A SMAD1/5-YAP signalling module drives radial glia self-amplification and growth of the developing cerebral cortex

Sonia Najas1,2,a†, Isabel Pijuan1,2,†, Anna Esteve-Codina3, Susana Usieto1, Juan D. Martinez1, An Zwijsen4, Maria L. Arbonès1,2, Elisa Martí1 and Gwenael Le Dréau1,§

ABSTRACT

The growth and evolutionary expansion of the cerebral cortex are defined by the spatial-temporal production of neurons, which itself depends on the decision of radial glial cells (RGCs) to self-amplify or to switch to neurogenic divisions. The mechanisms regulating these RGC fate decisions are still incompletely understood. Here, we describe a novel and evolutionarily conserved role of the canonical BMP transcription factors SMAD1/5 in controlling neurogenesis and growth during corticogenesis. Reducing the expression of both SMAD1 and SMAD5 in neural progenitors at early mouse cortical development caused microcephaly and an increased production of early-born cortical neurons at the expense of late-born ones, which correlated with the premature differentiation and depletion of the pool of cortical progenitors. Gain- and loss-of-function experiments performed during early cortical neurogenesis in the chick revealed that SMAD1/5 activity supports self-amplifying RGC divisions and restrains the neurogenic ones. Furthermore, we demonstrate that SMAD1/5 stimulate RGC self-amplification through the positive post-transcriptional regulation of the Hippo signalling effector YAP. We anticipate this SMAD1/5-YAP signalling module to be fundamental in controlling growth and evolution of the amniote cerebral cortex.

KEY WORDS: Cerebral cortex, Neurogenesis, Radial glial cells, Bone morphogenetic proteins (BMPs), SMAD transcription factors, YAP, Mouse

INTRODUCTION

The cerebral cortex is the region of the human brain responsible for higher cognitive functions. Errors during its formation provoke a vast array of brain disorders that affect intellectual ability and social behaviour (Hu et al., 2014; Jayaraman et al., 2018). This highlights the relevance of identifying the mechanisms that govern cortical development, in particular those controlling its growth and the production of neurons from cortical stem and progenitor cells.

Emerging in the dorsal part of the telencephalon (pallium), the mammalian cerebral cortex first consists of a pseudo-stratified epithelial layer (also called the ventricular zone, VZ) formed by primitive neural stem cells, the neuroepithelial cells, that rapidly mature into radial glial cells (RGCs) (Taverna et al., 2014). Like neuroepithelial cells, RGCs contact both the ventricle and the basal lamina, they divide at the apical membrane and they can expand through self-amplifying divisions that produce two daughter RGCs (De Juan Romero and Borrell, 2015; Taverna et al., 2014). This self-amplification of neuroepithelial cells and RGCs is the only mode of division that can ensure the tangential growth (i.e. along the rostral-caudal and medial-lateral axes) of the cortical VZ (De Juan Romero and Borrell, 2015; Taverna et al., 2014). Neurogenesis is initiated when RGCs start producing daughter cells committed to the neuronal lineage. Clonally-related cortical neurons (originating from a same original RGC) migrate basally with little tangential dispersion and appear to mature in a radial column, the position of which reflects that of their mother RGC, thereby ensuring the radial growth of the developing cerebral cortex (De Juan Romero and Borrell, 2015; Taverna et al., 2014). Neurogenesis can occur directly, when an RGC divides asymmetrically to generate another RGC and a daughter cell that differentiates directly into a neuron (De Juan Romero and Borrell, 2015; Taverna et al., 2014). Alternatively, neurogenesis can be indirect, whereby an RGC gives rise to an RGC and a basal progenitor (BP) that harbours a restricted lineage potential, delaminates from the VZ and divides basally. These BPs will increase the neuronal output, possibly self-amplifying for several rounds of divisions before generating two neurons through a final self-consuming division (Lui et al., 2011). Although intermediate progenitor cells (IPCs) possess a very limited self-amplifying potential and represent the vast majority of cortical BPs in lissencephalic species, basal (or outer) RGCs’ harbour a considerable self-amplifying potential and they are responsible for the tremendous production of neurons and the larger neocortex observed in gyrencephalic species (Lui et al., 2011; Shitamukai et al., 2011; Wang et al., 2011). Therefore, the decision of an RGC to self-amplify or to give rise to neurons, either directly or indirectly, has a huge impact on the final number of cortical neurons and represents the primary event that defines the tangential and radial growth of the tissue.

As highlighted by the gene mutations causing primary microcephaly, a variety of intracellular events regulate cerebral cortical size, including centrosome behaviour and centriole biogenesis, DNA replication and repair, cytokinesis and apical-basal polarity (Jayaraman et al., 2018; Saade et al., 2018). Moreover, the balance between RGC self-amplification and neurogenesis appears to be regulated by extrinsic cues emanating from the ventricular fluid, meninges, blood vessels and neighbouring cells (Llinares-Benadero and Borrell, 2019; Martynoga et al., 2012; Taverna et al., 2014). Thus, the molecular
events regulating RGC fate are complex and they are still not fully understood.

Seminal studies performed on cortical progenitors in vitro suggested that bone morphogenetic protein (BMP) signalling regulates their neurogenic potential (Li et al., 1998; Mabie et al., 1999). The microcephaly described in Bmp7 mutant mice, and the overproliferation and premature differentiation reported in the brains of transgenic mice expressing constitutively active forms of the type-1 BMP receptors Alk3 (also known as Bmpr1a) or Alk6 (Bmpr1b) supported this idea (Panchision et al., 2001; Segkla et al., 2012). However, definitive proofs of an instructive role for BMP signalling in cortical RGC fate decision in vivo are still lacking.

Here, we found that the activity of the transcription factors (TFs) SMAD1/5, two canonical effectors of BMP signalling, promotes cortical RGC self-amplification in both chick and mouse embryos, preventing their premature neurogenic switch and exhaustion, and thereby ensuring the appropriate production of the distinct classes of cortical excitatory neurons. In searching for the effectors of SMAD1/5 activity, we show that this role depends on the post-transcriptional regulation of YAP, a core component of the Hippo signalling pathway known to regulate cell growth and organ size. Together, our findings reveal an instructive and evolutionarily conserved role of the canonical BMP effectors SMAD1/5 in the control of RGC self-amplification in the developing cerebral cortex.

RESULTS

SMAD1/5 activity is required for brain growth and cortical neurogenesis in mouse

To determine the role played by the canonical BMP pathway during mammalian cerebral cortex development, we focused on the function of its canonical effectors SMAD1, SMAD5 and SMAD9 (human SMAD8) during mouse corticogenesis. In situ hybridization revealed that mSmad1 and mSmad5 transcripts are expressed throughout the rostral-caudal axis of the developing mouse cerebral cortex at embryonic day (E) 14.5 and are particularly enriched in the VZ, whereas mSmad8/9 transcripts were not detected (Fig. 1A). In agreement with previous observations (Saxena et al., 2018), immunostaining at mid-corticogenesis with an antibody that specifically recognizes the active carboxy-terminal phosphorylated form of SMAD1/5/8 (pSMAD1/5/8) revealed activity of these canonical BMP effectors in differentiating neurons as well as in both apically- and basally-dividing cortical progenitors (Fig. 1B). When quantified in phospho-Histone 3+ (pH3+) mitotic nuclei, pSMAD1/5/8 immunostaining revealed SMAD1/5 activity to be stronger in apically-dividing mouse RGCs than in basally-dividing IPCs (Fig. 1C), suggesting a correlation between high SMAD1/5 activity and RGC maintenance.

