YAP stabilizes SMAD1 and promotes BMP2-induced neocortical astrocytic differentiation

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ABSTRACT

YAP (yes-associated protein), a key transcriptional co-factor that is negatively regulated by the Hippo pathway, is crucial for the development and size control of multiple organs, including the liver. However, its role in the brain remains unclear. Here, we provide evidence for YAP regulation of mouse neocortical astrocytic differentiation and proliferation. YAP was undetectable in neurons, but selectively expressed in neural stem cells (NSCs) and astrocytes. YAP in NSCs was required for neocortical astrocytic differentiation, with no apparent role in self-renewal or neural differentiation. However, YAP in astrocytes was necessary for astrocytic proliferation. Yap (Yap1) knockout, Yap<sup>nestin</sup> conditional knockout and Yap<sup>GFAP</sup> conditional knockout mice displayed fewer neocortical astrocytes and impaired astrocytic proliferation and, consequently, death of neocortical neurons. Mechanistically, YAP was activated by BMP2, and the active/nuclear YAP was crucial for BMP2 induction and stabilization of SMAD1 and astrocytic differentiation. Expression of SMAD1 in YAP-deficient NSCs partially rescued the astrocytic differentiation deficit in response to BMP2. Taken together, these results identify a novel function of YAP in neocortical astrocytic differentiation. YAP stabilizes SMAD1 and promotes BMP2-induced astrocytic differentiation. Further mechanistic studies showed that YAP was required for BMP2-induced stabilization of SMAD1 and astrocytic differentiation. Taken together, these results identify a novel function of YAP that is crucial in promoting neocortical astrocytic differentiation and proliferation during mouse brain development.

KEY WORDS: YAP, BMP2, SMAD1, Astrocytes, Differentiation, Proliferation

INTRODUCTION

The conserved Hippo pathway regulates organ size and tumorigenesis by negatively regulating the oncogenic transcriptional co-activators yes-associated protein (YAP, or YAP1) and TAZ (WWTR1) (Pan, 2010; Mo et al., 2014; Piccolo et al., 2014). Upon stimulation, the Hippo pathway subsequently phosphorylates YAP, and phosphorylated YAP undergoes degradation or interacts with 14-3-3 for its cytoplasmic retention. When dephosphorylated, YAP enters the nucleus and interacts with TEAD family proteins to induce the transcription of genes that regulate diverse cellular processes, including cell survival, proliferation and differentiation (Zhao et al., 2007; Lian et al., 2010; Pan, 2010; Schlegelmilch et al., 2011; Tamm et al., 2011; Liu et al., 2012; Yu and Guan, 2013; Mo et al., 2014; Piccolo et al., 2014; Varelas, 2014; Yao et al., 2014; Ohgushi et al., 2015). Although the function of YAP in regulating the development of multiple organs has been investigated, its role in the developing nervous system is less well studied.

Astrocytes emerge as a type of glial cells crucial for a wide variety of functions in the CNS, including promoting neuronal survival (Sofroniew and Vinters, 2010). During brain development, astrocytes and neurons are derived from the same pool, namely the neural stem cells (NSCs) that reside in the ventricular zone (Temple, 2001; Kriegstein and Alvarez-Buylla, 2009). Rodent cortico-cerebral astrogliogenesis mainly takes place during the first postnatal week, following neurogenesis (Mallamaci, 2013), and comprises two concurrent regulatory processes: (1) determination of astrocyte progenitor cell fate (astrocyte differentiation); and (2) the local proliferation of astrocytes (Ge et al., 2012; Mallamaci, 2013). Although recent in vitro and mouse model studies indicate that the bone morphogenetic protein (BMP)-Smad (Gross et al., 1996; Bond et al., 2012; Mallamaci, 2013), Notch (Morrison et al., 2000; Mallamaci, 2013) and Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathways control the appropriate timing of astrogliogenesis (Bomni et al., 1997; He et al., 2005), exactly how these pathways regulate astrogliogenesis remains poorly understood.

Here, we provide evidence for YAP function in the regulation of mouse neocortical astrocytic differentiation and proliferation during brain development. YAP was selectively expressed in NSCs and astrocytes. Yap (Yap1) conditional knockout (CKO) mouse models, Yap<sup>nestin</sup>-CKO and Yap<sup>GFAP</sup>-CKO, displayed fewer neocortical astrocytes and impaired astrocytic proliferation, whereas YAP-deficient NSCs showed normal self-renewal activity and neural differentiation. Further mechanistic studies showed that YAP was required for BMP2-induced stabilization of SMAD1 and astrocytic differentiation. Taken together, these results identify a novel function of YAP that is crucial in promoting neocortical astrocytic differentiation and proliferation during mouse brain development.

