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# Coffin–Lowry syndrome: A role for RSK2 in mammalian neurogenesis

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ABSTRACT

Coffin–Lowry Syndrome (CLS) is an X-linked genetic disorder associated with cognitive and behavioural impairments. CLS patients present with loss-of-function mutations in the RPS6KA3 gene encoding the mitogen-activated protein kinase (MAPK)–activated kinase p90 ribosomal S6 kinase 2 (Rsk2). Although Rsk2 is expressed in the embryonic brain, its function remains largely uncharacterized. To this end, we isolated murine cortical precursors at embryonic day 12 (E12), a timepoint when neuronal differentiation is initiated, and knocked-down Rsk2 expression levels using shRNA. We performed similar experiments *in vivo* using *in utero* electroporations to express shRNA against Rsk2. Rsk2 knockdown resulted in a significant decrease in neurogenesis and an increase in the proportion of proliferating Pax6-positive radial precursor cells, indicating that Rsk2 is essential for cortical radial precursors to differentiate into neurons. In contrast, reducing Rsk2 levels *in vitro* or *in vivo* had no effect on the generation of astrocytes. Thus, Rsk2 loss-of-function, as seen in CLS, perturbs the differentiation of neural precursors into neurons, and maintains them instead as proliferating radial precursor cells, a defect that may underlie the cognitive dysfunction seen in CLS.



## Introduction

During mammalian development, the cerebral cortex arises from proliferating neural precursors that will sequentially generate neurons, astrocytes, and oligodendrocytes. In rodents, cortical neurogenesis occurs from E12 to E17, after which gliogenesis commences. The precise timing and extent of the genesis of these different cell types from cortical neural precursors are regulated by external cues such as growth factors, but the intracellular mechanisms that mediate these signals, and the ways that they impinge upon intrinsic cellular programs are just now being elucidated (reviewed in Miller and Gauthier (2007)). In this regard, we and others have previously shown that signalling proteins of the MAP kinase pathway such as H-Ras, MEK and Erk all play essential roles in embryonic cortical neural precursors (Ménard et al., 2002; Paquin et al., 2005; Liu et al., 2006; Gauthier et al., 2007; Samuels et al., 2008; Paquin et al., 2009). Despite these studies, however, the signals that are downstream of the MEK–Erk pathway that regulate cortical differentiation are incompletely understood.

Coffin–Lowry syndrome (CLS, OMIM 303600; Coffin et al., 1966; Lowry et al., 1971) is an X-linked genetic disorder caused by heterogeneous loss-of-function mutations in the RPS6KA3 gene that encodes the protein Rsk2. Over 75 different mutations spanning the 22 exons of this gene have been identified, most of them causing premature termination of protein translation resulting in a truncated, inactive protein (Zeniou et al., 2004; Falco et al., 2005). Patients diagnosed with CLS generally display thinning of the corpus callosum, asymmetry of lateral ventricles, and smaller brain volumes (Wang et al., 2006; Kesler et al., 2007). Males affected with CLS display cognitive deficits ranging from moderate to severe mental retardation whereas affected females display defects ranging from low-normal abilities to moderate mental dysfunction.

Rsk2, the protein that is mutated in CLS, is phosphorylated by the Erks and functions downstream of the Ras–MEK–Erk signalling pathway (Anjum and Blenis, 2008). Intriguingly, many CLS patients display a prominent forehead, broad and soft hands with stubby and tapering fingers, cardiovascular defects, and cognitive dysfunction (Reynolds et al., 1986; Hanauer and Young, 2002; Tartaglia and Gelb, 2005; Bentires-Alj et al., 2006; Zampino et al., 2007), characteristics that are also seen in patients with neuro-cranio-facial-cutaneous (NCFC) syndromes, a family of disorders caused by mutation of proteins in the MAP kinase pathway (reviewed in Aoki et al. (2008)). In this regard, two of the proteins that are mutated in NCFC syndromes, the protein tyrosine phosphatase SHP2

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(Noonan syndrome) and H-Ras (Costello syndrome), are essential for cortical precursor development (Aoki et al., 2005; Gauthier et al., 2007). Moreover, NCFC mutations in these proteins cause aberrant neurogenesis and gliogenesis in the developing mouse cortex (Gauthier et al., 2007; Paquin et al., 2009). These studies therefore support the idea that one way human mutations in the MAP kinase pathway cause cognitive dysfunction is by perturbing neural precursor development.

To test the idea that Rsk2 is also required for neural precursor development, we have examined a potential role for this protein in the embryonic mouse cortex. Here, we provide evidence that Rsk2 is essential for normal development of cortical precursors, and that genetic knockdown of Rsk2 decreases the differentiation of cortical radial precursors into neurons but not astrocytes. These findings strengthen the idea that the MAP kinase pathway provides one way that growth factors enhance neurogenesis, and suggest that the cognitive dysfunction observed in CLS is at least in part the consequence of perturbed neural precursor differentiation during development.

## Materials and methods

### *Animals*

CD1 mice were from Charles River Laboratories (Montreal, QC). This study was approved by The Hospital for Sick Children's Animal Care Committee, and use was in accordance with CCAC guidelines.

### *Culture of cortical precursor cells*

Cortical precursor cells were cultured as previously described (Barnabé-Heider and Miller, 2003; Paquin et al., 2005; Gauthier et al., 2007; Paquin et al., 2009; Dugani et al., 2009). Briefly, cortices were dissected from embryonic days 12 (E12) to E13 CD1 mouse embryos in ice-cold HBSS (Invitrogen, Gaithersburg, MD) and transferred to Neurobasal medium (Invitrogen) containing 500 μml glutamine (Cambrex Biosciences, Hopkinton, MA), 2% B27 supplement (Invitrogen), and 1% penicillin–streptomycin (Invitrogen). The medium was supplemented with 40 ng/ml FGF2 (Promega, Madison, WI). The tissue was mechanically triturated with a plastic pipette into single cells and cells were plated on four-well chamber slides (Nunc, Naperville, IL). Chamber slides were previously coated with 2% laminin and 1% poly-D-lysine (BD Biosciences, Bedford, MA) and cell density was 150,000 cells/well.

### *Transfection of cortical precursor cells*

For transfections, 1 to 2 h after plating, 1 μg of DNA and 1.5 μl of Fugene 6 (Roche, Welwyn Garden City, UK) were mixed with 100 μl of Opti-MEM (Invitrogen) and were incubated at room temperature for 45 min and then added to each well. The three Rsk2 shRNA constructs targeted three different regions on the Rsk2 mouse mRNA sequence (EZ Biolabs) and their sequences are: shRNA-1, 5′-CAGAAGAGATT-GAAATTCT-3′; shRNA-2, 5′-GAGGAAGATGTCAAATTCT-3′; shRNA-3, 5′-GAAGGTCACATCAAGTTAA-3′. A scrambled shRNA construct (5′-TTCTCCGAACGTGTCACGGT-3′) was used as a control, and a previously-described plasmid encoding EGFP under the CMV-promoter (pEGFP) was used as a marker for cotransfected cells (Paquin et al., 2005; Gauthier et al., 2007; Paquin et al., 2009; Dugani et al., 2009). For the rescue experiments, we used 2.33 μl of Fugene 6 and 1.55 μg of DNA in these proportions: 0.75 μg of HA-Rsk2 (kind gift from Dr. Deborah Lannigan, University of Virginia, Charlottesville, VA) or empty vector; 0.4 μg shRNA; and 0.4 μg EGFP. In some experiments, as specified, the day following plating, 50 ng/ml ciliary neurotrophic factor (CNTF; Peprotech, Rocky Hill, NJ) was added by changing one half of the medium.

### *In utero electroporation*

*In utero* electroporation was performed as previously described (Paquin et al., 2005; Bartkowska et al., 2007; Gauthier et al., 2007; Paquin et al., 2009; Dugani et al., 2009). Briefly, E13/E14 CD1 pregnant mice were anaesthesized with isoflurane, and a midline incision was performed to access the embryos. A total of 4 μg of DNA was injected in the lateral ventricle of each embryo with 0.05% trypan blue as a tracer. We used a previously-described nuclear EGFP expression plasmid driven from the EF1-promoter (pEF-GFP). The embryos were injected with nuclear EGFP expression plasmid driven from the EF1α-promoter at a 1:3 ratio with scrambled shRNA, Rsk2 shRNA, or HA-Rsk2, for a total of 4 μg DNA per embryo and 0.05% trypan blue as a tracer. After injection, electroporation was performed using a square electroporator CUY21 EDIT (TR Tech, Japan), delivering five 50 ms pulses of 40 V with 950 ms intervals per embryo. Embryos were then put back *in utero* and left to develop for 3–8 days. For BrdU, pregnant mice were injected with BrdU (Sigma) dissolved in PBS at a dose of 50 mg/kg body weight 3 days post-electroporation and sacrificed 24 h later, as previously described (Gauthier-Fisher et al., 2009). For analysis, brains were fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) at 4 °C overnight, cryoprotected in 30% sucrose at 4 °C overnight, and embedded in OCT compound (Sakura Finetek, Torrance, CA). The brains were kept at −80 °C until cryosectioned (16 μm) and immunostained.