To understand the role played by SMAD1/5 during mouse cerebral cortex development, we crossed Smad1wt/fl;Smad5wt/fl mice (Moya et al., 2012) with a Nestin:cre transgenic line that produces Cre-mediated recombination in neural progenitor cells and somites as early as E8.5 (Petersen et al., 2002), earlier than the more commonly used Nestin:cre transgenic line that produces efficient Cre-mediated recombination in cortical progenitors from mid-embryogenesis (Liang et al., 2012). In agreement with the early Cre-mediated recombination expected from this Nestin:cre transgenic line (Petersen et al., 2002), SMAD1 and SMAD5 protein levels were reduced by 54% and 32% in telencephalic extracts from E11.5 Smad1wt/fl;Smad5wt/fl;Nestin:Cre0/0 heterozygous embryos relative to their control Smad1wt/fl;Smad5wt/fl;Nestin:Cre−/− littermates (Fig. S1). These Smad1wt/fl;Smad5wt/fl;Nestin:Cre0/0 heterozygous mutant mice were viable and born following Mendelian ratios but were sterile, precluding the study of the homozygous compound mutants. Compared with their Smad1wt/fl;Smad5wt/fl;Nestin:Cre0/0 littermates (hereafter referred to as controls or +/+), the Smad1wt/fl;Smad5wt/fl;Nestin:Cre−/− heterozygous mutant mice (hereafter referred to as SmadNes mutants or +/-) presented an overall growth retardation, including a reduction in brain weight, detected from postnatal day (P)7 (Fig. 1D,E). The brain of the adult (P60) SmadNes mutants showed a 25% reduction in weight (+/-: 0.407 g ± 0.006 versus +/+: 0.544 g ± 0.021; mean ± s.d.; Fig. 1E), that correlated to a 29% decrease in volume (Fig. 1F,G) and an 18% reduction of its surface area in coronal sections (+/-: 42.19 mm² ± 2.30 versus +/+: 52.46 mm² ± 1.18, Fig. 1H-K). These reductions in brain weight and size were both greater than three standard deviations implying, according to clinical standards (Passemerd et al., 2013), that the SmadNes heterozygous mutants suffer a severe microcephaly. The coronal areas of the whole brain and cerebral cortex were similarly reduced in adult SmadNes mutants (18% and 19%, respectively; Fig. 1K,L), and this reduction was constant across the rostral-caudal axis (Fig. S2). Constant decreases in the length of the cortical VZ and pia along this axis were also observed in the SmadNes mutant brain (Fig. 1M,N and Fig. S2). Apart from these growth defects and a thinner corpus callosum (Fig. 1O), the brain of the SmadNes mutants did not present any major neuro-anatomical defects (Fig. 1J and Fig. S2B), thereby suggesting that SMAD1/5 inhibition impaired growth equally in all brain regions. Importantly, the brain area, cortical area and length of the cortical VZ were also reduced in E17.5 SmadNes mutant embryos (Fig. S3), indicating that these brain growth defects have an embryonic origin.

The cerebral cortices of the adult SmadNes mutants and their control littermates showed comparable thicknesses and presented similar densities of NeuN+ (also known as Rbfox3) neurons (Fig. 2A-C). They also presented similar densities of macroglial cells, including SOX9+ astrocytes (Fig. S4A,B; Sun et al., 2017), and oligodendroglial cells [SOX10+ or OLIG2+;CC1+ (APC) oligodendrocytes and OLIG2+;CC1+ progenitors, Fig. S4C-F; Bhat et al., 1996; Claus Stolt et al., 2002]. Taken together, these findings suggested that SMAD1/5 inhibition affects the tangential growth of the brain and the generation of its radial columns rather than its radial growth and the number of cells per radial unit. Nevertheless, we observed that the relative proportions of the different neuronal layers forming the cerebral cortex were altered in the adult SmadNes mutant (Fig. 2A,C). The number of early-born neurons forming the deep layer L6 and its thickness were increased, whereas these parameters were diminished in the superficial layer L2/3 containing late-born callosal projection neurons (Fig. 2A,C, D), consistent with the reduced thickness of the corpus callosum (Fig. 1O). This phenotype was observed at the early postnatal stage P7 (Fig. 2E-G), supporting the idea that these cortical defects originated during the embryonic phase.

Although the SmadNes mutant embryos did not present any obvious defect in telencephalic patterning (Fig. S5A), their programme of cortical neurogenesis did appear to be altered (Fig. 3). Around the onset of cortical neurogenesis (E11.5), the SmadNes mutant embryos presented RGCs [PAX6+;TBR2+ (EOMES) cells] in correct numbers per radial area and of a normal cell size (Fig. 3A-C and Fig. S5B). At this early neurogenic stage, the SmadNes mutant embryos also presented correct numbers of TBR2+ IPCs and a germinal zone of differentiating TBR1+ neurons in the SmadNes mutant cortex than in their control littermates from E11.5 onwards (Fig. 3H, I; Bulfone et al., 1995). The developing cerebral cortex of the SmadNes mutant
embryos contained fewer IPCs from E13.5 onwards and fewer RGCs at E17.5 (Fig. 3B-D). This decrease in cortical progenitors at E17.5 was associated with a reduced thickness of the germinal zones (Fig. 3E), a lower neuronal output (Fig. 3F,G) and fewer late-born CUX1+ and SATB2+ neurons (Fig. 3J,K and Fig. S6; Britanova et al., 2008; Nieto et al., 2004). Together, these data confirmed the developmental origin of the alterations in cortical projection neurons seen in the cerebral cortex of P7 and P60 SmadNes mutants (Fig. 2). The mitotic indices of the cortical RGCs and IPCs were comparable in SmadNes mutants and controls at all stages examined (Fig. S7), suggesting that the proliferation rate of the cortical progenitors is not severely affected in SmadNes mutants. We thus reasoned that the increased production of early-born neurons, the premature reduction in the number of RGCs and IPCs, and the decreased production of late-born neurons observed in the cerebral cortex of SmadNes heterozygous mutant embryos might likely be the result of a premature switch of RGCs from self-amplifying to neurogenic divisions during early corticogenesis.