RESULTS

Selective expression of YAP in NSCs and astrocytes in the developing mouse neocortex

To understand how YAP regulates brain development, we first examined its expression pattern in mouse brain by immunohistochemical staining. In P1 wild-type (WT) neocortex, YAP-positive signal was detected largely in BLBP<sup>+</sup> cells (Fig. 1A, arrows indicate typical examples; BLBP is also known as FABP7) and aldolase C<sup>+</sup> cells (Fig. 1B), both markers for postnatal...
neocortical astrocytes, as well as in nestin+ (an NSC marker) cells (Fig. 1C). Note that YAP was detected in a few BLBP− cells (Fig. 1A, arrowheads indicate typical examples). However, YAP was undetectable in NeuN+ (a marker for neurons, also known as RBFOX3) cells (Fig. 1D). These BLBP−/YAP+ cells might be endothelial cells of blood vessels. These results thus indicate YAP expression in astrocytes and NSCs, but not in neurons, in line with a previous report (Serinagaoglu et al., 2015). It is also important to note that the YAP/TAZ immunopositive signals in astrocytes and NSCs, but not blood vessels, were markedly reduced in Yapnestin−CKO neocortex (Fig. 1A,B; data not shown), demonstrating the specificity of YAP antibodies.

To further verify YAP expression in NSCs and astrocytes, but not neurons, we used primary cultured brain cells, including nestin+ NSCs and their derivatives, such as neurons and astrocytes. Indeed, double immunostaining of YAP and cell type-specific markers showed that YAP was detected in most of the GFAP+ astrocytes (Fig. 1E,F) and nestin+ NSCs (Fig. 1G), but not in MAP2+ neurons (Fig. 1H). Interestingly, YAP protein was mainly distributed in the cytoplasm of NSCs (Fig. 1G) but in the nuclei of astrocytes under the culture condition with serum (10% FBS+DMEM) (Fig. 1F). When cultured in serum-free DMEM, the astrocytic YAP was mainly distributed in the cytoplasm (Fig. 1E). YAP expression in NSCs and astrocytes, but not neurons, was further verified by western blot analysis (data not shown).

Finally, western blot analysis showed that YAP expression in the neocortex reached a peak level in postnatal weeks 2-3, which matched well with astrocyte development (Fig. S1A,B). Together, these results demonstrate selective YAP expression in NSCs and astrocytes in culture and in mouse brain.

Normal NSC proliferation in YAP-deficient NSCs
YAP is believed to be crucial for the self-renewal of embryonic stem cells (Lian et al., 2010; Pan, 2010; Schlegelmilch et al., 2011; Tamm et al., 2011; Liu et al., 2012; Mo et al., 2014; Piccolo et al., 2014; Pijuan-Galito et al., 2014; Varelas, 2014; Yao et al., 2014; Ohgushi et al., 2015). These observations and the high level of YAP in nestin+ NSCs led to the hypothesis that YAP might promote NSC self-renewal or proliferation. To test this hypothesis, we used neurosphere cultures derived from Yapf/f and Yapnestin-CKO neocortex (at E14.5). Again, YAP was expressed in NSCs from Yapf/f, but not Yapnestin-CKO embryos, and was largely distributed in the cytoplasm of NSCs, with a few nuclear YAP+ cells at the edge of neurospheres (Fig. 2A,B). In viewing the size of neurospheres of the two genotypes, no obvious difference was apparent even in the fifth passages of the NSC culture (Fig. 2A,C), implicating normal growth of NSCs.

We next examined cell proliferation in dissociated NSCs from neurospheres, which were plated onto poly-L-ornithine- and fibronectin-coated coverslips in media containing bFGF (FGF2) and EGF to keep the NSCs in a monolayer. Comparable numbers of Ki67+ and phospho-histone H3 (PH3)+ (markers of proliferative cells) were observed in Yapf/f and Yapnestin-CKO NSC cultures (Fig. 2D-G), suggesting little, if any, role of YAP in regulating NSC proliferation in culture. This view was further supported by in vivo immunohistochemical staining and stereological analyses of PH3+ cells in E14.5 Yapf/f and Yapnestin-CKO neocortex (Fig. 2H,I).
Consistently, deletion of Yap did not affect their mitotic division (Fig. S2A-D). Taken together, these observations suggest that YAP may not be required in nestin+ NSCs for their proliferation or self-renewal in culture and in vivo.

Impaired astrocytic differentiation in YAP-deficient NSCs

We next asked whether YAP plays a role in neurogenesis. Neurospheres were plated on coverslips coated with poly-L-ornithine and cultured in neurobasal medium plus 2% B27 without bFGF and EGF to induce neuronal differentiation. Tuj1+ (a marker for neurons, also known as TUBB3) cells were induced in both YAP+ and YAP-deficient cultures (Fig. 3A). No significant difference was detected between YAP+ and Yap mutant cultures based on the quantification of Tuj1+ neuron numbers and Tuj1 protein level (Fig. 3A-C), suggesting that YAP in nestin+ NSCs might not be required for neurogenesis in culture.

We then determined whether YAP regulates astrocytic differentiation. Neurospheres plated on coverslips coated with poly-L-ornithine were incubated with 10% FBS to induce astrocyte differentiation (Obayashi et al., 2009). As shown in Fig. 3D,E, GFAP+ astrocytes were induced from the NSCs of Yap+/- embryos, but they were obviously reduced in Yap/- mutant cultures based on the quantification of Tuji+ neuron numbers and Tuji protein level (Fig. 3A-C), suggesting that Yap in nestin+ NSCs might not be required for neurogenesis in culture.