### *Immunocytochemistry*

Immunocytochemistry of cultured cells and tissue sections was performed as described (Paquin et al., 2005; Paquin et al., 2009; Bartkowska et al., 2007; Gauthier et al., 2007; Paquin et al., 2009; Dugani et al., 2009). For immunocytochemistry of cultured cells, cells were washed with HEPES-buffered saline (HBS) and fixed with 4% PFA for 15 min, permeabilized with 0.2% NP-40 (USB Corporation, Cleveland, OH) in HBS, and blocked with buffer containing 6% normal goat serum (NGS) (Jackson ImmunoResearch, West Grove, PA) and 0.5% bovine serum albumin (BSA) (Jackson ImmunoResearch) for 1–2 h at room temperature. Cells were then incubated with primary antibodies in HBS containing 3% NGS and 0.25% BSA at 4 °C overnight. After washing with HBS, cells were incubated with secondary antibodies prepared in HBS containing 3% NGS and 0.25% BSA at room temperature for 1 h. Samples were then washed with HBS, counterstained with Hoechst 33258 (1:1000; Sigma, St-Louis, MO) for 2 min, and mounted with GelTol (Fisher Scientific, Houston, TX). For immunocytochemistry of tissue sections, sections were dried at 37 °C for 15 min, washed in phosphate buffer solution (PBS) (Hyclone, Logan, UT), and postfixed with 4% PFA for 10–15 min. They were then blocked and permeabilized with 10% BSA and 0.3% Triton X-100 (EMD Chemicals Inc., Gibbstown, NJ) for 1 h. The M.O.M. blocking kit (Vector Laboratories, Burlingame, CA) was then used according to the manufacturer's protocol. Sections were incubated with primary antibodies at 4 °C overnight, washed with PBS, and incubated with secondary antibodies at room temperature for 1 h. They were then counterstained with Hoechst 33258 for 2 min and mounted with GelTol. For BrdU immunohistochemistry, sections were incubated in 1 N HCL for 30 min at 55 °C before blocking in 10% horse serum and 0.3% Tx-100, based on our previous report (Gauthier-Fisher et al., 2009). The primary antibodies used were mouse anti-GFP (1:1000; Invitrogen), rabbit anti-GFP (1:500; Chemicon, Temecula, CA), rabbit anti-Rsk2 (1:200; Santa Cruz), rabbit anti-phospho-Rsk (Ser227; 1:200; Santa Cruz), rabbit anti-Erk (1:500; Santa Cruz) rabbit anti-phospho-Erk (Thr202/Tyr204; 1:500, Cell Signaling Technology), mouse anti-nestin (1:200, Chemicon), mouse anti-Ki67 (1:200; BD Biosciences), mouse anti-βIII-tubulin (1:800; Covance, Princeton, NJ), rabbit anti-GFAP (1:1000, Accurate Chemical & Scientific Corp., Westbury, NY), mouse anti-A2B5 (1:400; Chemicon), rabbit anti-Pax6 (1:2000; Covance), mouse anti-HuD (1:200; Invitrogen) and rat anti-BrdU (1:200; Accurate Chemicals). The secondary antibodies used for immunocytochemistry

were indocarbocyanine (Cy3)-conjugated goat anti-mouse and anti-rabbit IgG (1:400; Jackson ImmunoResearch), FITC conjugated anti-mouse and anti-rabbit IgG (1:200; Jackson ImmunoResearch), goat anti-rat IgG AlexaFluor 555 (1:1000; AlexaFluor), dichlorotriazinyl amino fluorescein-conjugated streptavidin (1:1000; Jackson ImmunoResearch), and (Cy3)-conjugated streptavidin (1: 1000; Jackson ImmunoResearch). The specificity of the Rsk2 antibody for immunostaining was confirmed using a blocking peptide supplied by the manufacturer (Santa Cruz).

*Microscopy and confocal analysis*

For quantification of immunocytochemistry on cultured cells, approximately 200–300 cells per condition per experiment were counted and analyzed in at least 15 randomly selected fields spanning the culture well. Digital image acquisition was performed with Northern Eclipse software (Empix, Mississauga, Ontario, Canada) using a Sony (Tokyo, Japan) XC-75CE CCD video camera. In all cases at least 3 independent experiments were performed and these data were pooled unless otherwise indicated. For quantification of immunocytochemistry on tissue sections, brains were chosen with a similar anatomical distribution and level of EGFP expression. Images of the electroporated dorsal telencephalon were taken and covered the ventricular zone, subventricular zone, and cortical plate of each coronal section. A total of four to five sections were analyzed per animal and 4–6 littermate pairs were analyzed per condition. Images represented a mean of four scans taken with a 40× objective and were analyzed using a Zeiss Pascal confocal microscope and the manufacturer's software (Oberkochen, Germany). Error bars indicate s.e.m., and the statistics were performed using Student's t test.

*Western blot analysis*

To assess the expression of Rsk2 and phospho-Rsk, embryonic cortical tissue was rinsed twice in ice-cold phosphate-buffered saline (PBS) and digested in lysis buffer containing protease inhibitors (Roche). To assess the efficacy of Rsk2 shRNA to knockdown overexpressed Rsk2, HEK293 cells were cotransfected with mouse HA-Rsk2 and either non-silencing shRNA or Rsk2 shRNA. 30 μg of protein lysates was run on SDS-PAGE, and Western blots were performed as described (Barnabé-Heider et al., 2005; Dugani et al., 2009; Gauthier-Fisher et al., 2009). Blots were reprobed for ERK1/2 as a loading control. The primary antibodies were rabbit anti-Rsk2 (1:200; Santa Cruz), rabbit anti-phospho-Rsk (Ser227; 1:200; Santa Cruz), anti-HA (1:1000; Hospital for Sick Children Core Facility, Toronto), and rabbit anti-Erk (1:3000; Santa Cruz). Secondary antibodies were HRP-conjugated goat anti-mouse or anti-rabbit (1:10,000; Boehringer Mannheim). Densitometry was carried out using Image J software, and the density of HA-Rsk2 was expressed relative to Erk expression obtained by reprobing the same blot.

## Results

*Rsk2 is present and active in embryonic cortical radial precursor cells*

To address a potential role for Rsk2 during cerebral cortex development, we first determined its timecourse of expression in the embryonic cortex. To do this, we isolated cortices at timepoints ranging from embryonic days 12–13 (E12–13), when the cortex is largely comprised of proliferating precursors, until postnatal day 2 (P2), when it also contains postmitotic neurons and glial cells. Western blot analysis of tissue lysates with an antibody that recognizes both the unphosphorylated and the activated, phosphorylated forms of Rsk2 demonstrated that this protein was present at the highest levels at the earliest timepoints examined, and that its levels decreased substantively by P2 (Fig. 1A). To determine if Rsk2 was active, we performed a similar analysis with an antibody that recognizes only the phosphorylated form of Rsk. Western blots revealed that phospho-Rsk levels were highest at E12–13, and that the amount of phospho-Rsk decreased coincidentally with the decrease in Rsk2 levels (Fig. 1B).