**SMAD1/5 activity is required for RGC self-amplification during chick cortical neurogenesis**

There is increasing evidence that the basic gene regulatory networks, progenitor cell types and cellular events governing the generation of neurons during corticogenesis are evolutionarily
Fig. 2. Inhibiting SMAD1/5 activity in mouse neural progenitors causes an increase in early-born neocortical neurons at the expense of late-born ones. (A,B) NeuN+ neurons present in coronal sections of the brains of SmadNes mutant mice (Smad1wt/fl;Smad5wt/fl;Nestin:Cre0/0, +/-) and their control littermates (Smad1wt/fl;Smad5wt/fl;Nestin:Cre0/0, +/+ ) at P60 (A), and their mean number±s.d. quantified in a 100 µm-wide cortical area, obtained from 3 +/- and 4 +/- animals (B). (C,D) Mean thickness of the cortical neuronal layers±s.e.m. (C) and mean number of NeuN+ neurons±s.e.m. in the different layers in a 100 µm-wide cortical area at P60 (D), obtained from 4 +/- and 3 +/- animals. (E-G) Early- and late-born neocortical neurons present in coronal sections of the brains of SmadNes mutant pups and their control littermates at P7. Early-born L6 (TBR1+), L5 (CTIP2+) and late-born L4-2/3 (CUX1+) projection neurons (E), mean thickness of the layers±s.e.m. (F) and mean number of neurons±s.e.m. in the different layers (G) in a 100 µm-wide cortical area, obtained from 5-7 +/- and 5 +/- animals. Significance was assessed using the non-parametric Mann–Whitney test (B, C, D, and G for the total cumulated thickness) or a two-way ANOVA+Sidak’s test (C, D, F, G). *P<0.05, **P<0.01, ***P<0.001; n.s., P>0.05. See also Fig. S3. L1-6, cortical neuronal layers 1-6; SP, subplate; C.C, corpus callosum. Scale bars: 100 µm.

Conserved between mammals and sauropsids, particularly birds (Cardenas et al., 2018; Le Dreau et al., 2018; Nomura et al., 2013; Suzuki et al., 2012; Yamashita et al., 2018). To assess whether SMAD1/5 regulate the mode of division of cortical RGCs, we turned to the developing chick cerebral cortex, as this avian model offered greater possibilities than the mouse to accurately manipulate SMAD1/5 activity at the onset of cortical neurogenesis.

The production of neurons in the developing chick cerebral cortex spans from E3 to E8 (Fig. 4A,B; Suzuki et al., 2012). As in mammals, cortical neurogenesis in the chick is initiated by the onset of neurogenic divisions of PAX6+;TBR2+ RGCs that divide apically in the VZ (Fig. 4A,B). From E5 onwards, the production of cortical neurons is enhanced by symmetric neurogenic divisions of basally-dividing TBR2+ IPCs (Fig. 4A,B; Suzuki et al., 2012). In situ hybridization revealed that both cSmad1 and cSmad5 transcripts are expressed throughout the neurogenic period, mostly in the VZ where cSmad8 transcripts were essentially absent (Fig. S8). Immunostaining with the pSMAD1/5/8 antibody at E5 revealed activity of SMAD1/5 in both apically- and basally-dividing cortical progenitors as well as in differentiating neurons (Fig. 4C), as previously observed in the developing mouse cortex (Fig. 1B). When quantified in pH3+ mitotic nuclei, SMAD1/5 activity was weaker in basal IPC divisions than in RGC divisions (Fig. 4D). When quantified after in ovo electroporation of a pTis21(Btg2):RFP reporter that is specifically activated during neurogenic divisions (Fig. S9; Le Dreau et al., 2014; Saade et al., 2013; Saade et al., 2017), SMAD1/5 activity was diminished in pTis21:RFP+ neurogenic divisions relative to pTis21:RFP- self-amplifying divisions (Fig. 4E,F). Therefore, a positive correlation exists between SMAD1/5 activity and the potential for RGC self-amplification during chick cortical neurogenesis.

Endogenous SMAD1/5 activity was inhibited from the onset of cortical neurogenesis by in ovo electroporation of sh-RNA plasmids that specifically target cSmad1 or cSmad5 and reduced their transcript levels by 40% and 60%, respectively (sh-S1/5; Le Dreau et al., 2012). Inhibiting SMAD1 or SMAD5 activity resulted in a similar phenotype, nearly doubling the proportion of electroporated RGCs undergoing neurogenic pTis21:RFP+ divisions (Fig. 4G,H). This precocious switch to neurogenic divisions provoked a premature and accelerated depletion of electroporated PAX6+; TBR2- RGCs, their accelerated progression towards a committed TBR2+ fate and, ultimately, their differentiation into SOX2+;HuC/D+ (Elavl3/4) and TBR1+ neurons (Fig. 4I-K). Thus, full SMAD1/5 activity is required to support RGC self-amplification during chick cortical neurogenesis.

Conversely, enhancing SMAD5 activity through in ovo electroporation of a constitutively active SMAD5 mutant (SMAD5-S/D; Le Dreau et al., 2012) produced the opposite phenotype and reduced by 50% the proportion of electroporated RGCs undergoing neurogenic pTis21:RFP+ divisions (Fig. 5A,B). This reduction in neurogenic divisions was associated with the electroporated cells remaining as PAX6+; TBR2-; HuC/D+ neurons (Fig. 5C-D). Thus, the SMAD5-S/D construct rescued the phenotype caused by sh-S5 (Fig. 5E,F). In 5 out of 20 electroporated embryos, SMAD5-S/D electroporation itself caused the abnormal generation of ectopic rosettes of cortical progenitors, which developed an apical-basal polarity (Fig. S10). Together, these data revealed that the fine tuning of SMAD1/5 activity is required to properly balance the self-amplification of RGCs with the production of cortical excitatory neurons.
Fig. 3. Inhibiting SMAD1/5 activity in mouse cortical progenitors causes premature neurogenesis and depletion of RGCs and IPCs. (A) Timeline of cortical neurogenesis and BrdU injections during mouse embryonic development. (B-J) Analysis of corticogenesis in the developing cerebral cortex of SmadNes mutant embryos (Smad1wt/fl;Smad5wt/fl;Nestin:Cre+/0, +/−) and their control littermates (Smad1wt/fl;Smad5wt/fl;Nestin:Cre0/0, +/+). (B-D) Immunostaining of cortical progenitors and mean number±s.d. of PAX6+/TBR2− RGCs (C) and TBR2+ IPCs (D) quantified in a 100 µm-wide cortical area, obtained from 5, 4, 4 +/+ and 5, 3, 4 +/− animals analyzed at E11.5, E13.5 and E17.5, respectively. (E) Mean thickness of VZ+SVZ±s.d., obtained from 5, 4, 4 +/+ and 5, 4, 5 +/− animals at E11.5, E13.5 and E17.5, respectively. (F,G) Neuronal output defined as the mean percentage±s.d. of BrdU+/Tuj1+/BrdU− cells quantified in a 100 µm-wide cortical area 24 h after a BrdU pulse (see A), obtained from 5, 4, 3 +/+ and 5,4,4 +/− animals at E11.5, E13.5 and E17.5, respectively. (H-K) Immunostaining and mean number±s.d. of early-born (TBR1; H,I) and late-born (CUX1; J,K) projection neurons quantified in a 100 µm-wide cortical area, obtained from 5, 4, 4 +/+ and 5, 3, 4 +/− animals at E11.5, E13.5 and E17.5, respectively (I), and 5 +/+ and 5 +/− animals at E17.5 (K). Each dot represents the value of one animal. Significance was assessed using the two-sided unpaired t-test (C,D,G,I), the non-parametric Mann–Whitney test (K) or a two-way ANOVA+Sidak’s test (E). *P<0.05, **P<0.01, ***P<0.01; n.s, P>0.05. CP, cortical plate; IZ, intermediate zone; L1-6, cortical neuronal layers 1-6; SP, subplate; SVZ, sub-ventricular zone; VZ, ventricular zone. See also Figs S4-S6. Scale bars: 50 µm.
Fig. 4. Inhibiting SMAD1/5 activity in chick cortical RGCs increases neurogenic divisions and causes their premature depletion and differentiation.