To determine whether YAP is involved in astrocytic proliferation in vivo, we carried out BrdU injection experiments to mark proliferative cells. First, BrdU was injected into pregnant mothers at ~E14.5, and P1 pups were examined (Fig. 4E). Coinmunostaining analysis of Ki67 and BrdU revealed a marked reduction of Ki67+ cells in the neocortex of Yap+/- and Yap+/-,CKO mice compared with Yap+/- controls (Fig. 4F,G). These results indicate impaired astrocytic differentiation in YAP-deficient NSC cultures. Together, these results suggest that YAP expression in nestin+ NSCs is necessary for astrocytic differentiation but not for NSC proliferation or neurogenesis.

Reduced proliferation of neocortical astrocytes in Yap+/-, CKO brain

In addition to NSCs, YAP is expressed in astrocytes (Fig. 1A,B,E,F). To examine YAP function in astrocytes, we first examined whether it is required for astrocytic proliferation. When primary astrocytes from P1 Yap+/- and Yap+/-,CKO mice were cultured, the YAP-deficient astrocytes appeared to proliferate at a slower rate than Yap+/- astrocytes. To avoid cell density effects on astrocytic proliferation, we replated YAP+ and YAP-deficient astrocytes at the same density on coverslips and after overnight culture stained for GFAP, Ki67 and PH3. Indeed, marked reductions in Ki67+ and PH3+ astrocytes were detected in Yap mutant culture, as compared with controls (Fig. 4A-D), indicating that YAP is necessary for astrocytic proliferation in culture.
shown in D. (F) Western blot analysis of GFAP expression in astrocytes
local proliferation of astrocytes is impaired by
the postnatal neocortex (Ge et al., 2012), we next examined whether
NSCs. postnatal cell proliferation but not for the proliferation of embryonic
mutant mice (Fig. 4F,H), suggesting that YAP might be required for
postnatal cell proliferation but not for the proliferation of embryonic NSCs.

Since local proliferation constitutes a major astrocyte source in
the postnatal neocortex (Ge et al., 2012), we next examined whether
local proliferation of astrocytes is impaired by Yap deletion. BrdU
was injected into P1 and P7 mice and 2 h after injection the brains
were examined (Fig. 4I, Fig. S3A). Co-immunostaining analysis of
BrdU with BLBP (a marker not only for radial glia during
embryonic development, but also for astrocytes in the postnatal
neocortex) (Guo et al., 2009; Ge et al., 2012) showed a significant
reduction of BrdU+ cells and BLBP+BrdU double-positive cells in the
neocortex of Yapnestin-CKO mice compared with WT controls
(Fig. 4J-L, Fig. S3B-D). Note that our results also showed fewer
Ki67+ (P0 staining; Fig. 4F,G) and BrdU+ (P1 injection; Fig. 4I,K)
cells in the intermediate zone-subventricular zone layers of the
neocortex. These proliferative cells include NSCs and astrocyte
precursors. Thus, it is possible that YAP regulates both astrocyte
and postnatal NSC proliferation. Taken together, these results
suggest that YAP is also required for the local proliferation of
neocortical astrocytes.

**Impaired neocortical astrogliogenesis in Yapnestin-CKO and YapGFAP-CKO mice**

To further determine YAP function in astrogliogenesis in vivo, we
compared astrocyte number and morphology in Yapflox/flox and Yapnestin-
CKO brain sections. As shown in Fig. 5A,B, BLBP+ cells were markedly reduced in the neocortex of Yapnestin-CKO mice. However, BLBP+ cells were not decreased in the hippocampus of Yapnestin-CKO mice (Fig. 5A,B). Moreover, BLBP protein was selectively reduced in the mutant neocortex, but not hippocampus (Fig. 5C,D). Aldolase C+ cells (another astrocyte marker) (Molofsky et al., 2012) were also markedly reduced in the neocortex, but not hippocampus, of Yapnestin-CKO mice (Fig. 5E-G). These results suggest that YAP might be selectively involved in neocortical astrogliogenesis.

To further confirm YAP functions in neocortical astrogliogenesis, we generated another Yap knockout allele, YapGFAP-CKO, by crossing Yapflox with GFAP-Cre. GFAP-Cre drives Cre expression under the control of the Gfap promoter, which is more selectively expressed in astrocytes than elsewhere (Gavéraux-Ruff and Kieffer, 2007). As in Yapnestin-CKO mice, YAP expression was largely decreased in brain regions in YapGFAP-CKO mice, including the olfactory bulb, midbrain, neocortex, hippocampus and cerebellum (Fig. S4A,B), and abolished in cultured YapGFAP-CKO astrocytes (Fig. S4C,D). The primary astrocytes from YapGFAP-CKO mice displayed reduced cell proliferation, compared with WT controls (Fig. S5A-D). In agreement with the phenotypes detected in Yapnestin-CKO mice, YapGFAP-CKO mice showed decreased neocortical astrocytic proliferation (Fig. S5E,G) and fewer neocortical astrocytes (Fig. S5F,H). These observations thus provide additional support for the proposal that YAP is necessary to promote neocortical astrogliogenesis. Taken together, these results suggest that YAP may selectively regulate neocortical astrogliogenesis, but not hippocampal astrogliogenesis or neuronal differentiation.