The decrease in levels of Rsk2 coincides temporally with the differentiation of cortical precursors into neurons and glial cells. To ask if, as suggested by these findings, Rsk2 was expressed in precursor cells and/or their progeny *in vivo*, we performed immunocytochemistry for Rsk2 on the E13.5 embryonic cortex, double-labelling sections with an antibody for the proliferation marker Ki67 to identify proliferating precursor cells. This analysis (Fig. 1C) demonstrated that Rsk2 was expressed in Ki67-positive cells of the ventricular zone/subventricular zone (VZ/SVZ) which contains various precursor populations. In these cells, Rsk2 immunoreactivity was primarily localized to the cell soma, with some immunoreactivity in nuclei, consistent with its known subcellular localization (Anjum and Blenis, 2008). Rsk2 was also expressed in cells that had migrated out of the VZ/SVZ into the cortical

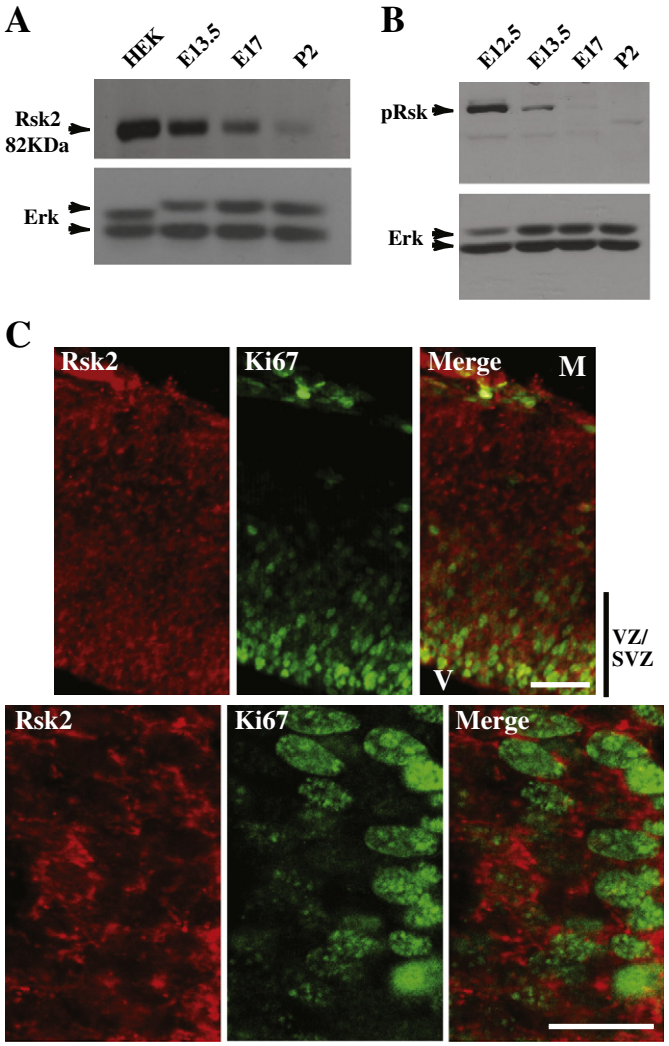



**Fig. 1.** Rsk2 is present and active in precursor cells of the embryonic cortex. (A) Western blot analysis for Rsk2 in HEK293 cells and cortices isolated at different developmental stages. The blot was reprobed for Erk protein as a loading control. (B) Western blot analysis for phospho-Rsk protein (pRsk) in cortices isolated at different developmental stages. The blot was reprobed for total Erk. (C) Immunocytochemical analysis for Rsk2 (red) and the proliferation marker Ki67 (green) in coronal sections of E13.5 cortex. The top panels show the entire width of the cortex from the ventricle (V) to the meninges (M). The VZ/SVZ, which contains precursor cells, is denoted. The bottom panels show the VZ/SVZ at higher magnification. In both cases, the right panels show the merged image. Scale bars = 50 μm (upper panels); 20 μm (bottom panels).

mantle, consistent with expression in postmitotic neurons, as previously reported (Zeniou et al., 2002; Kohn et al., 2003). Detectable Rsk2 immunoreactivity was decreased at later embryonic timepoints, consistent with the Western blot analysis (data not shown).

To ask about a potential role for Rsk2 in cortical precursors, we cultured primary cells from E12–13 cortex, and plated them in the presence of FGF2. Upon plating, these cells are almost all dividing, nestin-positive radial precursor cells. Over the next 1–7 days in culture, these cells will exit the cell cycle and sequentially differentiate into neurons, astrocytes, and oligodendrocytes, as we have previously described (Barnabé-Heider and Miller, 2003; Barnabé-Heider et al., 2005; Gauthier et al., 2007; Paquin et al., 2009). Initially, we performed Western blot analysis to confirm that Rsk2 was expressed in cortical precursor cells in culture as it was *in vivo*. Western blots at 2, 4 and 6 days in culture (Fig. 2A) showed that Rsk2 was expressed at the highest levels at 2 days, when cultures consist predominantly of precursors, and decreased somewhat by 6 days, when cultures contain a mixed population of precursors and neurons. To identify the cell types

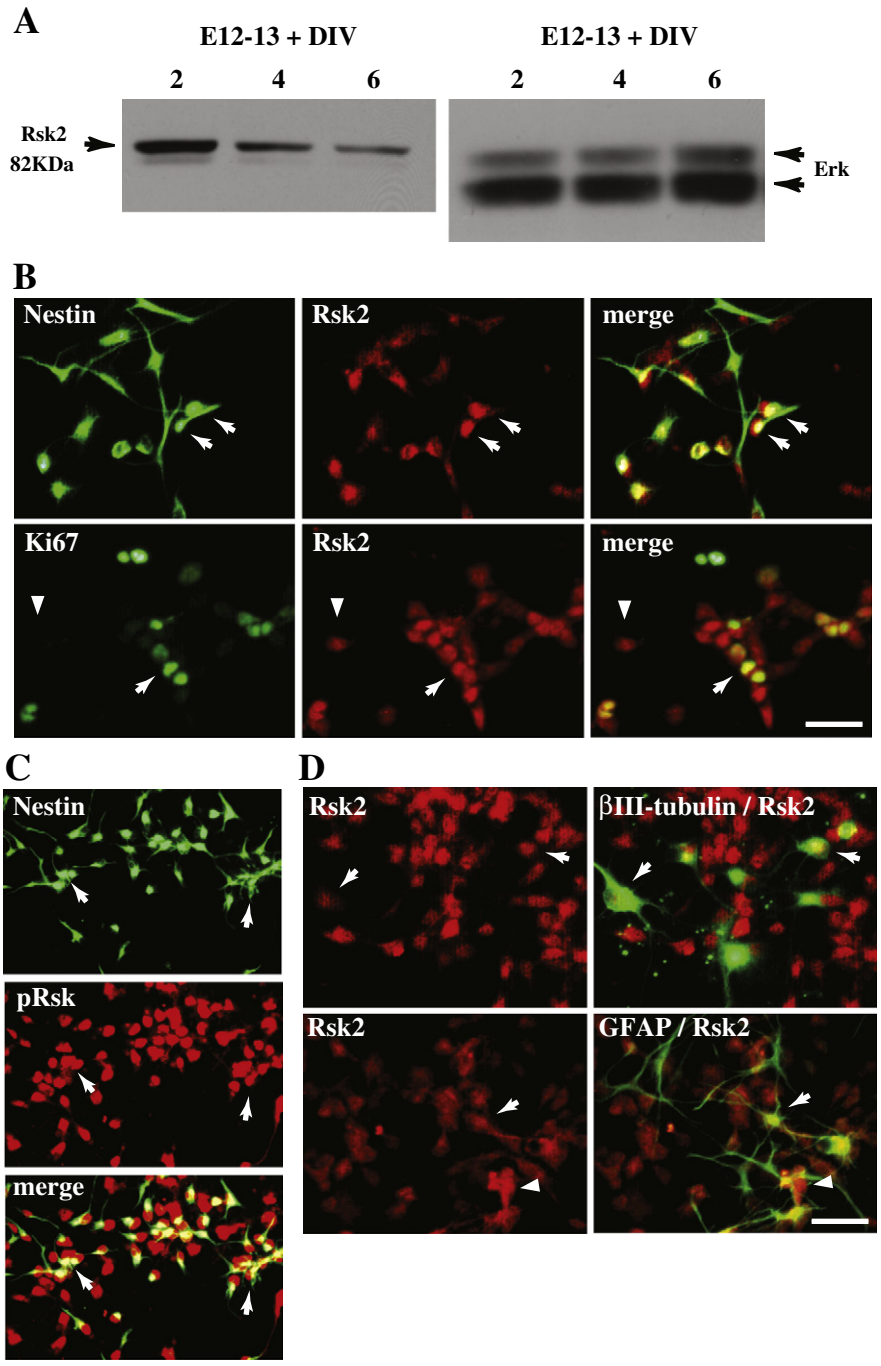



**Fig. 2.** Rsk2 is present and active in cortical precursors and their neuronal and glial progeny in culture. (A) Western blot analysis for Rsk2 in E12–13 cortical precursor cells cultured for 2, 4 and 6 days. The Rsk2 blot was reprobed for total Erk as a control for equal protein loading. (B) Immunocytochemical analysis of cortical precursors cultured for 2 days, and double-labelled for Rsk2 (red) and the precursor marker nestin (green, top panels) or the proliferation marker Ki67 (green, bottom panels). Right panels show the merged micrographs. Arrows denote double-labelled cells and the arrowhead a cell that is only positive for Rsk2. Scale bar = 100 μm. (C) Immunocytochemical analysis of cortical precursors cultured for 2 days, and immunostained for nestin (green) and phospho-Rsk (pRsk, red). Arrows denote double-labelled cells. Scale bar = 100 μm. (D) Immunocytochemical analysis of cortical precursors cultured for 4 days (top panels) or 6 days (bottom panels), and immunostained for Rsk2 (red) and the neuron-specific protein βIII-tubulin, or the astrocyte protein GFAP (all in green). Arrows denote double-labelled cells. Scale bar = 100 μm.

that expressed this kinase, cultures were immunostained for Rsk2 and the precursor marker nestin or the proliferation marker Ki67. At 2 days, Rsk2 was expressed in virtually all nestin-positive, Ki67-positive precursors in these cultures (Fig. 2B). Some Rsk2 was present in the cytosol of these precursors, but much of it was localized to nuclei, potentially because cells are cultured in saturating FGF2, and Rsk proteins translocate to the nucleus when activated (Anjum and Blenis, 2008). Support for this idea was obtained by immunostaining for phospho-Rsk and nestin; activated Rsk was detectable in the nuclei of almost all nestin-positive precursors (Fig. 2C).

