p27^{Kip1} independently promotes neuronal differentiation and migration in the cerebral cortex

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The generation of neurons by progenitor cells involves the tight coordination of multiple cellular activities, including cell cycle exit, initiation of neuronal differentiation, and cell migration. The mechanisms that integrate these different events into a coherent developmental program are not well understood. Here we show that the cyclin-dependent kinase inhibitor p27^{Kip1} plays an important role in neurogenesis in the mouse cerebral cortex by promoting the differentiation and radial migration of cortical projection neurons. Importantly, these two functions of p27^{Kip1} involve distinct activities, which are independent of its role in cell cycle regulation. p27^{Kip1} promotes neuronal differentiation by stabilizing Neurogenin2 protein, an activity carried by the N-terminal half of the protein. p27^{Kip1} promotes neuronal migration by blocking RhoA signaling, an activity that resides in its C-terminal half. Thus, p27^{Kip1} plays a key role in cortical development, acting as a modular protein that independently regulates and couples multiple cellular pathways contributing to neurogenesis.

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During development of the vertebrate CNS, cycling progenitors generate post-mitotic precursors that rapidly migrate out of germinal zones and initiate neuronal differentiation. The key steps of cell cycle exit, cell migration, and neuronal differentiation are tightly linked during neurogenesis. Proliferation and differentiation are largely incompatible cellular states in the CNS, and cells that initiate neuronal differentiation while remaining mitotic often die (Lee et al. 1992). Conversely, neuronal differentiation and neuronal migration involve a common process of cytoskeleton remodeling and are usually coupled (Luo 2000). A number of molecules that play important roles in regulating individual steps of neurogenesis, including cell cycle exit (Ohnuma and Harris 2003), cell migration (Bielas et al. 2004), and neuronal differentiation (Bertrand et al. 2002) have been identified. Little is known, however, of how these different cellular steps are coordinated in a coherent program of neurogenesis.

Cyclin-dependent kinase inhibitors (CKIs) are good candidates to regulate multiple aspects of neurogenesis. Two families of CKIs promote cell cycle withdrawal by blocking the activity of cyclin/cyclin-dependent kinase (CDK) complexes: the Cip/Kip family, including p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, and the INK4 family, including p15^{Ink4b}, p16^{Ink4a}, p18^{Ink4c}, and p19^{Ink4d} (Elledge and Harper 1994). INK4 proteins act by inhibiting the activity of CDK4 and CDK6. Cip/Kip proteins have broader activities, as they interact with all cyclin/CDK complexes (Sherr and Roberts 1999). CKIs play an essential role in regulating cell cycle in neural tissues. In particular, p27^{Kip1} has been implicated in promoting cell cycle arrest of neural progenitors during embryogenesis (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996; Carruthers et al. 2003), in regulating the division of transit amplifying progenitors in the adult subventricular zone (Doetsch et al. 2002), and, together with p19^{Ink4d}, in maintaining differentiated neurons in a nonmitotic state (Zindy et al. 1999).

Interestingly, there is accumulating evidence that Cip/Kip proteins have activities that go beyond their well-characterized control of cell division. The three Cip/Kip proteins have been shown to regulate differentiation of

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muscle cells (Zhang et al. 1999; Vernon and Philpott 2003) and white blood cells (Casini and Pelicci 1999; Steinman 2002). Cip/Kip proteins have also been implicated in fate specification and differentiation of glial cells, including oligodendrocytes (Durand et al. 1997; Zezula et al. 2001) and retinal Müller glia cells (Ohnuma et al. 1999). Less is known, however, of the role of these factors in neuronal differentiation, although $p27^{Kip1}$ has been implicated in primary neurogenesis in *Xenopus* embryos (Vernon et al. 2003), and $p21^{Cip1}$ has been shown to regulate neurite outgrowth in retinal cells (Tanaka et al. 2002).