**Increased neocortical neurodegeneration in the Yapnestin.
CKO brain**

In addition to astrocytes, we examined neocortical and neuronal morphology by Nissl staining analysis. At P0-P1, neocortical morphology and the neural layers appeared to be normal (Fig. 6A,B). However, at P7, the neocortex became thinner in Yapnestin-CKO brain compared with control (Yapflox) mice, and the neuronal density, particularly in neocortical layers IV-VI, was significantly reduced (Fig. 6A,B). Additionally, CUX1+ (a marker of neocortical layers II-IV) and NeuN+ cells in layers IV-VI were significantly decreased in P7 Yapnestin-CKO mice compared with controls (Yapflox) (Fig. 6C-E). Again, this phenotype was not observed in P0-P1 mutant brain, as NeuN+, TBR1+ and TBR2+ (neocortical layer marker, also known as EOMES) cells appeared to be unchanged in Yapnestin-CKO compared with Yapflox mice (Fig. S6A,B). These results suggest that Yap deletion might cause neocortical neuronal loss.

This view was further supported by the observations that Fluoro-Jade C (a maker of degenerating neurons) signal was detected in P7 Yapnestin-CKO mice, but not in the controls (Fig. 6F,G), and that active caspase 3 (a marker of apoptosis) staining was also elevated in mutant neocortex (Fig. 6H,I). Moreover, the neocortex became thinner in YapGFAP-CKO brains compared with control (Yapflox) mice,
and the neuronal density, particularly in neocortical layers IV-VI, was significantly reduced (Fig. S7A,B). Taken together, these results demonstrate neocortical neurodegeneration in the mutant brain, which may be a consequence of impaired astrogliogenesis.

Requirement of YAP for BMP2-induced astrocytic differentiation

The BMP2 signaling pathway is crucial for astrocytic differentiation (Gross et al., 1996; Mallamaci, 2013) and YAP is required for BMP2 signaling in HaCaT cells or mouse embryonic stem cells (Alarcon et al., 2009; Aragon et al., 2011; Yao et al., 2014). We thus examined whether YAP is required for BMP2-induced astrocytic differentiation in NSCs. Cultured NSCs plated on coverslips were treated with BMP2 to induce astrocytic differentiation. Interestingly, GFAP+ astrocytes were induced from NSCs of control embryos at day 3 after BMP2 treatment, whereas they were reduced in YAP-deficient cultures (Fig. 7A,B). These results support the view that YAP in NSCs is required for BMP2-induced astrocytic differentiation.

We next examined whether BMP2 downstream signaling (e.g. pSMAD1/5/8) in NSCs is impaired by Yap deletion. As shown in Fig. 7C-F, pSMAD1/5/8 was induced by BMP2 in control NSCs in a time-dependent manner, and YAP protein was also increased by BMP2 stimulation, whereas the ratio of pYAP/YAP was decreased by BMP2 stimulation. By contrast, the BMP2-induced pSMAD1/5/8 and SMAD1 levels were reduced in YAP-deficient NSCs, compared with control NSCs (Fig. 7C,F,G). The decrease in pSMAD1/5/8 is likely to be due to the reduction of SMAD1 protein. The reductions in BMP2-induced pSMAD1/5/8 and SMAD1 were also detected in YAP-deficient astrocytes (Fig. S8A-D). Furthermore, the reduced pSMAD1/5/8 immunosignal was apparent in aldolase C+ astrocytes in P1 Yap−/− mutant neocortex (Fig. S8E,F). These results suggest that YAP in both NSCs and astrocytes is required to maintain BMP2-induced SMAD1 protein levels, and that YAP in NSCs is also necessary for BMP2-induced astrocytic differentiation.

YAP is necessary for BMP2 induction and stabilization of SMAD1 signaling

How does YAP regulate BMP2-Smad signaling? We first examined whether BMP2 ‘activates’ YAP by promoting YAP nuclear translocation. Indeed, double immunostaining analysis showed increased nuclear translocation of YAP in both WT NSCs and WT astrocytes stimulated by BMP2 (Fig. 7H, Fig. 8A,B), where YAP colocalized with pSMAD1/5/8 (Fig. 7H, Fig. 8A). Again, the pSMAD1/5/8 level was significantly decreased in YAP-deficient cells in response to BMP2 (Fig. 7H, Fig. 8A,B). We further tested...
whether the nuclear YAP forms a complex with SMAD1 by nuclear complex co-immunoprecipitation experiments. YAP was detected in the SMAD1 immuno-complex, which was stimulated by BMP2 in astrocytes (Fig. 8C). Because BMP2 promoted pSMAD1/5/8 and YAP nuclear translocation, there was more SMAD1 and YAP at 30 min compared with time zero. These results suggest YAP ‘activation’ by BMP2.