(A) Schematic showing cortical neurogenesis in the chick, its main cortical progenitor subtypes and their modes of division. (B) Coronal sections from 3, 5 and 8 day-old chick embryos showing the PAX6+ developing cerebral cortex, formed of SOX2+ neural progenitors, differentiating HuC/D+ and TBR1+ neurons, PAX6+; TBR2− RGCs undergoing mitosis at the apical surface (black arrowheads) and TBR2+ IPCs dividing mostly basally (white arrowheads). Panels on right show magnification of boxed areas in left panels. (C,D) The active, phosphorylated form of SMAD1/5/8 (pSMAD1/5/8) immunoreactivity at E5 (C), and its mean intensity±s.d. measured in 276 apical and 93 basal mitoses obtained from five embryos (D). Panels on right show magnification of boxed area in left panel. Dotted circles indicate pH3+ mitotic nuclei. (E,F) The pSMAD1/5/8 immunoreactivity in mitotic cortical progenitors 24 h after in ovo electroporation (IOE) with the pTis21:RFP reporter along with a control H2B-GFP-producing plasmid (E), and its mean intensity±s.d. quantified in 137 pTis21:RFP+ and 107 pTis21:RFP− divisions derived from eight embryos (F). Dotted circles indicate pH3+ mitotic nuclei. (G,H) The mean proportion±s.d. of electroporated (H2B-GFP+) cortical progenitors undergoing pTis21:RFP+ divisions (white arrowheads) or pTis21:RFP− divisions (black arrowheads) after IOE of shRNAs targeting cSmad1 or cSmad5 (sh-S1/5, n=12 embryos) or their control (n=9). (I,J) Representative images (I) and mean proportions±s.e.m. (J) of electroporated (H2B-GFP+) cells marked as SOX2+/HuC/D−/TBR2−/− (top), PAX6+/−/−/TBR2+/− (middle) and HuC/D+/−/−/TBR1+/− (bottom), assessed 48 h after IOE with sh-S1 (n=8, 7, 5 embryos), sh-S5 (n=12, 5, 6) or their control (n=13, 8, 9). (K) The mean proportion±s.d. of electroporated cells identified as PAX6+; TBR2− RGCs (top), TBR2+ committed cells (middle) and HuC/D+ neurons (bottom), assessed 24 (E4), 48 (E5) and 72 (E6) h after IOE and obtained from n≥6 embryos per condition and stage. Significance was assessed using the non-parametric Mann–Whitney test (D,F), the two-sided unpaired t-test (H) or a two-way ANOVA+Tukey’s (J) or Sidak’s (K) test. **P<0.01, ***P<0.001; ns, not significant. D, dorsal; L, lateral; M, medial; MZ: mantle zone; N, neuron; NN, symmetric neurogenic division; PN, asymmetric division; PP, self-amplifying division; SVZ, sub-ventricular zone; V, ventral; VZ, ventricular zone. See also Figs S7 and S8. Scale bars: 50 µm (B,C,I); 10 µm (E,G).
Fig. 5. Increasing SMAD1/5 activity in chick cortical RGCs impedes neurogenic divisions and restrains differentiation. (A,B) Representative images (A) and mean proportion±s.d. (B) of electroporated (H2B-GFP⁺) cortical progenitors undergoing pTis21:RFP⁺ divisions (white arrowheads) or pTis21:RFP⁻ divisions (black arrowheads) after in ovo electroporation (IOE) of a constitutively active SMAD5 mutant (5-S/D, n=8 embryos) or its control (n=9). (C,D) Representative images (C) and mean proportion±s.e.m. (D) of electroporated (H2B-GFP⁺) cells marked as PAX6−/−:TBR2−/− and SOX2+/−:HuC/D+/− cells 48 h after IOE of 5-S/D (n=15 and 14 embryos) or its control (n=7 and 10). (E,F) Representative images (E) and mean proportion±s.e.m. (F) of electroporated (H2B-GFP⁺) cells marked as SOX2+/−:HuC/D+/− cells 48 h after IOE of a control plasmid (n=13), or sh-S5 combined with a control plasmid (sh-S5, n=12) or with the constitutively active SMAD5-S/D mutant (sh-S5+S-S/D, n=8). Significance was assessed using the two-sided unpaired t-test (B), a two-way ANOVA+ Sidak’s (D) or +Tukey’s (F) tests. **P<0.01, ***P<0.001. See also Fig. S9. Scale bars: 10 µm (A), 50 µm (C,E).

SMAD1/5 regulate RGC self-amplification and early cortical neurogenesis through YAP

To identify the gene regulatory networks controlled by SMAD1/5 during cortical neurogenesis, cortical RGCs from SmadNes mutant and control E12.5 embryos were purified by fluorescence-activated cell sorting (FACS) based on their prominin 1 expression (Corti et al., 2007), and their transcriptomes were compared by genome-wide RNA-sequencing (RNA-seq; Fig. 6A). A shortlist of differentially expressed transcripts (DETs) was identified (90 DETs with adjusted P<0.05 and 128 with adjusted P<0.1; Fig. 6B and Table S1). A gene ontology (GO) term enrichment analysis correlated this DET signature especially to biological processes related to the regulation of neurogenesis and cell biosynthesis (Fig. 6C and Table S2), these two categories containing 33 and 44 genes, respectively, including 15 in common (Table S2). The genes retrieved in these two GO categories code for proteins playing various functions, the most frequent one being related to DNA-binding and the regulation of transcription (Fig. 6D and Table S2). A Transfac/Jaspar analysis revealed that the promoter regions of these DETs are enriched in binding motifs for TFs of the TEAD and SP families (Fig. 6E and Table S3). We considered particularly interesting these latter results pointing to an altered TEAD activity in response to SMAD1/5 inhibition, as the TEAD TFs indeed represent the transcriptional effectors of the Hippo signalling pathway, which regulates cell growth and organ size (Yu et al., 2015). Of the 42 DETs found to be related to either TEAD2 or TEAD4, 15 and 20 were retrieved in the GO terms related to neurogenesis and biosynthesis, respectively (Table S3). Using the genepaint database, we observed that the genes encoding these DETs are indeed expressed in the mouse developing cerebral cortex at mid-corticogenesis, mainly in the VZ (such as Cnm2, Cct3, Cd2hp2, Glce, Hmgn2, Phf21a, Spata13 and Trim24), the cortical plate (Ctnna2, Islr2, Nav1, Ndn and Reln) or in both (Agrn, Arid1a, Ehmt2, Hnrnpk, Klhl25, Spire1 and Slc25a51; Fig. S11).