$p27^{Kip1}$ also appears to be an important regulator of cell migration in a variety of cell culture models, including fibroblasts, vascular smooth muscle cells, and endothelial cells (Sun et al. 2001; Diez-Juan and Andres 2003; McAllister et al. 2003). $p27^{Kip1}$ promotes migration of fibroblasts by blocking the activity of the small GTPase RhoA, and absence of $p27^{Kip1}$ results in increased number of stress fibers and focal adhesions, and reduced cell motility (Besson et al. 2004). Whether $p27^{Kip1}$ regulates cell migration in vivo, in particular in the nervous system, has not yet been addressed.

The embryonic cortex is an excellent model to study how cell cycle exit, differentiation, and migration are coordinately regulated during neurogenesis. Cortical projection neurons are generated over a 7-d period in the mouse, from progenitor cells located in the germinal zone of the dorsal telencephalon. Newborn neurons migrate radially to reach the cortical plate, where they settle in distinct neuronal layers. Early-born neurons occupy deep cortical layers while later born neurons occupy progressively more superficial layers, resulting in an “inside-out” pattern of cortical histogenesis (Sidman and Rakic 1973). $p27^{Kip1}$ has been shown to play an important role in development of the cerebral cortex, by controlling the birth date of cortical neurons. In $p27^{Kip1}$ -null mutant mice, there is a decrease in neuronal production during mid-corticogenesis and an increase in production of late-born neurons, resulting in an enlargement of upper cortical layers (Goto et al. 2004). Conversely, overexpression of $p27^{Kip1}$ in cortical progenitors results in a reduction in number of upper layer neurons (Tarui et al. 2005). $p27^{Kip1}$ expression levels in cortical progenitors appear to determine both cell cycle length and the probability of cell cycle re-entry, and differences in $p27^{Kip1}$ expression levels between areas of the developing primate cortex have been implicated in area-specific levels of neuronal production (Lukaszewicz et al. 2005).

Here, we have asked whether $p27^{Kip1}$ regulates aspects of cortical neurogenesis other than neuronal production. By analyzing $p27^{Kip1}$ -null mutant embryos and performing overexpression and knockdown experiments, we have shown that $p27^{Kip1}$ promotes both the radial migration and differentiation of newborn cortical neurons. These activities are cell cycle-independent and are independently regulated by distinct domains of the $p27^{Kip1}$ protein. Altogether, our results demonstrate that $p27^{Kip1}$ is a modular protein that regulates multiple pathways

during neurogenesis and thereby plays a key role in coordinating cell cycle exit, differentiation, and radial migration during cortical development.

Results

p27^{Kip1} is the predominant Cip/Kip protein in cortical progenitors and neurons

To investigate the role of Cip/Kip proteins in cortical neurogenesis, we first examined the expression of $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ by RNA in situ hybridization and immunocytochemistry in embryonic day 14.5 (E14.5) mouse cortex. Only $p27^{Kip1}$ transcripts were detected at a significant level in the ventricular zone (VZ), subventricular zone (SVZ), and intermediate zone (IZ), while transcripts for all three genes were present in the cortical plate (CP) (Fig. 1A–C). Similarly, $p27^{Kip1}$ is the only Cip/Kip protein expressed throughout the cerebral cortex, at a moderate level in a subset of VZ progenitors

Figure 1. $p27^{Kip1}$ is expressed in cortical progenitors and neurons. (A–C) In situ RNA hybridization with $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ probes on coronal sections through E14.5 dorso-lateral cortex. The three genes are expressed at low ($p21^{Cip1}$) or high levels ($p27^{Kip1}$, $p57^{Kip2}$) in the cortical plate (CP), while only $p27^{Kip1}$ transcripts are significantly present in the ventricular zone (VZ), subventricular zone (SVZ), and intermediate zone (IZ). (D–F) Immunofluorescent staining at the same stage reveals a pattern of Cip/Kip protein expression comparable to that of their transcripts, with $p27^{Kip1}$ highly expressed from the SVZ to the CP and at lower level in the VZ, while $p21^{Cip1}$ and $p57^{Kip2}$ are only expressed at low levels in the CP. (G–I) Note the predominantly cytoplasmic localization of $p27^{Kip1}$ in VZ and IZ cells and its nuclear localization in CP cells.