Note that total SMAD1 was decreased in YAP-deficient cells before and after BMP2 treatment (Fig. 7C,G, Fig. S7A,D). We thus speculate that YAP as a transcription factor might regulate Smad1 transcription. However, Yap deletion did not affect the basal levels of Smad1 mRNA or in response to BMP2 (Fig. 8D), excluding regulation at the transcriptional level. We then asked whether YAP regulates SMAD1 protein stability. Astrocytes were incubated with cycloheximide (CHX), an inhibitor of protein synthesis, for various times. Cell lysates were analyzed for SMAD1 expression by western blotting. SMAD1 protein levels were decreased more rapidly in YAP-deficient astrocytes than in controls (Fig. 8E,F), suggesting that SMAD1 is unstable in YAP-deficient cells. We further examined SMAD1 stability in BMP2-stimulated YAP+ and YAP-deficient astrocytes. As shown in Fig. 8G,H, SMAD1 protein levels were also degraded more rapidly in YAP-deficient cells than in controls. These results suggest that YAP is required to maintain SMAD1 protein stability in both basal and BMP2-stimulated culture conditions.

We further asked whether YAP regulation of SMAD1 in NSCs is essential for YAP promotion of astrocytic differentiation. Flag-tagged SMAD1 was expressed in YAP+ and YAP-deficient NSCs, which were subjected to astrocytic differentiation by BMP2. As shown in Fig. 8I,J, 3 days after BMP2 treatment, more GFAP+ astrocytes were detected in YAP+ and YAP-deficient NSCs expressing Flag-SMAD1 than in the untransfected YAP+ and YAP-deficient NSCs. The impaired astrocytic differentiation in YAP-deficient NSCs was diminished by expressing Flag-SMAD1 (Fig. 8I,J). Taken together, these results suggest that SMAD1 is crucial downstream of YAP in NSCs to promote BMP2-induced astrocyte differentiation.

**DISCUSSION**

Here, we present evidence for YAP function in neocortical astrocytic differentiation and proliferation and propose a working model, as depicted in Fig. 8K. In this model, YAP is suggested to promote astrocytic proliferation and differentiation by stabilizing BMP2-SMAD1 signaling. This study thus not only identifies a novel function of YAP in neocortical astrocytic proliferation and differentiation during brain development, but also provides new insights into the molecular pathways underlying astrocytic differentiation.

In light of reports that YAP is crucial for the proliferation or self-renewal of embryonic stem cells (Lian et al., 2010; Pan, 2010; Schlegelmilch et al., 2011; Tamm et al., 2011; Liu et al., 2012; Ramos and Camargo, 2012; Mo et al., 2014; Piccolo et al., 2014; Varelas, 2014; Yao et al., 2014), we first tested the hypothesis that Yap deletion in NSCs might impair their proliferation or self-renewal. However, to our surprise, several lines of evidence suggest...
Fig. 6. Increased neocortical neurodegeneration in Yapnestin-CKO brain. (A) Nissl stained images (sagittal sections) showing the brain phenotype of P0 and P7 Yapff and Yapnestin-CKO mice. Selected regions in P7 Yapff (a) and Yapnestin-CKO (b) mice are shown at higher magnification. (B) Quantitative analysis of neocortical thickness of P0 and P7 Yapff and Yapnestin-CKO mice (n=25 per group, normalized to WT group). (C) Double immunostaining analysis of CUX1 (green) and NeuN (red) in neocortex of P7 Yapff and Yapnestin-CKO mice (sagittal sections). (D,E) Quantitative analysis of the thickness of the CUX1+ cell layer (D, n=9 in Yapff mice, n=8 in Yapnestin-CKO mice) and the density of NeuN+ cells of layers IV-VI (E, n=8 per group) as shown in C. (F) Fluoro-Jade C (green) staining in the neocortex of P7 Yapff and Yapnestin-CKO mice. (G) Quantitative analysis of the relative intensity of Fluoro-Jade C (normalized to WT group, n=6 per group) shown in F. (H) Immunostaining analysis of cleaved caspase 3 (green) in layer IV-VI neocortex of P7 Yapff and Yapnestin-CKO mice. (I) Quantitative analysis of the number of cleaved caspase 3+ cells in each field (n=7 per group) as shown in H. Boxed regions are shown at higher magnification. Data are mean ±s.e.m. **P<0.01, compared with control group, Student’s t-test. Scale bars: 20 μm.
little to no role for YAP in regulating NSC proliferation. First, in the EGF- and bFGF-dependent neurosphere culture system, neurosphere formation and cell proliferation appeared to be normal in YAP-deficient NSCs, as compared with WT controls (Fig. 2). Second, PH3 staining and BrdU injection into embryonic Yap

