinsic stimuli or if G1/S cells also  
contribute to the reactivity of this cell population remains to be investigated. The latter are certainly an  
518 intriguing cell population that is not apparent in post-mitotic brain ependymal cells analysed in the  
Fucci2a mouse (Ford et al., 2018).

520 Early expression pattern studies in a range of amniote embryos suggest that the progenitor cell  
population expressing *Pax6* and *Nkx6-1*, but not *Nkx2-2*, is retained during development and gives rise  
522 to the adult central canal (Fu et al., 2003, Yu et al., 2013). Accumulating evidence, however, shows that  
the origin of spinal cord central canal cells is rather heterogeneous, including cells derived from the roof  
524 plate (Xing et al., 2018, Shinozuka et al., 2019) and from the floor plate (Khazanov et al., 2017, Ghazale  
et al., 2019) (and this study). Here, we found that cells expressing the floor plate marker ARX, but not  
526 FOXA2, are present in the adult spinal cord central canal and that Shh-GFP expressing cells are found  
integrated in the forming central canal at late embryonic stages.

528 An outstanding question is whether cells derived from the roof plate and floor plate remain as  
signalling centres during late embryonic development and into the adult spinal cord, as they do in  
530 regenerative species such as the axolotl and zebrafish (Schnapp 2005, Reimer 2009). Here, we have  
shown that Shh signalling declines from the ventral midline/floor plate at late embryonic stages and that  
532 Shh-GFP is no longer detected in central canal cells of the adult spinal cord. While it is possible that  
the detection of GFP in Shh-GFP and GBS-GFP transgenic embryos reflects the relatively long half-life  
534 of GFP rather than persistence of SHH, the detection of Shh targets *Ptch1* (this study) and *Gli* (Yu et  
al., 2013), support the possibility of dwindling Shh signalling at these late stages. This would also be  
536 consistent with studies using transgenic mice to delete *Shh* from the floor plate or its mediator  
*Smoothed* from the neural tube (Yu et al., 2013), as well as *Gli2* mutants (Ding et al., 1998, Matisse  
538 et al., 1998), all of which fail to form a proper central canal, although confirmation of a persisting role  
for Shh signalling requires interference with this pathway specifically at late stages or in the adult.

540 In contrast, we found that the activity of the BMP signalling pathway, which opposes Shh  
signalling in the developing spinal cord, although initially dorsal expands as the central canal forms,  
542 with pSMAD1/5 detected extensively in more ventral regions as well as in dorsal roof plate-derived  
cells. This is further reflected by BMPR1B expression, which was detected in all cells in the adult central  
544 canal albeit at a lower level in the most ventral cells. A recent study has shown that Wnt signalling first  
from roof plate cells and later from the ependymal cells is required to maintain the adult central canal  
546 (Xing et al., 2018), while Wnt promotion of BMP signalling (found at earlier stages (Ille et al., 2007)),  
would be further consistent with the expansion of dorsal signalling. Taken together, this investigation of  
548 these key signalling pathways in the late embryonic and adult spinal cord suggests that dorsal signalling  
comes to prevail in cells of the ventricular layer as the central canal forms and ependymal cells  
550 differentiate. The involvement of BMP signalling in the regulation of adult neural stem cell quiescence

552 in the brain (Llorens-Bobadilla et al., 2015, Mira et al., 2010) now prompts further experiments to  
 investigate whether this spread of BMP activity promotes acquisition of the quiescent cell state,  
 including changes in cell cycle and thereby sets aside the elusive spinal cord neural stem cell as the  
 554 central canal forms.



558 **Figure 8 Summary of steps contributing to spinal cord ventricular layer attrition** Schematic of key changes  
 in cell arrangement and cell cycle in the spinal cord ventricular layer at daily intervals leading up to the formation  
 of central canal. Glial cell migration precedes dorsal collapse, which is accompanied by appearance of the dorsal  
 560 commissure and reduced proliferation of ventricular layer cells. This is then followed by ventral dissociation of the  
 floor plate cell population, accompanied by appearance of the ventral commissure and an increase in percentage  
 562 of cells in G1/S phase of the cell cycle.

564

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572

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582 **Author contributions**

KGS and PF designed the project. MAC, GS, NS and MG designed and performed experiments. MAC,  
584 ARA and KGS interpreted the data and wrote the paper. All authors read and approved the final  
manuscript.

586 **Competing interests**

The authors declare that they have no competing interests.

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