To ask whether Rsk2 was also present and active in the differentiated progeny of cortical precursors, we immunostained cultures for Rsk2 or phospho-Rsk and the neuronal marker βIII-tubulin, the astrocyte marker GFAP or the early oligodendrocyte marker A2B5 at 3, 5 and 7 days, respectively. This analysis revealed detectable expression of both Rsk2 and phospho-Rsk in almost all of these differentiated cells (Fig. 2D, data not shown), although expression was apparently lower than in cortical precursors, consistent with the *in vivo* timecourse (Figs. 1A, B).

### *Decreased levels of Rsk2 disrupt neurogenesis in cultured cortical precursors*

These data demonstrated that Rsk2 is expressed and active in cortical precursor cells. We therefore asked whether Rsk2 is essential for the genesis of neurons from cortical precursors. To do this, we utilized 3 different shRNA plasmids to knockdown endogenous Rsk2 mRNA. As a control, we utilized an shRNA against a mismatched mouse sequence. To confirm the efficacy and specificity of these shRNAs, we cotransfected them into HEK293 cells with a plasmid encoding an HA-tagged form of murine Rsk2, and then analyzed these cells 2 days later by Western blotting for the HA tag. This analysis (Fig. 3A) demonstrated that all three shRNAs were able to decrease the expression of the HA-tagged murine Rsk2, and densitometry showed that the approximate level of knockdown was 60–80% (Fig. 3A) in each case.

Having established the efficacy of these shRNAs, we asked whether Rsk2 was essential for normal cortical precursor development. To do this, we cotransfected cultured cortical precursors with a plasmid encoding nuclear EGFP and either the control shRNA or one of the three Rsk2 shRNAs. Initially, we examined cell survival by immunostaining these cultures for EGFP and quantifying the percentage of EGFP-positive transfected cells with condensed, fragmented nuclei. We have previously shown that this is an accurate measurement of apoptosis in cultured cortical precursors, and that it gives results similar to those obtained by immunostaining for the apoptotic marker cleaved caspase-3 (Bartkowska et al., 2007; Dugani et al., 2009). This analysis demonstrated that Rsk2 knockdown had no effect on cell survival at either 2 (Fig. 3B) or 5 (Fig. 3C) days in culture. We then asked whether Rsk2 knockdown had any effect on cell proliferation by immunostaining similar transfected cultures for EGFP and the proliferation marker Ki67 (Fig. 3D). Quantification demonstrated that Rsk2 shRNA knockdown had no effect on the percentage of Ki67-positive, EGFP-positive precursors at 2 days in culture (Fig. 3E).

Having established that Rsk2 knockdown had no effect on cell survival or proliferation in these cultures, we asked whether it affected neurogenesis. To do this, we cotransfected cortical precursors with plasmids encoding EGFP and one of the three shRNAs, and then immunostained the cultures at 5 days for the early neuronal marker βIII-tubulin (Fig. 3F). Quantification revealed that all three of the Rsk2 shRNAs decreased the percentage of neurons by approximately 20–30% (Fig. 3G). Since cell survival is unaffected at this timepoint (Fig. 3C), then these data imply that there must be a commensurate increase in the relative proportion of precursors and/or another differentiated cell type in these cultures. To address this issue, we immunostained cultures for the radial precursor marker Pax6 at 5 days. Quantification demonstrated that the proportion of radial precursors increased from approximately 25% to 40–45% in these cultures (Fig. 3H). Since the proportion of neurons in these cultures decreases from approximately 80% to 60% when Rsk2 is knocked-down (Fig. 3I), then this indicates that when Rsk2 is knocked-down radial precursors are maintained at the expense of newly-born neurons.

Finally, to ensure that this decrease in neurogenesis was due to knockdown of Rsk2, we performed rescue experiments. Cortical precursors were cotransfected with EGFP and the control or Rsk2 shRNAs, along with an expression construct encoding HA-tagged Rsk2. As a control, we used the empty vector instead of HA-Rsk2. Immunostaining five days later revealed that expression of the HA-Rsk2 construct, but not the empty vector, was able to completely rescue the decrease in transfected βIII-tubulin-positive neurons following knockdown of Rsk2 (Fig. 3I).

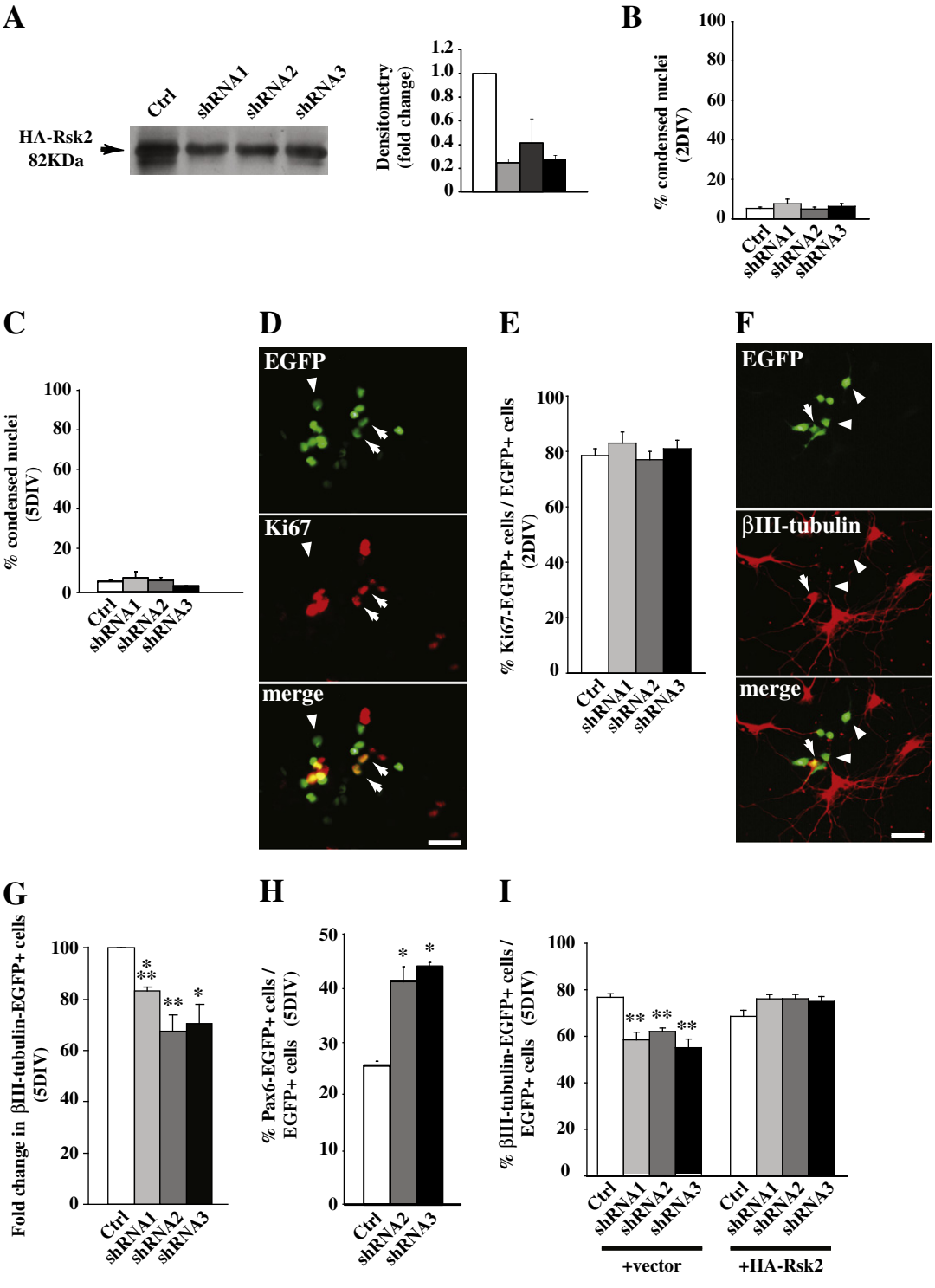
### *Rsk2 knockdown increases the number of proliferating radial precursor cells in vivo*