and at elevated levels in neurons migrating through the IZ and into the CP (Fig. 1E). $p27^{Kip1}$ expression switches from predominantly cytoplasmic in VZ progenitors and IZ neurons to a predominantly nuclear localization in CP neurons (Fig. 1G–I). $p21^{Cip1}$ and $p57^{Kip2}$ proteins were detected at low levels in a subset of CP cells (Fig. 1D,F). $p27^{Kip1}$ is therefore the predominant Cip/Kip protein in the E14.5 cerebral cortex.

p27^{Kip1} mutant mice exhibit defects in cortical neuron migration and differentiation that are unrelated to cell cycle exit deficits

Given the broad cortical expression of $p27^{Kip1}$, including in post-mitotic neurons, we asked whether $p27^{Kip1}$ may have additional roles in cortical development beyond its well-established function in promoting cell cycle exit (Caviness et al. 2003). We first examined the radial migration of newly born neurons in $p27^{Kip1}$ -null mutant embryos ($p27^{-/-}$) by conducting birth-dating experiments with bromodeoxyuridine (BrdU). Pregnant females were injected with a single dose of BrdU at E14.5, and embryos were harvested at E17.5. Brightly labeled BrdU-positive cells (neurons born at E14.5) were aberrantly distributed in the cortex of E17.5 $p27^{-/-}$ embryos (Fig. 2A). A significantly greater number of BrdU-labeled cells accumulated

in the VZ/SVZ and the IZ, and fewer cells reached the CP than in cortices from wild-type littermates (Fig. 2A). To determine if this defect in cortical neuron migration was related to the cell cycle regulation function of $p27^{Kip1}$, we examined cortical neuron migration in embryos homozygous for a mutant version of $p27^{Kip1}$ that no longer binds to cyclins and CDKs and does not promote cell cycle exit ($p27^{CK-}$ allele; Besson et al. 2006). Interestingly, no defect in the distribution of BrdU-labeled cells was observed in E17.5 $p27^{CK-}$ cortices injected with BrdU at E14.5 (Fig. 2A). Thus $p27^{Kip1}$ is required for the normal migration of cortical neurons, and this function is independent of its role in promoting cell cycle exit.

Cortical neurons begin to express the neuronal differentiation marker HuC/D soon after exiting the cell cycle and before they migrate out of the VZ/SVZ (Fig. 2B). To determine whether $p27^{Kip1}$ also regulates the differentiation of cortical neurons, we examined the expression of HuC/D in the same embryos as above. The cortex of $p27^{-/-}$ embryos showed a reduced number of E14.5-born neurons (BrdU-labeled) that expressed HuC/D at E17.5 compared with $p27^{CK-}$ embryos and wild-type littermates (Fig. 2B). Most of the reduction in neuronal differentiation was observed in the VZ/SVZ ($44.8\% \pm 5.1\%$ HuC/D+ cells among BrdU+ cells in the VZ/SVZ of $p27^{-/-}$ embryos, compared with $81.3\% \pm 3.5\%$ in wild-