nestin-CKO mice showed comparable levels of proliferative NSCs in the mutant neocortex and controls (Figs 2 and 4). Third, YAP was largely distributed in the cytoplasm of NSCs (Fig. 1C,G, Fig. S2), whereas it is believed that nuclear YAP is essential for cell proliferation (Yu and Guan, 2013; Piccolo et al., 2014). The view that YAP is not necessary for NSC proliferation is also in line with recent observations of unchanged proliferation of stem cells in the mutant neocortex and controls (Figs 2 and 4). Third, YAP was largely distributed in the cytoplasm of NSCs (Fig. 1C,G, Fig. S2), whereas it is believed that nuclear YAP is essential for cell proliferation (Yu and Guan, 2013; Piccolo et al., 2014). The view that YAP is not necessary for NSC proliferation is also in line with recent observations of unchanged proliferation of stem cells in mammmary glands (Chen et al., 2014), pancreas (Zhang et al., 2014) and intestines (Azzolin et al., 2014) upon conditional genetic inactivation of Yap (Piccolo et al., 2014). Also noteworthy is that this view appears to differ from reports that EGF activates YAP to promote cell proliferation through activation of Ras-MAPK and PI3K-PDK1 signaling in immortalized mammary cells (Fan et al., 2013; Gumbiner and Kim, 2014). Such differing conclusions might be a result of the different cell types tested. Indeed, we found that YAP was required for astrocytic differentiation in mouse NSCs (Fig. 3), whereas in astrocytes it was necessary for cell proliferation (Figs 4, S3 and S5).

YAP appeared to be required for neocortical, but not hippocampal, astrogliogenesis (Fig. 5), although YAP was expressed in astrocytes in both brain regions. The mechanism underlying such a regional effect of YAP is unclear. One recent study has shown that gain of function of YAP by aberrant YAP activation leads to overexpansion of dorsal root ganglion glial populations (Serinagaoglu et al., 2015). In cultured human NSCs, YAP mediates astrocyte differentiation as a downstream signaling protein of the stretch-activated ion channel PIEZO1 (Pathak et al., 2014). Our results are consistent with these previous studies. We are also aware of reports that YAP regulates neural progenitor cell numbers in the developing neural tube of chick and Xenopus embryos and the mouse hippocampus, as shown in YAP gain- or loss-of-function assays (Cao et al., 2008; Gee et al., 2011; Lavado et al., 2013; Piccolo et al., 2014). However, our results showed normal neocortical neurogenesis in culture and in P0-PI Yap

nestin-CKO mice (Figs 3, 5, 6 and S6). Again, the differing observations

Fig. 7. YAP is required for BMP2-induced astrocytic differentiation. (A) Immunostaining analysis of GFAP (red) in astrocytes differentiated from WT and YAP-deficient NSCs induced by BMP2 treatment (100 ng/ml) for 3 days. (B) Quantitative analysis of the percentage of GFAP+ cells among total cells in one field shown in A (n=12 fields for each group). (C) Western blot to detect signaling downstream of BMP2 in WT and YAP-deficient NSCs before and after BMP2 treatment (100 ng/ml) at the indicated time points. (D-G) Quantitative analysis of the relative ratio of pYAP/YAP (D), relative YAP (E), pSMAD1/5/8 (F) and SMAD1 (G) as shown in C (n=3 per group, normalized to 0 min). (H) Double immunostaining analysis of pSMAD1/5/8 (green) and YAP (red) in WT and YAP-deficient NSCs before and after BMP2 treatment (100 ng/ml). Data are means±s.e.m. *P<0.05, **P<0.01, compared with control group, Student’s t-test. Scale bars: 20 μm.
might result from the different species (mouse versus chick) or brain regions (neocortex versus hippocampus) examined. Astrocytes play a key role in promoting neuronal survival (Sofroniew and Vinters, 2010). Although Yap deletion did not affect neurogenesis at birth, our results showed death of neocortical neurons at P7, which might be a consequence of decreased astrogliogenesis in the Yap mutant brain, as YAP was not expressed in neocortical neurons (Fig. 1C,G).

BMPs are members of the transforming growth factor beta (TGFβ) superfamily of signaling ligands (Bond et al., 2012) and play dynamic roles in neurogenesis and astrogliogenesis (Gross et al., 1996; Bond et al., 2012; Mallamaci, 2013). During late embryonic and early postnatal periods, BMP signaling promotes astroglial differentiation (Gross et al., 1996; Mehler et al., 2000; Mallamaci, 2013). Our results indicate that YAP in NSCs and astrocytes was activated by BMP2 and stabilized SMAD1 in the nucleus. This effect was required for neocortical astrogliogenesis. It is of interest to note that, in HEK293 or Eph4 cells, YAP is found to interact with SMADs in the nucleus to modulate BMP-SMAD1 or TGF-SMAD2 signaling (Alarcón et al., 2009; Aragon et al., 2011; Nallet-Staub et al., 2015; Narimatsu et al., 2015). In mouse embryonic stem cells, YAP promotes SMAD1-dependent transcription and is required for BMP2 suppression of neural differentiation (Alarcón et al., 2009). Our results are in line with these reports, revealing the importance of YAP regulation of BMP2-SMAD1 signaling in various cell types.

In summary, we provide evidence for YAP function in promoting neocortical astrocyte proliferation and differentiation during mouse development, which is likely to be due to YAP stabilizing BMP2-SMAD1 signaling.