The *in vitro* data suggest that Rsk2 serves to promote the genesis of neurons from cortical radial precursor cells during embryogenesis. To test this hypothesis *in vivo*, we acutely manipulated precursors in the VZ/SVZ of the E13/14 cortex *in vivo* using *in utero* electroporation. We have previously shown that 1 day following electroporation, all of the transfected cells reside in the VZ/SVZ, and most of them are proliferating radial precursors (Paquin et al., 2005; Bartkowska et al., 2007; Gauthier-Fisher et al., 2009). Over the next few days, many of these transfected precursor cells differentiate into neurons and migrate to the cortical plate, while, of those that remain, many differentiate into astrocytes and some into oligodendrocytes during late embryogenesis and early neonatal life. We therefore coelectroporated plasmids encoding nuclear-localized EGFP and Rsk2 shRNAs

**Fig. 3.** Rsk2 knockdown decreases the genesis of neurons and maintains radial precursors in culture. (A) Western blot analysis of HEK293 cells cotransfected with HA-tagged wild type mouse Rsk2 and a control non-silencing shRNA, or one of three Rsk2 shRNAs (shRNA-1, 2 and 3). The membrane was probed for the HA tag to detect the transfected mouse Rsk2. The graph on the right shows quantification of two similar experiments where the blots were reprobed for total Erk to monitor protein loading, and then analyzed by scanning densitometry. Numbers represent the relative HA-Rsk2/Erk ratio normalized to cells transfected with control shRNA. (B–I) Cultured cortical precursors were cotransfected with plasmids encoding nuclear EGFP, to identify transfected cells, and the control shRNA, or one of the three Rsk2 shRNAs. (B,C) Quantification of cortical precursors cultured for 2 days (B) or 5 days (C), immunostained for EGFP and counterstained with Hoechst 33258 to analyze the percentage of transfected cells with condensed, fragmented apoptotic nuclei. Results are pooled data from 3 independent experiments. Error bars represent s.e.m. (D) Fluorescence photomicrographs of cortical precursors immunostained at 5 days for EGFP (green) and Ki67 (red). Arrows denote double-labelled cells, while the arrowhead indicates a cell that is only positive for EGFP. Scale bar = 50 μm. (E) Quantification of the percentage of EGFP-positive cells that were also positive for Ki67 cells in experiments similar to that shown in D, after 2 days in culture. The graphs represent combined data from 3 independent experiments. Error bars denote s.e.m. (F) Fluorescence photomicrographs of cortical precursors immunostained at 5 days for EGFP (green) and βIII-tubulin (red). Arrow denotes a double-labelled cell, while arrowheads indicate cells that are only positive for EGFP. Scale bar = 50 μm. (G) Quantification of cultures similar to that shown in F for the relative percentage of EGFP-positive cells that were also positive for βIII-tubulin after 5 days in culture, normalized to sister cultures that were transfected with the control shRNA (considered as 100%). Graphs represent combined data from 3 independent experiments. Error bars denote s.e.m. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ relative to control shRNA-transfected cultures. (H) Quantification of cortical precursors immunostained for EGFP, to detect transfected cells, and the radial precursor marker Pax6 after 5 days in culture, in experiments similar to that shown in F. Results are expressed as the percentage of EGFP-positive cells that were also positive for Pax6. Graphs represent combined data from 3 independent experiments. Error bars denote s.e.m. $^{*}p<0.05$ relative to control shRNA-transfected cultures. (I) Quantification of the percentage of EGFP-positive, βIII-tubulin-positive cells after 5 days in culture, in experiments similar to that shown in F, where cortical precursor cells were also transfected with HA-Rsk2 or empty vector. The graphs represent combined data from 4 independent experiments. Error bars denote s.e.m. $^{**}p<0.01$ relative to control shRNA-transfected cultures.

into cortical precursors lining the lateral ventricles of E13/14 embryos, and asked whether Rsk2 knockdown had any effect on these precursor cells. Initially, we examined cell proliferation; brains were coronally sectioned 3 to 4 days post-electroporation and immunostained for EGFP and the proliferation marker Ki67. Confocal microscopy and quantification of these sections demonstrated a significant increase in the percentage of Ki67-positive cells that were transfected with Rsk2 shRNA versus control shRNA at 3 days post-electroporation (Figs. 4A–C). Similar results were obtained at 4 days post-electroporation, with 13% Ki67-GFP-positive for the control compared to 19% Ki67-GFP-positive cells with Rsk2 shRNA-transfected precursor cells. To confirm this finding, we electroporated embryos at E13/14 with EGFP and control or Rsk2 shRNA, injected the mothers with BrdU 3 days post-electroporation, and then analyzed the embryonic cortices 24 h later. Double-labelling for BrdU and EGFP demonstrated that the proportion of precursor cells entering S-phase over the previous 24 h was increased almost two-fold when Rsk2 was knocked-down (Fig. 4D).

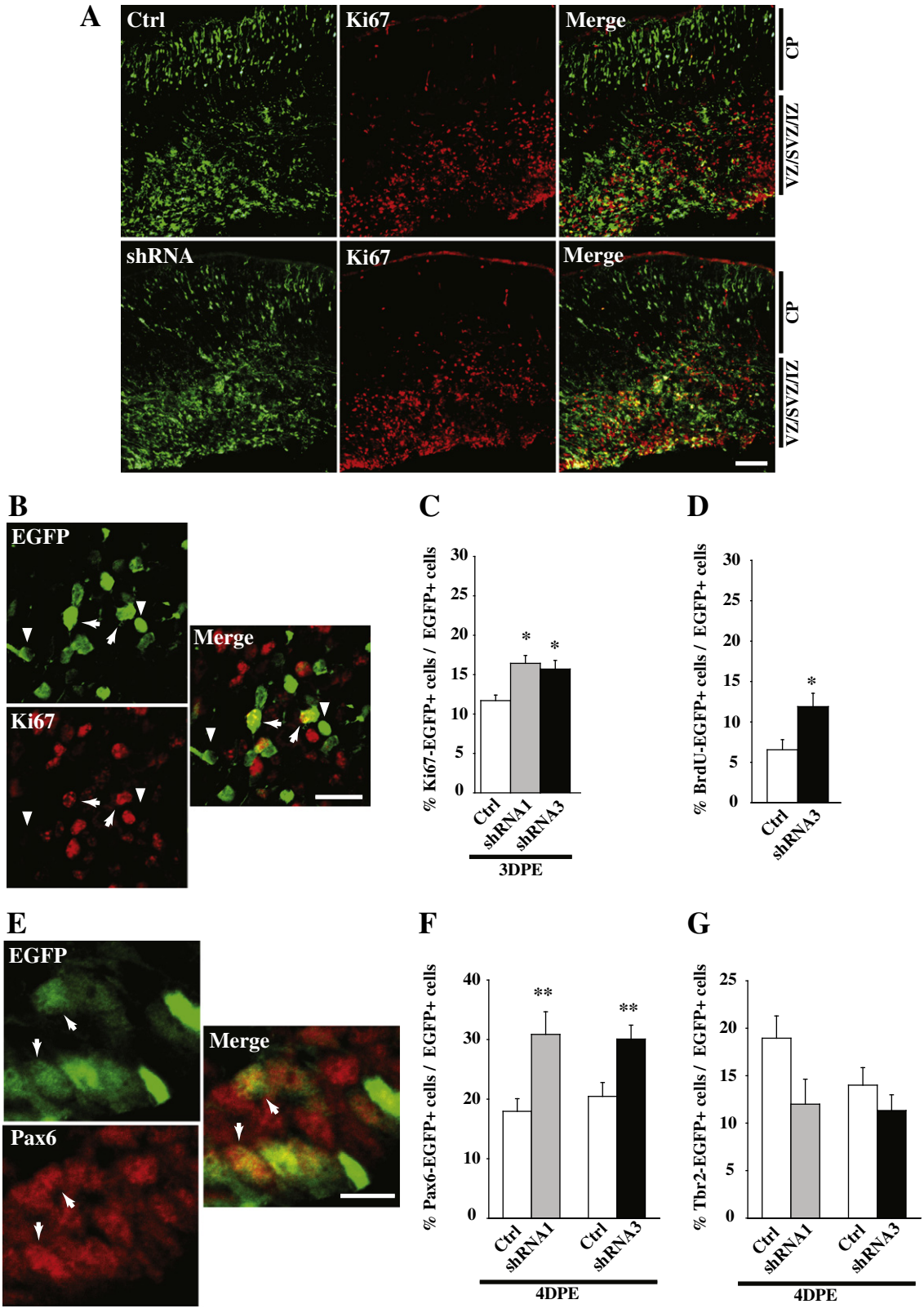
To define the population of precursors that was maintained when Rsk2 was knocked-down, we performed similar experiments, and immunostained cortical sections 4 days post-electroporation for the radial precursor transcription factor Pax6, or for the intermediate progenitor marker Tbr2. This analysis revealed a 1.5-to-2-fold increase in the percentage of Pax6 positive precursor cells when



Rsk2 was knocked-down (Figs. 4E, F). By contrast, the percentage of Tbr2-positive intermediate progenitors was unaffected, although there was a trend towards a decrease in their numbers (Fig. 4G). Since intermediate progenitors are biased neuronal progenitors (Noctor et al., 2004; Gal et al., 2006; Attardo et al., 2008), then these findings are consistent with the conclusion that Rsk2 knockdown specifically promotes maintenance of proliferating radial precursors, potentially by inhibiting their differentiation along a neuronal pathway.