The activity of TEADs depends directly on the availability of their co-factors YAP/TAZ, which are themselves regulated by upstream kinases of the Hippo pathway (Yu et al., 2015). Cortical RGCs from the SmadNes embryos did not present any alteration in the transcript levels of the TEAD TFs, nor of other factors known to participate in Hippo signalling (Table S4). Thus, SMAD1/5 does not appear to regulate any member of the Hippo signalling pathway at the transcriptional level. However, previous findings established that YAP can physically interact with SMAD1/5 and that its activity is required for optimal SMAD1/5 activity in several cellular contexts, including cultured mouse embryonic stem cells, the Drosophila wing imaginal disc and mouse astrocyte differentiation during postnatal development (Fig. 6F; Alarcón et al., 2009; Huang et al., 2016). On the other hand, BMP-induced SMAD1/5 signalling has been reported to stimulate YAP protein stability, hence its activity (Fig. 6F; Huang et al., 2016). We thus reasoned that YAP might participate together with SMAD1/5 in controlling RGC self-amplification during cortical neurogenesis. A luciferase assay performed after in ovo electroporation of a TEAD-responsive reporter (p8xGTIIC; Dupont et al., 2011) in the chick dorsal telencephalon confirmed that the ability of YAP overexpression to stimulate TEAD transcriptional activity was markedly impaired when SMAD1 activity was concomitantly inhibited (Fig. 6G).
Therefore, we analysed YAP expression and activity relative to SMAD1/5 activity during cortical neurogenesis. In agreement with recent reports (Kostic et al., 2019; Saito et al., 2018), immunostaining for the YAP protein revealed that the active (nuclear) YAP was more intensely expressed in mitotic apical RGCs than in basally-dividing IPCs, and its expression was strongly correlated with SMAD1/5 activity during both chick and mouse cortical neurogenesis (Fig. 7A-D and Fig. S12A-D). Inhibiting SMAD1/5 during chick cortical neurogenesis impaired YAP activity, as witnessed through the reduced nuclear YAP intensity in mitotic cortical RGCs and the global increase in the proportion of the phosphorylated form of YAP (pYAP), which is primed for proteasomal degradation (Fig. 7E,F and Fig. S12E,F). Similarly, the pYAP/YAP ratio increased in the cortex of E11.5 SmadNes mutant embryos, as shown by immunohistochemistry and western blotting (Fig. 7G,H and Fig. S12G,H). These latter findings indicate that SMAD1/5 positively regulate YAP protein levels and activity both during mouse and chick cortical neurogenesis.

Finally, we tested whether increasing YAP activity could compensate for the phenotype caused by SMAD1/5 inhibition. In ovo electroporation of a wild-type form of YAP rescued the premature exhaustion of RGCs driven by sh-S1, reverting it to control levels and impeding their progression towards differentiation into SOX2−;HuC/D+ neurons (Fig. 7I-L). Intriguingly, YAP overexpression forced the vast majority of electroporated cells to remain SOX2+ (Fig. 7I,J), with more than 30% being found ectopically in the mantle zone irrespective of SMAD1 inhibition (Fig. 7I,M and Fig. S12I). Together, these results support a model whereby SMAD1/5 promote RGC self-amplification and orchestrate cortical growth and neurogenesis through YAP (Fig. 7N).

DISCUSSION

In this study, we identify a novel role for the canonical BMP effectors SMAD1/5 in the regulation of brain growth and cortical neurogenesis. More specifically, our findings demonstrate that SMAD1/5 activity stimulates cortical RGC self-amplification and impedes their premature switch to neurogenic divisions. By altering the balance between these modes of divisions, impairing SMAD1/5 activity during early corticogenesis has two main consequences.
First, the cerebral cortex of adult SmadNes mutant mice is smaller along the medial-lateral and rostral-caudal axes, although its thickness and the cell density are nearly normal. These observations suggest that reducing SMAD1/5 activity affects more severely the generation of radial columns than the number of cells per radial unit, emphasizing the importance of SMAD1/5 activity for cortical RGC self-amplification, which represents the main event ensuring the tangential growth of the cerebral cortex and which is

Fig. 7. SMAD1/5 regulate early cortical neurogenesis through YAP. (A) YAP expression during early mouse corticogenesis (E11.5) relative to SMAD1/5 activity (pSMAD1/5). Dotted circles indicate pH3+ mitotic nuclei. (B-D) The mean intensities±s.d. of YAP (B) and pSMAD1/5 (C) immunoreactivities, quantified in 70 apical and 28 basal pH3+ mitotic nuclei (n=3 embryos), and their Pearson’s correlation coefficient r (D). (E,F) Total YAP immunostaining in the developing chick cerebral cortex (E) and its ratio±s.d. (F) quantified in electroporated pH3+ mitotic cells relative to non-electroporated mitotic cells 24 h after in ovo electroporation (IOE) with a control (Ctrl: 147 pH3+;GFP+ cells, 154 pH3+;GFP− cells, n=7 embryos) or sh-S1/5 (sh-S1/5: 162 pH3+;GFP+ cells and 140 pH3−;GFP− cells, n=7 embryos). Right panels show magnification of boxed area in left panels. Dotted circles indicate pH3+ mitotic nuclei. (G,H) Immunostaining of pYAP and total YAP (G) and the quantification of the pYAP/YAP intensity ratio±s.d. (H) in the developing cerebral cortex of E11.5 SmadNes mutants (Smad1wt/fl; Smad5wt/fl;Nestin:Cre+/0, +/−, n=4 embryos) and control littermates (Smad1wt/fl; Smad5wt/fl; Nestin:Cre0/0, +/+, n=4). (I-M) Representative sections (I) and mean proportion±s.e.m. (J-M) of electroporated (GFP+) cells identified as SOX2+/−;HuC/D+/− (J), PAX6+;TBR2−RGCs (K), TBR2+ (L) and ectopic SOX2+ (M) 48 h after IOE of sh-S1 or its control electroporated alone (Ctrl, n=9 embryos; sh-S1, n=8) or together with a wild-type YAP1 construct (Ctrl+YAP, n=8; sh-S1+YAP, n=8). In I, dashed lines delimitate the ventricular zone and black arrowheads highlight ectopic SOX2+ cortical progenitors. (N) Model proposing that, upon BMP signalling, the activated SMAD1/5 recruit YAP to promote RGC self-amplification. When SMAD1/5 activity is reduced or abrogated and YAP is inactivated and primed for degradation, their transcriptional activity is suppressed, enabling RGCs to undergo neurogenic divisions. N, neuron; S1, SMAD1; S4, SMAD4; S5, SMAD5; VZ, ventricular zone. Significance was assessed using the non-parametric Mann–Whitney test (B,C,F,H) or a two-way ANOVA+Tukey’s multiple comparisons test (J-M). *P<0.05, ***P<0.001; ns, P>0.05. See also Fig. S11. Scale bars: 25 µm.
particularly crucial before and during the early stages of cortical neurogenesis (Cardenas and Borrell, 2019). Of note, crossing the Smad1<sup>+/−</sup>,Smad5<sup>+/−</sup> mice with the Nestin:cre line from Jackson Laboratory, which produces an efficient Cre-mediated recombination in cortical progenitors only from late embryogenesis (around E17.5; Liang et al., 2012), did not cause any obvious alteration in cortical neurogenesis nor any apparent brain growth defects (Dr Eve Seuntjens, unpublished observations). These observations further support our conclusion that the role of SMAD1/5 in sustaining RGC self-amplification is especially crucial during the early steps of corticogenesis. As the SmadNes heterozygous mutant mice present an overall reduction in brain size and weight, it is likely that SMAD1/5 play a similar role throughout the developing brain. We previously reported that SMAD1/5 promote neural progenitor self-amplification during spinal neurogenesis (Le Dreau et al., 2014, 2018). Altogether, these data thus suggest that SMAD1/5 promote stem cell maintenance and growth throughout the developing CNS.