MATERIALS AND METHODS

Mouse breeding and genotyping

Yap<sup>nestin-CKO</sup> and Yap<sup>GFAP-CKO</sup> mice were generated by crossing the floxed Yap allele (Yap<sup>F</sup>) with nestin-Cre or GFAP-Cre transgenic mice (from The Jackson Laboratory; donated by Dr Rudiger Klein and Dr Albee Messing, respectively), maintained in a C57BL/6 strain background. Yap<sup>ET</sup>...
mice were generated as previously described (Zhang et al., 2010; Wang et al., 2014). Yap
test−CKO mice survived the early postnatal stages, but after P8 they displayed marked growth retardation and died at ~P25. The use of experimental animals has been approved by the IACUC at Georgia Regents University in accordance with NIH guidelines.

**Primary culture of astrocytes, neurons and NSCs and transfection**

Primary astrocyte cultures were prepared from the cerebral neocortex of P0-P3 neonatal mice (after genotyping) as described previously with slight modifications (Su et al., 2009). Briefly, cerebral neocortex was removed, demembranated, chopped, and then incubated with 0.125% trypsin at 37°C for 20 min, and dissociated into a single-cell suspension by mechanical disruption. The cells were seeded on poly-L-lysine (0.1 mg/ml, Sigma-Aldrich)-coated culture flasks and incubated in DMEM containing 10% fetal bovine serum (FBS, Gibco). After 6-10 days, the cultures became confluent. Microglia and oligodendrocytes were removed by shaking at 250 rpm for 4-6 h. Astrocytes were subsequently detached and plated into poly-L-lysine-coated dishes or coverslips. The purity of GFAP+ cells in our culture system is greater than 94%.

For NSC culture, pregnant mice (E14.5) were sacrificed following an established protocol (Wang and Yu, 2013). Mouse neocortex was dissociated to achieve a single-cell suspension. The single-cell suspensions were grown in Neurobasal-A Medium (NB, Life Technologies) supplemented with B27 (Life Technologies), 2 mM L-glutamine (Life Technologies), bFGF (20 ng/ml, Gibco) and EGF (20 ng/ml, Gibco). In cases where monolayer NSCs were needed for immunostaining or other treatments, neurospheres at passage two or three were dissociated into single cells and seeded onto poly-L-ornithine- and fibronectin-coated plates (Sigma). For NSC differentiation experiments, neurospheres were plated on poly-L-ornithine-coated coverslips under DMEM+10% FBS for 24 h or NB+2% B27 for 48-72 h. For NSC transfection, we used the NSC Nucleofector Kit (Amaxa) according to the manufacturer’s instructions. The Flag-SMAD1 plasmid was purchased from Addgene.

**Western blot**

Brain tissues or cultured cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 5 mM sodium fluoride, 2 mM sodium orthovanadate and protease inhibitor cocktail (Sigma, P8340)] for 30 min on ice and centrifuged at 12,000 rpm (15,100 g) for 20 min, and protein concentration was determined using a BCA protein assay kit (Thermo). Proteins were separated by 8%-12% SDS-PAGE and transferred onto nitrocellulose membrane. Blotted membranes were blocked in 10% skimmed milk at room temperature for 1 h and incubated with primary antibody overnight at 4°C, rinsed and incubated for 1 h at room temperature with an appropriate horseradish peroxidase-conjugated secondary antibody [1:5000, Bio-Rad, #170-6515 (goat anti-rabbit), #170-6516 (goat anti-mouse)] or Alexa Fluor 680/790 fluorescent secondary antibody [1:200, Molecular Probes, A21207, A21206 (donkey anti-mouse)]. Primary antibodies included mouse monoclonal anti-YAP (1:1000, WW0010432M1, Sigma), anti-GFP (1:1000, MAB360, Millipore), anti-Tuj1 (1:500, T8660, Sigma), anti-nestin (1:1000, MAB353, Millipore), rabbit polyclonal anti-BLB2 (1:1000, Ab32423, Abcam), anti-pYAP Ser127 (1:1000, #4911, Cell Signaling Technology (CST)), anti-pSMA1/5/8 (1:1000, #13820, CST) and anti-SMAD1 (1:1000, #9743, CST), β-actin as a loading control was detected alongside the experimental samples (1:7000, A2228, Sigma). For chemiluminescence, protein bands detected by ECL (Pierce) were scanned alongside the experimental samples (1:7000, A2228, Sigma). For fluorescence (Fig. 7C and Fig. 8E,G), images were acquired using the Odyssey Imaging System (LI-COR) and analyzed by Image-Pro analysis software (Media Cybernetics).

**Nuclear complex co-immunoprecipitation (co-IP)**

For co-IP experiments of SMAD1 and YAP, primary cultured astrocytes were starved with DMEM without serum for at least overnight before BMP2 treatment. After BMP2 (100 ng/ml) treatment, nuclear extracts were harvested for co-IP assays with anti-SMAD1 antibody (1:100, CST, #9743) using the Nuclear Complex co-IP Kit (Active Motif) according to the provided protocol.

**Immunostaining**

For brain tissue section staining, brains of E14-E16 and P0-P1 mice were removed and fixed in fresh 4% paraformaldehyde (PFA) for 2 days, and older mouse brains were removed and fixed in 4% PFA for 2 days after transcardial perfusion. Then brains were dehydrated in 15%, then 30% sucrose in PBS for 1-2 days, and cryopreserved in O.C.T. compound (SAKURA, #4583) for brain sections. Sections (20-30 μm) were cut on a freezing microtome and immediately processed for immunostaining by 1 h blocking in 10% BSA plus 0.3% Triton X-100 at room temperature, overnight incubation with primary antibodies at 4°C, and 1 h incubation at room temperature with appropriate secondary antibodies [1:500, Molecular Probes, A21202, A10036 (donkey anti-mouse), A21207, A21206 (donkey anti-rabbit), A11055 (donkey anti-goat)].