*Rsk2 is essential for cortical neurogenesis, but not astrogenesis*

To ask whether Rsk2 knockdown decreases neurogenesis *in vivo*, as it does in culture, we performed similar experiments, electroporating



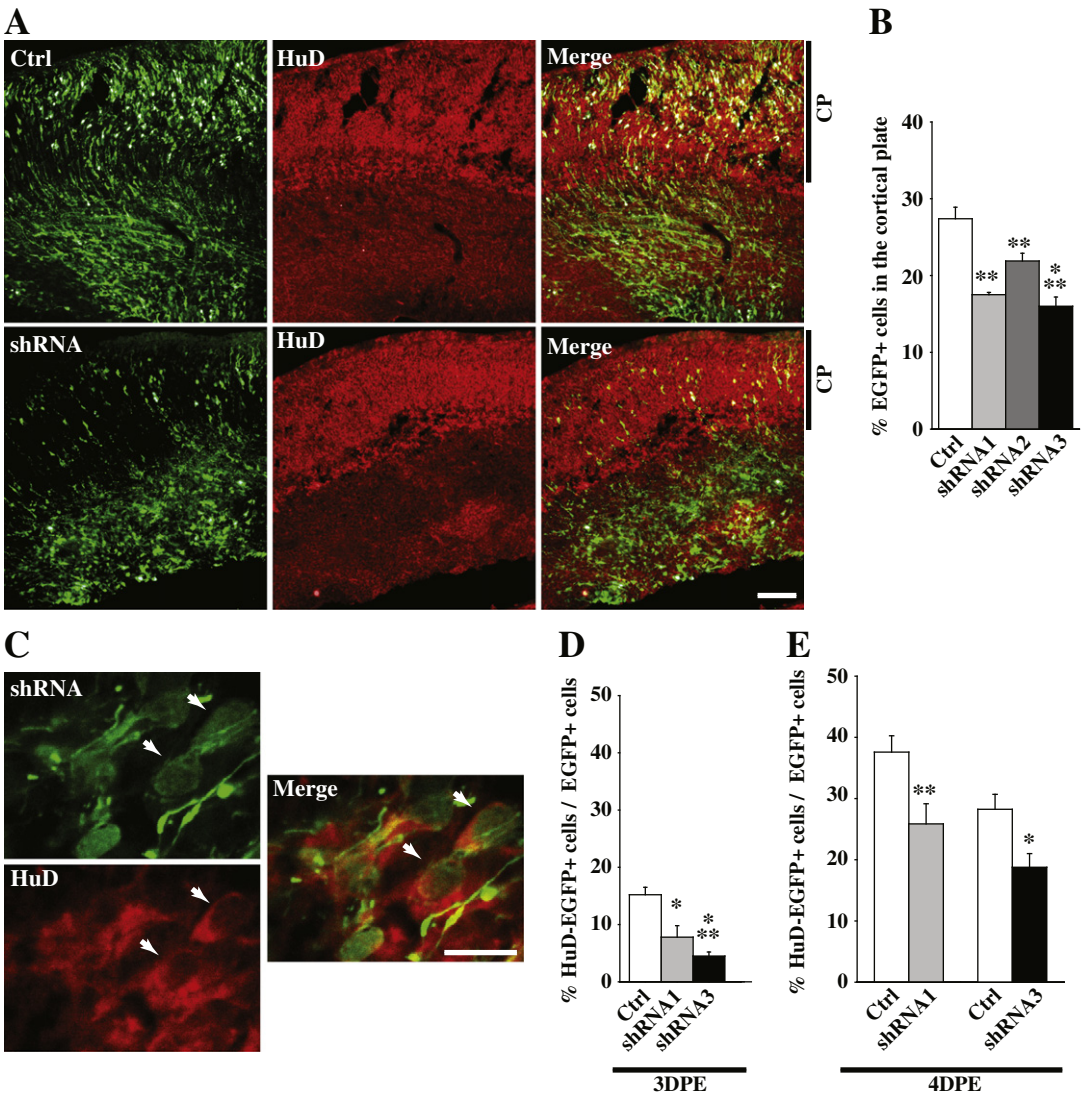



**Fig. 5.** Rsk2 knockdown decreases neurogenesis *in vivo*. Precursor cells of the embryonic cortex were transfected by *in utero* electroporation at E13/14 with plasmids encoding nuclear EGFP and a control shRNA or Rsk2 shRNAs, and then analyzed 3 or 4 days later. (A) Confocal micrographs of cortical sections 3 days post-electroporation (3DPE) that were immunostained for EGFP (green) and HuD (red), a neuron-specific marker primarily expressed in neurons of the cortical plate (CP). The right panels show the merged images. Scale bar = 100 μm. (B) Quantification of EGFP-positive cells located within the cortical plate as a percentage of total EGFP transfected cells. Data are combined from 3–5 embryos per condition. Error bars denote s.e.m. $^{**}p<0.01$, $^{***}p<0.001$, relative to the scrambled shRNA-electroporated cortices. (C) High magnification confocal images of a section similar to those shown in panel A, immunostained for EGFP (green) and HuD (red). Arrows denote double-labelled cells. Scale bar = 10 μm. (D, E) Quantification of the percentage of EGFP-positive cells also positive for HuD in experiments similar to that shown in panel C at 3 (D) and 4 (E) days post-electroporation. The graphs represent combined data from 3–5 embryos per condition. Error bars denote s.e.m. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, relative to the scrambled shRNA-electroporated cortices.

E13/14 cortices with constructs encoding nuclear EGFP and either control or Rsk2 shRNAs and analyzing them 3 or 4 days later (Fig. 5A). Analysis of coronal sections through these cortices demonstrated a decrease of as much as 43% in the percentage of EGFP-positive cells that had migrated from the VZ/SVZ to the cortical plate (which contains newly-born cortical neurons) when Rsk2 expression was knocked-down (Fig. 5B). Since cortical precursors normally generate neurons in the VZ/SVZ, and these newly-born neurons then migrate to the cortical plate, then this decrease suggests that fewer neurons are generated when Rsk2 is knocked-down. To test this idea, we

**Fig. 4.** Rsk2 knockdown increases the number of proliferating radial precursor cells *in vivo*. Precursor cells of the embryonic cortex were transfected by *in utero* electroporation at E13/14 with plasmids encoding nuclear EGFP and either a control shRNA or Rsk2 shRNAs, and then analyzed 3 or 4 days later. (A) Confocal micrographs of electroporated cortical sections that were immunostained for EGFP (green) and the proliferation marker Ki67 (red). The right panels show the merged image. Scale bar = 100 μm. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate. (B) High magnification confocal images of a section similar to that shown in A, for Ki67. Arrows denote double-labelled cells, while arrowheads indicate cells that were only positive for EGFP. Scale bar = 25 μm. (C) Quantification of the percentage of EGFP-positive cells also positive for Ki67 in experiments similar to that shown in B at 3 days post-electroporation (3DPE). The graphs represent combined data from 3–5 embryos per condition. Error bars denote s.e.m., $^{*}p<0.05$ relative to cortices electroporated with the control shRNA. (D) Quantification of the percentage of EGFP-positive cells that were also positive for BrdU in experiments where pregnant mothers were injected with BrdU 3 days after their embryos were electroporated with control shRNA or Rsk2 shRNA-3, and embryonic cortices were analyzed 1 day later. The graphs represent combined data from 5 brain sections per embryo and 2 embryos per condition. Error bars denote s.e.m., $^{*}p<0.05$ relative to cortices electroporated with control shRNA. (E) High magnification confocal images of a section similar to those shown in (A) for the radial precursor marker Pax6. Arrows denote double-labelled cells. Scale bar = 10 μm. (F) Quantification of the percentage of EGFP-positive cells also positive for Pax6 in experiments similar to that shown in E. The graphs represent combined data from 3–4 embryos per condition. Error bars denote s.e.m., $^{**}p<0.01$ relative to cortices electroporated with the control shRNA. (G) Quantification of the percentage of EGFP-positive cells also positive for Tbr2 4 days following electroporation. The graphs represent combined data from 3–4 embryos per condition. Error bars denote s.e.m. $p>0.05$.

immunostained these sections for the neuron-specific protein HuD (Figs. 5A, C). Confocal microscopy demonstrated that fewer HuD-positive neurons were generated when Rsk2 was knocked-down with either of two different Rsk2 shRNAs (Figs. 5D, E). The decrease in the percentage of neurons was comparable to the decrease in cells within the cortical plate; at 3 days, the decrease was as much as 75%, while by 4 days, it was approximately 30%. Since no cells expressing HuD were mislocalized to the VZ/SVZ, then these data indicate that the primary effect of Rsk2 knockdown was on the genesis of neurons, not on the migration of those newly-born neurons.