Second, the premature switch from RGC self-amplification to neurogenic divisions caused by SMAD1/5 inhibition altered the generation of the distinct classes of cortical projection neurons. Reducing SMAD1/5 activity during early mouse corticogenesis caused cortical progenitors to prematurely enter neurogenesis, enhancing the generation of early-born cortical neurons and prematurely exhausting the RGC and IPC pools, subsequently limiting the production of late-born cortical neurons. Related to this aspect, a recent study revealed that SMAD1/5 activity also orchestrates the transition from an early to late phase of neurogenesis during mouse cerebellum development, by repressing the late-born interneuron fate determinant Gsx1 (Ma et al., 2020). Importantly, our results strongly suggest that the dependence on SMAD1/5 activity to maintain RGC self-amplification and ensure appropriate neuronal production during corticogenesis is evolutionarily conserved in amniotes, at least between mammals and birds. This leads us to hypothesize that the TFs SMAD1/5 are part of the core ancestral gene regulatory network that governs corticogenesis throughout the amniote lineage, together with PAX6, the proneural bHLH proteins, and the NOTCH and SLIT/ROBO signalling pathways (Cardenas et al., 2018; Le Dreau et al., 2018; Nomura et al., 2013; Suzuki et al., 2012; Yamashita et al., 2018).

The endogenous activity of SMAD1/5 was assessed using an antibody that specifically recognizes the active carboxy-terminal phosphorylated form of SMAD1/5/8. This phosphorylation targeting the three carboxy-terminal serine residues is mediated by type-1 BMP receptors, activation of which depends on their interaction with type-2 BMP receptors, which itself depends on the binding of BMP ligands (Massague et al., 2005). Thus, the activity of SMAD1/5 described herein should reflect the activity of BMP ligands. Various members of the BMP family (including Bmp2, Bmp4, Bmp5, Bmp6 and Bmp7) are expressed in the developing mouse cerebral cortex (Mehler et al., 1997). To our knowledge BMP7, deletion of which causes microcephaly in the mouse (Segkli et al., 2012), is the sole BMP ligand with a reported role in cortical neurogenesis. BMP7 is expressed by the hem, the meninges and the choroid plexus and can be detected in the cerebrospinal fluid (Segkli et al., 2012). BMP7 activity might thus be transduced to SMAD1/5 within RGCs through either their apical membrane or basal foot.

In agreement with previous studies (Alarcon et al., 2009; Saxena et al., 2018), the endogenous pSMAD1/5/8 immunoreactivity was particularly obvious in mitotic RGCs and IPCs. Although we do not rule out that the low levels of pSMAD1/5/8 immunoreactivity that we observed in other phases of the cell cycle might correspond to low levels of SMAD1/5 transcriptional activity, our data favour the idea that SMAD1/5 are activated immediately before or during mitosis. A growing body of literature reveals that many, if not most, of the genes transcribed during interphase are also transcribed during mitosis, albeit to low levels (Palozola et al., 2019). This low transcriptional activity occurring during mitosis, termed mitotic bookmarking, is believed to ensure transcriptional memory propagation from a mother cell to its daughters. Such activity has been observed for general promoter TFs, and for a growing list of tissue-specific TFs (Palozola et al., 2019). The detection of active pSMAD1/5 immunoreactivity in mitotic RGCs and IPCs observed herein thus supports the idea that SMAD1/5 could play a role in mitotic bookmarking.

Our RNA-seq approach correlated SMAD1/5 impairment with an altered activity of the Hippo signalling effectors TEADs. However, none of the TEAD family members nor other factors known to participate in Hippo signalling presented an altered transcriptional expression in mouse cortical RGCs in response to SMAD1/5 inhibition (Table S4), thereby pointing to a transcription-independent regulation of TEAD activity by SMAD1/5. To test whether SMAD1/5 and TEADs might cooperatively regulate the same target genes, we analysed <em>in silico</em> the presence of SMAD1/5 binding motifs in the proximal promoter region (~2000bp ≥ TSS ≥ +500 bp) of the genes retrieved from our RNA-seq that possess TEAD binding motifs. SMAD1/5 binding motifs were found only in 23% (6 out of 26) of the gene promoters containing TEAD2/4 binding motifs (Table S3). Although these results suggest that SMAD1/5 and TEADs might not directly cooperate at the promoter level, it is worth mentioning that these two families of TFs appear to preferentially regulate transcription by binding to distal enhancers (Morikawa et al., 2011; Stein et al., 2015), thereby leaving the question about their direct cooperation open. By contrast, our findings clearly established that the functional cooperation between SMAD1/5 and YAP, a central actor in the Hippo signalling pathway (Yu et al., 2015), is crucial for the regulation of cortical growth and neurogenesis, whereby SMAD1/5 regulate YAP activity in early cortical RGCs. Accordingly, the tight regulation of YAP activity appears to be crucial for correct brain formation and growth. Cortical and general brain development is affected when YAP activity is enhanced, either directly or indirectly (Lavado et al., 2013, 2018; Liu et al., 2018; Saio et al., 2018). More importantly, an aberrant increase in YAP activity has been linked to various types of cortical heterotopia, such as those observed in the Van Maldergem syndrome (Cappello et al., 2013; Liu et al., 2018). Our observation that YAP overexpression causes ribbon-like heterotopias of SOX2<sup>+</sup> and PAX6<sup>+</sup> progenitors in the developing chick cerebral cortex is therefore reminiscent of these severe mammalian cortical defects. Conversely, a reduction in YAP activity is plausibly one of the mechanistic events contributing to primordial dwarfism syndromes, the defining features of which include microcephaly and general growth defects (Klingseisen and Jackson, 2011). Of note, the SmadNes heterozygous mutant mice present both microcephaly and growth retardation. Interestingly, the levels of YAP activity also determine the abundance and proliferative ability of neocortical basal progenitors (Kostic et al., 2019), such that its regulation might have contributed to the evolutionary diversification and expansion of the mammalian neocortex. Our findings suggest that the regulation of YAP activity by SMAD1/5 in RGCs might be evolutionarily conserved. It is thus tempting to speculate that modulations of the canonical BMP activity might have influenced the growth and...
expansion of the cerebral cortex during amniote evolution, a hypothesis that remains to be investigated further.