For cultured cell staining, cells were fixed with fresh 4% PFA in 0.1 M PBS (pH 7.4) for 20 min. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 in 0.1 M PBS for 5 min, followed by incubation in blocking buffer (5% BSA and 0.1% Triton X-100 in 0.1 M PBS, pH 7.4) for 1 h, and incubated overnight at 4°C with primary antibodies diluted in the blocking buffer. Cells were washed three times with PBS and incubated for 1 h at room temperature with an appropriate fluorescence-conjugated secondary antibody [1:500, Molecular Probes, A21202, A10036 (donkey anti-mouse), A21207, A21206 (donkey anti-rabbit)]. Primary antibodies were rabbit polyclonal antibodies against nestin (1:200, SAB4200394, Sigma), BLBP (1:300, Ab32423, Abcam), Ki67 (1:200, AB9260, Millipore), Ph3 (1:200, 06-570, Millipore), GFAP (1:500, AB804, Millipore), GLAST (SLC1A3 or EAAT1; 1:200, AB416, Abcam), pSMAD1/5/8 (1:200, #13820, CST), FLAG (1:2000, #14793, CST), YAP (1:200, #8418/D24AE, CST), CUX1 (1:200, M222, Santa Cruz), cleaved caspase 3 (1:100, #9661, CST), TBR1 (1:200, AB10554, Millipore) or TBR2 (1:400, AB2283, Millipore), or with monoclonal antibodies against YAP (1:200, WH0010431M1, Sigma), GFAP (1:500, MAB360, Millipore), NeuN (1:500, MAB377, Millipore), MAP2 (1:500, AB5622, Millipore), TuJ1 (1:500, T8660, Sigma) and nestin (1:1000, MAB353, Sigma), or with goat polyclonal antibodies against aldolase C (1:500, SC-12065, Santa Cruz). Sections or cells were stained with DAPI (1:1000, Molecular Probes) to visualize the nucleus. Fluoro-Jade C was purchased from Millipore, and staining followed the provided protocol. Images were acquired on a Zeiss confocal system (FM300) using a multi-track configuration and processed using Zeiss confocal software and Adobe Photoshop CS 8.0 software.

**BrdU injection and staining**

For BrdU incorporation experiments, BrdU (100 μg/kg in PBS, Sigma) was administered by intraperitoneal injection at E14.5 (sacrificed at P0), P1 or P7 (sacrificed after 2 h). Brain sections were treated with 1 M HCl on ice for 10 min, then 2 M HCl at 37°C for 30 min, followed by treatment with 0.1 M Na2Ba4O7 (pH 8.5) for 30 min at room temperature. After washing with PBS, brain sections were blocked with 10% BSA plus 1% Triton X-100. Monoclonal anti-BrdU antibody (1:200, Developmental Studies Hybridoma Bank) was incubated at 4°C overnight. Sections were then incubated with fluorescently conjugated Alexa Fluor 488 monoclonal IgG secondary antibody (1:500, Molecular Probes, A21202) for 45 min at room temperature. Images were acquired on a Zeiss confocal system using a multi-track configuration and processed using Adobe Photoshop CS 8.0 software.

**Quantitative real-time PCR (qRT-PCR) analysis**

For qRT-PCR, total RNA was extracted from astrocytes with Trizol reagent (Invitrogen). 1 μg of RNA was converted to cDNA using the Revert Aid first strand cDNA synthesis kit (Thermo) in a 20 μl reaction, and then 0.5 μl product was used in a 20 μl reaction mixture containing SYBR GreenER qPCR SuperMix Universal (Invitrogen) with Smad1 primers: forward, 5′-ACCTGCTACGTGCCCTCCTG-3′; reverse, 5′-CATAGCAACCCGCT-GAACACA-3′. The amplification cycle consisted of an initial step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min, and extension at 72°C for 30 s. Samples were amplified.
independently at least three times. Relative gene expression was converted using the 2^{-ΔΔCt} method against the internal control hypoxanthine guanine phosphoribosyltransferase 1 (Hprt); forward, 5'-TGGCCCTCTGTTGTCATCAA-3'; reverse, 5'-TGATCCATACAGTACCTCTAGTGTGA-3'.

Statistical analysis
All data presented represent results from at least three independent experiments. Statistical analysis was performed using Student's t-test or using ANOVA with pairwise comparisons. Statistical significance was defined as P<0.05.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
L.M., W.-C.X., Z.H. and J.Z. designed experiments and interpreted results; Z.H., W.-C.X., Z.H. and J.Z. designed experiments and interpreted results; Z.H., W.-C.X. and J.Z. designed experiments and interpreted results; Z.H., W.-C.X. and J.Z. designed experiments and interpreted results.

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