Together, these data indicate that Rsk2 is essential for efficient cortical neurogenesis, and that when it is knocked-down, many cortical precursors do not differentiate appropriately into neurons, but instead remain as cycling radial precursors. To ask whether Rsk2 was also essential for astrocyte differentiation, we first performed experiments in culture. Cultured cortical precursors were cotransfected with constructs encoding nuclear EGFP and either control shRNA or Rsk2 shRNA, and then treated the next day with ciliary neurotrophic factor (CNTF), which induces astrogenesis (Bonni et al., 1997; Rajan and McKay, 1998; Barnabé-Heider et al., 2005). The cultures were then immunostained for the astrocyte marker GFAP after 5 days (Fig. 6A). Quantification demonstrated that the knockdown of Rsk2 had no significant effect on the generation of astrocytes, and that the percentage of GFAP-positive cells ranged between 35% and 45% in cells transfected with either the scrambled shRNA or Rsk2 shRNAs (Fig. 6B). We then asked the same question *in vivo*, using *in utero* electroporation to cotransfect E13/14 cortices with plasmids encoding nuclear GFP and either control shRNA or Rsk2 shRNA, allowing the animals to develop until P3, at which point astrogenesis is ongoing. Immunostaining of coronal sections for the astrocyte marker GFAP (Fig. 6C) revealed no differences in the percentage of GFAP-positive cells that were transfected with Rsk2 versus control shRNAs (Fig. 6D). Thus, while Rsk2 is essential for efficient neurogenesis, it is apparently dispensable for normal astrogliogenesis.

These data demonstrate that Rsk2 knockdown causes decreased neurogenesis, but has no effect on the proportion of astrocytes, suggesting that even after birth, Rsk2 knockdown might increase the proportion of radial precursors that are present. To address this

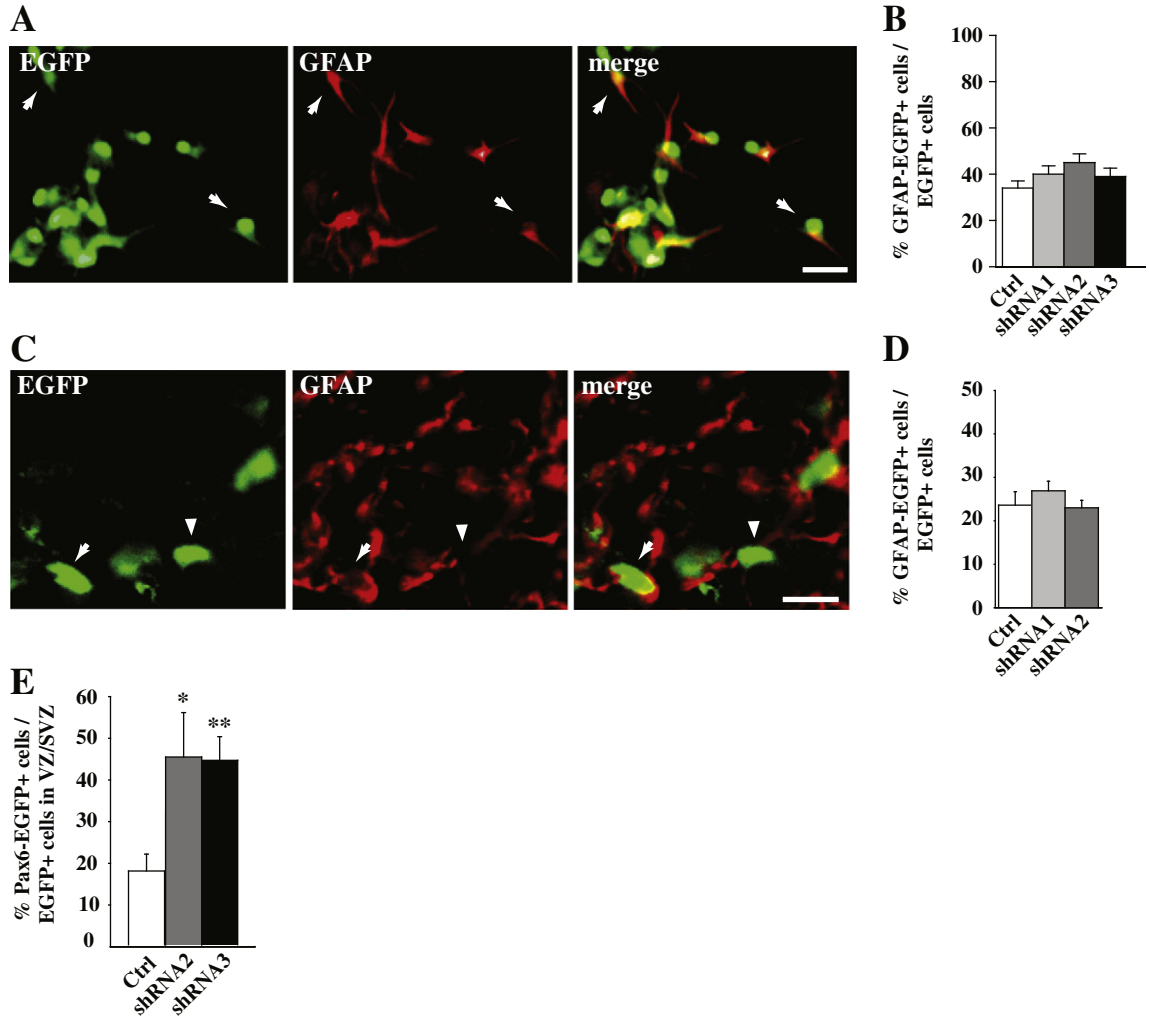



**Fig. 6.** Rsk2 knockdown does not affect astrogenesis but increases the size of the radial precursor pool in the neonatal brain. (A) Cortical precursors cotransfected with plasmids encoding nuclear EGFP and a control shRNA, or one of the three Rsk2 shRNAs, cultured for 5 days in CNTF, and immunostained for EGFP (green) and the astrocyte marker GFAP (red). Arrows denote double-labelled cells. Scale bar = 15 μm. (B) Quantification of the percentage of EGFP-positive cells that expressed GFAP in experiments similar to that shown in panel A. The graph represents combined data from three independent experiments, and error bars are s.e.m. (C) Confocal micrographs of a cortical section where electroporation was performed at E13/14 and the cortex was analyzed 10 days later at P3. Cortices were electroporated with plasmids encoding nuclear EGFP and a control shRNA or Rsk2 shRNAs, and immunostained for EGFP (green) and GFAP (red). The right panel shows the merged image, and the arrow denotes a double-labelled cell while the arrowhead indicates a cell that is positive for only EGFP. Scale bar = 10 μm. (D) Quantification of the percentage of EGFP-positive cells also positive for GFAP in experiments similar to that in panel C. The graph represents combined data from 3–4 animals per condition. Error bars denote s.e.m. (E) Quantification of the percentage of EGFP-positive cells that were also positive for Pax6 in the VZ/SVZ of cortices electroporated as for the experiments shown in panel C, except that sections were immunostained for Pax6 rather than GFAP. The graph represents combined data from 3–4 animals per condition. Error bars denote s.e.m. *$p<0.05$, **$p<0.01$.

possibility, we immunostained sections from the same electroporated P3 cortices for the radial precursor marker Pax6. This analysis (Fig. 6E) showed that Rsk2 knockdown increased the proportion of EGFP-positive radial precursors in the postnatal VZ/SVZ approximately 3-fold. Thus, radial precursors that would normally have generated neurons during embryogenesis are not ultimately subverted to a glial fate by Rsk2 knockdown, but are instead maintained as radial precursors at least until the neonatal period.