MATERIALS AND METHODS

Animals

Smad1fl/fl;Smad5fl/fl;Nestin:Cre0/0 embryos and postnatal mice were obtained by crossing Smad1fl/fl;Smad5fl/fl mice (Moya et al., 2012) with transgenic mice that express Cre-recombinase in neural progenitor cells and somites from E8.5 (NesCre8 mice: Petersen et al., 2002). Smad1fl/fl; Smad5wt/fl;Nestin:Cre0/0 littersmates were used as controls. The days of the vaginal plug and birth were defined as E0.5 and P0, respectively. Smad5fl/fl, Smad5wt/fl and NesCre8 mice were maintained in their original mixed genetic backgrounds (CD1, 129/ola and C57BL6). All the experimental procedures were carried out in accordance with the European Union guidelines (Directive 2010/63/EU) and the protocols were approved by the ethics committee of the Parc Científic de Barcelona.

Fertilized white Leghorn chicken eggs were provided by Granja Gibert, rambla Regueral, S/N, 43850 Cambrils, Spain. Eggs were incubated in a humidified atmosphere at 38°C in a Javier Masalles 240N incubator for the appropriate duration and staged according to the method of Hamburger and Hamilton (HH; Hamburger and Hamilton, 1951). According to EU animal care guidelines, no IACUC approval was necessary to perform the experiments described herein, as the embryos used in this study were always harvested at early stages of embryonic development. Sex was not identified at these stages.

In ovo electroporation

Unilateral in ovo electroporations were performed in the developing chick dorsal telencephalon at stage HH18 (E3, 69-72 h of incubation). Analyses were performed specifically in the dorsal-medial-lateral region of the developing chick cerebral cortex to minimize any possible variability along the medial-lateral axis. Plasmids were diluted in RNase-free water at the required concentration (0 to 4 µg/µl) and injected into the right cerebral ventricle using a fine glass needle. Electroporation was triggered by applying five pulses of 50 ms at 22.5 V with 50 ms intervals using an Intracel Dual Pulse (TSS10) electrorator. Electroporated chicken embryos were incubated back at 38°C and recovered at the times indicated.

Plasmids

Inhibition of cSmad1 and cSmad5 expression was triggered by electroporation of short-hairpin constructs inserted into the pSuper (Oligoengine) or pSHIN vectors together with a control H2B-GFP-producing plasmid as previously reported (Kojima et al., 2004; Le Dreau et al., 2012, 2018). Electroporation of 2-4 µg/ml of these sh-Smad1 and sh-Smad5 constructs caused specific and reproducible inhibition (40% and 60%, respectively) of the target gene expression (Le Dreau et al., 2012). The pCAGGS_SMAD5-SD_ires_GFP, its control pCAGGS_ires_GFP (pCIG), the pCAGGS_SMAD5-SD_ires_GFP (pCIG), as well as the pTis21:RFP reporter used to assess the modes of divisions undergone by spinal progenitors, have been previously described in detail (Le Dreau et al., 2012; Megason and McMahon, 2002; Saade et al., 2013). The pCAGGS_Flag-YAP1_ires_GFP construct was obtained by subcloning from a pCNA3:Flag-YAP1 (Addgene plasmid #18881, deposited by Yosef Shaul; Levy et al., 2008) kindly provided by Conchi Estarás (Lewis Katz School of Medicine, Temple University, Philadelphia, PA, USA). The TEAD-responsive p8xGT1IC-luciferase reporter (Addgene plasmid #34615, deposited by Stefano Piccolo; Dupont et al., 2011) was kindly provided by Sebastian Pons (Instituto de Biologia Molecular de Barcelona, Barcelona, Spain).

 Luciferase assay

TEAD transcriptional activity was assessed following electroporation of the pSxGT1IC-luciferase reporter together with a Renilla luciferase reporter used for normalization, in combination with the indicated plasmids required for experimental manipulation. Embryos were harvested 24 h later, the electroporated telencephalic region carefully dissected and homogenized in a Passive Lysis Buffer on ice (Promega). Firefly- and Renilla-luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega).

In situ hybridization

Chicken embryos were recovered at the indicated stages, fixed overnight at 4°C in 4% paraformaldehyde (PFA), rinsed in PBS and processed for whole-mount RNA in situ hybridization following standard procedures. Probes against chick Smad1 (#chEST899n18) and Smad8 (#chEST222h17) were purchased from the chicken EST project (UK-HGMP RC; www.chick. manchester.ac.uk). The probe against cSmad5 was kindly provided by Dr Marian Ros (Instituto de Biomedicina y Biotecnología de Cantabria, Santander, Spain). Hybridized embryos were post-fixed in 4% PFA and washed in PBS containing 0.1% Triton X-100 (PBT), and 45 µm-thick vibratome sections (VT1000S, Leica) were mounted and photographed under a microscope (DC300, Leica). The data show representative images obtained from three embryos for each stage and probe. The images of mSmad5, mSmad3 and mSmad8 expression in the developing mouse dorsal telencephalon at E14.5 and those of candidate target genes selected from the RNA-seq analysis were all obtained from the Genepaint database (https:// gp3.mpmp.de).

Histology and immunohistochemistry

Mouse embryos were recovered at the indicated stages and their heads fixed by immersion in 4% PFA for 24 h at 4°C, cryoprotected with 30% sucrose in PBS, embedded in Tissue-Tek O.C.T. (Sakura Finetek), frozen in isopentane at -30°C and sectioned coronally on a cryostat (Leica). Cryosections (14 µm) were collected on Starfire pre-coated slides (Knittel Glasser) and distributed serially. Postnatal and adult mice were deeply anaesthetized in a CO2 chamber and transcardially perfused with 4% PFA. The brains were removed, post-fixed and vibratome sections (40 µm) were then distributed serially. Chicken embryos were carefully dissected, fixed for 2 h at room temperature (RT) in 4% PFA, rinsed in PBS and cryoprotected with 30% sucrose in PBS, and 16 µm-thick coronal sections prepared with a cryostat.

For both species, immunostaining was performed following standard procedures. After washing in PBT, the sections were blocked for 1 h at RT in PBT supplemented with 10% bovine serum albumin (BSA). When necessary, sections were submitted to an antigen retrieval treatment before blocking by boiling sections for 10 min in sodium citrate buffer (2 mM citric acid monohydrate, 8 mM tri-sodium citrate dehydrate (pH 6.0)). For BrdU immunostaining, sections were incubated before blocking in 50% formamide in 2× SSC at 64°C for 10 min followed by an incubation in 2N HCl at 37°C for 30 min and finally 10 min in 0.1 M boric acid (pH 8.5) at RT. Sections were then incubated overnight at 4°C with the appropriate primary antibodies (Table S5) diluted in a solution of PBT supplemented with 10% BSA or sheep serum. After washing in PBT, sections were incubated for 2 h at RT with the appropriate secondary antibodies diluted in PBT supplemented with 10% BSA or sheep serum. Alexa488-, Alexa555-, Alexa633 and Cy5-conjugated secondary antibodies were obtained from Invitrogen (#A-11078; #A-21202; #A-31570; #A-21050; #A-31572; #A-21070; #A-2108; #A-21094) and Jackson Laboratories (#115-175-146; #712-175-150) and all used at dilution 1:1000. Sections were then stained with 1 µg/ml DAPI and mounted in Mowiol (Sigma-Aldrich).

Cell cycle exit assay

Pregnant female mice received an intra-peritoneal injection of BrdU (100 mg/kg; Sigma-Aldrich) and were sacrificed 24 h later. Embryos were collected and processed as described above. Sections were immunostained for BrdU and Tuj1 and the cell cycle exit rate (neuronal output) was estimated by quantifying the proportion of BrdU/ immunolabelled cells that were Tuj1+ (% of Tuj1+/BrdU+/BrdU− cells).

Image acquisition and treatment

Optical sections of mouse and chick embryo fixed samples (coronal views) were acquired at RT with the Leica LAS software, in a Leica SP5 confocal microscope using 10× (dry HC PL APO, NA 0.40), 20× (dry HC PL APO, NA 0.70), 40× (oil HCX PL APO, NA 1.25-0.75) or 63× (oil HCX PL APO, NA 1.40-0.60) objective lenses. Maximal projections obtained from 2 µm-stack images were processed in Photoshop CSS (Adobe) or ImageJ for image merging, resizing and cell counting. Optical sections of postnatal and
adult mouse samples were acquired with a Leica AF7000 motorized wide-field microscope. Cell counting in embryo, postnatal and adult mouse samples was performed in a 100 μm-wide column of the lateral cortical wall, as indicated in the figures. Cell counts were performed in a minimum of three sections of the same rostral-caudal level per embryo or postnatal mouse sample. Cell counting and measurements of the layer thickness in the P7 and P60 mouse cerebral cortex was carried out based on DAPI staining combined with CUX1 (Nieto et al., 2004), CTIP2 (Bol11b; Arliotta et al., 2005), TBR1 (Bulfone et al., 1995) and NeuN immunostaining. Quantification of pSMAD1/5/8, PAX6, TBR2, YAP and pYAP intensities was assessed using the ImageJ software. Cell nuclei of mitotic pH3+ cells or H2B-GFP + electroporated and neighbouring non-electroporated cells were delimited by polygonal selection, and the mean intensity was quantified as mean grey values.

Brain morphometry

Morphometric parameters were estimated from serial coronal sections stained with DAPI, using the ImageJ software. Images were collected in a Leica AF7000 microscope using 5× or 10× objective. The brain volume was calculated according to the Cavalieri principle (Gundersen and Jensen, 1987), which consists of multiplying the distance between sections by their area, using a set of serial consecutive sections spanning from Bregma +1.10 mm to −1.82 mm (Paxinos and Franklin, 2001).

Western blot

Total protein extracts (~40 μg) were resolved by SDS-PAGE following standard procedures and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences) that was probed with the primary antibodies (Table S5), binding of which was detected by infra-red fluorescence using the LI-COR Odyssey IR Imaging System V3.0 (LI-COR Biosciences).

Purification of mouse Prominin1+ RGCs

The purification of Prominin1+ cortical RGCs was achieved by FACS of dissociated cells obtained from the forebrain of Smad1wt/+;Smad5wt/+; Nestin:Cre+/- and Smad1wt/+;Smad5wt/+;Nestin:Cre+/- E12.5 embryos. Forebrains of littermates with the same genotype were pooled and dissociated by gentle trituration and collected by centrifugation at 300 g for 5 min at 37°C. Cells were mechanically dissociated by gentle trituration and collected by centrifugation at 300 g for 10 min at 4°C. Cells were resuspended in 200 µl of incubation media (PBS, pH 7.4, containing 0.6% glucose, 2% foetal bovine serum and 0.02% NaN3) and kept for 10 min at 4°C with mild agitation. Cells were then incubated for 30 min at 4°C with an APC-conjugated anti-Prominin1 antibody (eBioscience, #17-4301-82, diluted at 0.2 mg/ml) and IgG1-APC antibody (eBioscience, #17-1331-81) diluted at 0.2 mg/ml. Incubation with a rat anti-mouse IgG antibody (Hybond-ECL, Amersham Biosciences) was followed by generation of FASTQ sequence files. RNA-seq paired-end reads were mapped against Mus musculus reference genome (GRCm38) using STAR version 2.5.3a with ENCODE parameters for long RNA. Isoforms were quantified using RSEM version 1.3.0 with default parameters for stranded sequencing and the gencode version M15. Differential isoform analysis was performed using DESeq2 version 1.18.66 with default parameters. We considered differentially expressed transcripts to be those showing an adjusted P-value <0.05 or adjusted P-value <0.1 (extended list). Fold-change values between genotypes (Smad/Nes mutants over controls) are expressed in Log2 (Table S1). The GO term enrichment analysis (biological process) of the extended DE signature was performed using the PANTHER classification system (http://pantherdb.org) and the Transfac/Jasper analysis using Enrichr. The binding motifs for SMAD1 and SMAD5 were obtained from Jaspar. The proximal promoter region (~2000bp to TSS±500bp) of each gene of interest was obtained from the UCSC Genome Browser (https://genome.ucsc.edu/) and the presence of the distinct TF binding motifs in the promoters was determined using the software FIMO (http://meme-suite.org/tools/fimo) (Table S3).

Statistical analyses

No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments. Statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software, Inc.). Unless noted otherwise (see quantifications), cell counts were typically performed on 3–5 images per embryo and n values correspond to different embryos or animals. The normal distribution of the values was assessed by the Shapiro–Wilk normality test. Significance was then assessed with a two-sided unpaired t-test, one-way ANOVA+Tukey’s test or two-way ANOVA+Sidak’s or Tukey’s test for data presenting a normal distribution, or alternatively with the non-parametric Mann–Whitney test for non-normally distributed data. The following convention was used: n.s.: P > 0.05; *P < 0.05, **P < 0.01, ***P < 0.001. The detailed information related to quantifications are detailed in the figure legends.

Acknowledgements

We thank the members of M.L.A.’s and E.M.’s laboratories for their discussion of this study. We thank E. Rebollo and the IBMB Molecular Imaging platform, J. Comas and the Parc Cientific de Barcelona flow cytometry facility, and the CNAG-CRG Sequencing Unit for their assistance. We are grateful to E. J. Robertson and W. Zhong for providing the Smad1/5 and Nestin:cre mice, to E. Seuntjens for sharing information, and to C. Estarls, S. Pons, M. Ros and M. Wegner for providing reagents.

Competing interests

The authors declare no competing or financial interests.

Author contributions


Funding

The work in M.L.A.’s and E.M.’s laboratories was supported by the Ministerio de Ciencia e Innovacion, Gobierno de España (MCINN; grants SAF2016-77971-R, RED2018-102553 and BFU2016-77498-P). I.P. received a PhD fellowship from the Ministerio de Economía, Industria y Competitividad, Gobierno de España (MINECO, BES2014-069217). A.E.-C. was supported by Instituto de Salud Carlos III (MINECO, PT17/0009/019) and the European Regional Development Fund (FEDER); G.L.D. was supported by the Fundación Científica Asociación Española Contra el Cáncer (AI01414205LED).

Data availability

RNA-seq data have been deposited in GEO under accession number GSE153751.
Supplementary information
Supplementary information available online at https://dev.biologists.org/lookup/doi/10.1242/dev.187005.supplemental

References