## Discussion

The results from this study support a number of major conclusions. First, we show that Rsk2 is expressed in embryonic cortical precursors and their neuronal and glial progeny, and that both Rsk2 and activated phosphorylated Rsk decrease during cortical development, coincidentally with precursor differentiation. Second, our experiments using shRNAs to acutely knockdown Rsk2 in culture show that Rsk2 is not required for precursor survival, but that it is instead required for radial precursors to efficiently differentiate into neurons. Third, our *in utero* electroporation experiments confirm that Rsk2 is essential for radial precursors to generate neurons *in vivo*, and show that when its levels are decreased, a greater percentage of precursors are maintained as proliferating radial precursors throughout embryonic development. Finally, we show that both in culture and *in vivo*, acute knockdown of Rsk2 has no apparent effect on the genesis of astrocytes. Thus, our data indicate that Rsk2 is essential for radial precursors to exit the cell cycle and generate neurons, and that Rsk2 therefore likely provides a key downstream target for extracellular cues such as growth factors that regulate the timing and number of neurons that are generated. Moreover, these findings suggest that the dysregulated neurogenesis seen when levels of Rsk2 are decreased may explain the neuroanatomical and cognitive deficits observed in individuals with CLS, providing support for the idea that some genetic disorders that cause cognitive dysfunction do so by perturbing neural precursor development.

During embryonic cortical development, various intrinsic and extrinsic factors regulate the genesis of neurons (Miller and Gauthier, 2007; Corbin et al., 2008; Rakic, 2009). We and others have previously provided evidence that one way growth factors regulate neurogenesis is via the SHP2–MEK–Erk–C/EBP pathway (Ménard et al., 2002; Paquin et al., 2005; Liu et al., 2006; Gauthier et al., 2007; Samuels et al., 2008). In particular, neurogenesis was decreased in cortical precursors following SHP2 knockdown (Gauthier et al., 2007), or expression of dominant-negative MEK (Ménard et al., 2002) and mice with a conditional loss of Erk in the cortex show blunted neurogenesis (Samuels et al., 2008). Moreover, the transcription factor C/EBP was shown to be essential for efficient cortical neurogenesis both in culture and *in vivo*, and phosphorylation of C/EBP downstream of the MEK–Erk pathway was essential for C/EBP to enhance neurogenesis (Ménard et al., 2002; Paquin et al., 2005). Intriguingly, the neurogenic bHLH neurogenin has also recently been shown to be downstream of Erk5 (Cundiff et al., 2009), raising the possibility that the MEK–Erk pathway activates multiple transcription factors to promote the genesis of neurons from neural precursors.

While Erk can itself directly phosphorylate and regulate transcription factors that are important for cortical development, our data indicate that it also likely regulates neurogenesis by phosphorylating and activating Rsk2 (Roux et al., 2003; Kang et al., 2007). We show that in culture, cortical precursors express Rsk2, and that much of this is activated and localized to the nucleus, likely as a consequence of their exposure to saturating quantities of FGF2, a growth factor that we have previously shown activates the MEK–Erk pathway in these cells (Barnabé-Heider and Miller, 2003). Our *in vivo* Western blot and immunocytochemical analyses indicate that Rsk2 is also expressed in dividing precursors in the embryonic cortex and that at least a fraction of this Rsk2 is phosphorylated and activated under physiological conditions. However, in contrast to the culture situation, much of this Rsk2 is localized to the cytosol of precursors *in vivo*, with only some of it being nuclear-localized. Since Rsk proteins translocate to the nucleus when activated (Anjum and Blenis, 2008), then these findings are consistent with the concept that activation of Rsk2 is not saturating *in vivo*, and that small changes in growth factors within the embryonic environment might cause large changes in cortical precursor biology *in vivo*, in part by acting via signalling kinases such as Rsk2.

How then does Rsk2 promote neurogenesis? We propose that it does so by phosphorylating key transcription factors and potentially by directly regulating chromatin structure. In this regard, we have previously shown that the Rsk phosphorylation site on C/EBP is important for its ability to enhance transcription of neuron-specific genes such as Tα1-tubulin (Ménard et al., 2002; Paquin et al., 2005). A second potential Rsk2 neurogenic target is ATF5, a transcription factor that is highly expressed in cortical precursors, and that functions to inhibit differentiation (Angelastro et al., 2003, 2005; Mason et al., 2005). While Rsk2 has not been shown to phosphorylate ATF5, it directly phosphorylates the related family member ATF4 to regulate osteoblast differentiation (Yang et al., 2004). With regard to the potential regulation of chromatin structure, Rsk2 has been shown to interact with and phosphorylate the transcriptional coactivator CBP and to directly regulate its histone acetylase activity (Merienne et al., 2001). Since CBP-mediated histone acetylation is essential for cortical precursors to express lineage-specific genes and differentiate into neurons and glia (Wang et al., 2010), then a MEK–Rsk2–CBP pathway would provide one way that growth factors could globally regulate important neurogenic genes.

What growth factors might utilize this pathway to regulate neurogenesis? PDGF enhances cortical neurogenesis (Williams et al., 1997) at least in part via a MEK–C/EBP pathway (Ménard et al., 2002; Paquin et al., 2005). In addition, BDNF, which binds to the TrkB receptor, causes increased cortical neurogenesis *in vivo*, while knockdown of the TrkB receptor decreases neurogenesis (Bartkowska et al., 2007). Since both PDGF and BDNF bind to receptor tyrosine kinases, and since the MEK–Erk pathway is robustly activated in cortical precursors by both of these growth factors (Ménard et al., 2002; Barnabé-Heider and Miller, 2003), then we suggest that Rsk2 likely provides a downstream proneurogenic convergence point for these and many other growth factors that are encountered within the cortical environment.

While this model focuses on a proneurogenic action for Rsk2, our data also show that Rsk2 knockdown maintains radial precursors in a proliferating precursor state. One explanation for this phenotype is that the precursor phenotype is the default state and that in the absence of an Rsk2-mediated neurogenic signal, precursors are maintained. However, an alternative, and not mutually-exclusive explanation is that Rsk2 is a direct negative regulator of radial precursor proliferation, and that when it is knocked-down, precursors continue to cycle, something that is incompatible with neurogenesis. Our data do not distinguish these two possibilities, and it is even likely that Rsk2 may act in both ways, given the obligate coupling between cell cycle exit and expression of a neuronal phenotype.

Findings reported here provide additional support for the idea that genetic disorders that cause cognitive dysfunction do so, at least in part, by perturbing neural precursor development. Individuals with loss-of-function mutations in Rsk2 that are diagnosed with CLS display thinning of the corpus callosum, asymmetry of the lateral ventricles, and smaller brain volumes (Wang et al., 2006; Kesler et al., 2007), in addition to cognitive deficits. While the brains of Rsk2−/− mice have not been characterized extensively, these mice also display cognitive impairments (Dufresne et al., 2001; Poirier et al., 2007) that are thought to be due to a necessity for Rsk2 in neuronal circuit formation/function (Fisher et al., 2009). However, our findings indicate that these behavioural abnormalities could also be explained by deficits in neurogenesis within the developing cortex and perhaps in other regions of the brain. Intriguingly, Rsk2−/− mice have been

shown to display dysregulation of the CNS dopaminergic system (Marques-Pereira et al., 2008), a problem that could potentially arise because of perturbations in genesis of dopaminergic neurons.

The perturbations in cell genesis reported here for Rsk2 knockdown have also been reported for mutations in signalling proteins upstream of Rsk2. For example, SHP2 mutations cause Noonan syndrome, where approximately half of individuals show some cognitive dysfunction, and when SHP2 mutations are knocked-in to the mouse SHP2 gene, this causes enhanced neurogenesis and decreased astrogenesis (Gauthier et al., 2007). Costello syndrome, which is associated with mental retardation, is caused in part by hyperactivation of H-Ras, and Costello syndrome H-Ras mutants cause aberrant differentiation and proliferation of cortical precursors and their glial progeny (Paquin et al., 2009). Haploinsufficiency for CBP causes Rubinstein–Taybi syndrome and when this haploinsufficiency is modeled in mice, it causes a global decrease in cortical precursor differentiation and behavioural deficits within days of birth (Wang et al., 2010). Thus, while previous studies have largely focused upon how these human mutations affect neural circuit function in the adult brain (Zeniou et al., 2002; Hanauer and Young, 2002; Wang et al., 2006; Poirier et al., 2007), this body of work argues that many of these mutations also dysregulate embryonic neural precursors at much earlier developmental stages. Such findings do not negate the impact of these mutations upon neural circuit establishment and function. Instead, these studies define an additional pathological mechanism, and perhaps even more intriguingly, suggest that human mutations that cause cognitive dysfunction may also define previously-unsuspected pathways that are important for neural precursors, thereby providing a novel and unbiased window into neural development.

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