Figure 2. $p27^{Kip1}$ -null mutant cortices present defects in neuronal differentiation and radial migration. Pregnant females carrying $p27^{Kip1}$ mutant embryos were injected with a single dose of BrdU at E14.5 of gestation to mark neurons born at that date, and embryos were harvested at E17.5 and analyzed with antibodies to BrdU and HuC/D. (A) Loss of $p27^{Kip1}$ reduces radial migration of BrdU+ cortical neurons, as shown by the accumulation of labeled cells in the VZ/SVZ and IZ and the loss of labeled cells in the CP of $p27^{-/-}$ mutant embryos compared to wild-type littermates. Embryos homozygous for the cell cycle mutant allele $p27^{CK-}$ do not present defects in distribution of BrdU+ neurons. The histogram shows the distribution of BrdU+ cells in the different compartments of the cortex (VZ/SVZ, IZ, and CP) as a way to quantify radial migration in the different genotypes. Asterisks indicate significant differences in percentages of BrdU+ cells in a given cortical zone in mutant and wild-type cortices ($n = 2-3$, * $p < 0.05$, ** $p < 0.01$). Panels on the right illustrate the distribution of BrdU+ cortical cells in the different genotypes. (B) Loss of $p27^{Kip1}$ also reduces the differentiation of BrdU+ cortical neurons, as marked by expression of HuC/D, while $p27^{CK-}$ embryos do not present this defect. The histogram shows the percentage of BrdU+ cells expressing HuC/D in the different genotypes ($n = 2-3$ brains; ** $p < 0.01$). Panels illustrate double immunostaining for BrdU (green) and HuC/D (red). Arrows in insets indicate double-labeled cells.

type littermates and $82\% \pm 3.8\%$ in $p27^{CK-}$ cortices; data not shown). Our results thus indicate that $p27^{Kip1}$ is required for the normal differentiation and migration of cortical cells and that these activities are cell cycle-independent.

p27^{Kip1} is the only Cip/Kip protein that promotes cortical neuron differentiation and migration

We next asked whether all members of the Cip/Kip family shared the same cell cycle-independent activities in the cortex. For this, we performed overexpression experiments by in utero electroporation of cortices at E14.5 and analyzed electroporated cells and their progeny at E17.5 (Fig. 3A). To determine whether any activity of Cip/Kip proteins is dependent on their cell cycle regulatory function, we used both wild-type and cell cycle mutant versions of these proteins (Supplementary Fig. 1; Welcker et al. 1998; Ohnuma et al. 1999).

Strikingly, overexpression of $p27^{Kip1}$ together with GFP enhanced the radial migration of electroporated cells away from the VZ/SVZ, resulting in an increased number of GFP⁺ cells accumulating in the CP compared to control electroporated cells (Fig. 3B–D). $p27^{Kip1}$ was the only CKI promoting cell migration in this assay (Fig. 3B). Conversely, radial migration was impaired when $p27^{Kip1}$ expression was acutely down-regulated by electroporation of two distinct siRNAs, similar to the phenotype observed in the $p27^{-/-}$ cortex, while a control siRNA had no activity (Supplementary Fig. 2A,B). The radial glia scaffold was unaffected by $p27^{Kip1}$ knockdown, indicating a cell autonomous role for $p27^{Kip1}$ in radial migration (Supplementary Fig. 2C). Regulation of cell migration by $p27^{Kip1}$ was independent of its cell cycle activity since (1) the cell cycle mutant version of $p27^{Kip1}$ ($p27^{ck-}$) promoted radial migration as efficiently as wild-type $p27^{Kip1}$ (Fig. 3B,E) and (2) $p21^{Cip1}$ and $p57^{Kip2}$ had no activity on radial cell migration (Fig. 3B), although they promote cell cycle exit as efficiently as $p27^{Kip1}$ (Supplementary Fig. 1B).

In addition to its effect on cell migration, overexpression of $p27^{Kip1}$ also produced an increased number of GFP⁺ cells expressing HuC/D or β III-tubulin compared to GFP control (Fig. 3F–H). This effect of $p27^{Kip1}$ on differentiation was mostly observed in the VZ/SVZ, where undifferentiated progenitors cells reside ($47.8\% \pm 1.3\%$ HuC/D⁺ cells and $34.9\% \pm 5.7\%$ β III-tubulin⁺ cells among $p27^{Kip1}$ electroporated cells in the VZ/SVZ, compared with $27.3\% \pm 3.3\%$ and $18.3\% \pm 1.6\%$ in control experiments, respectively; data not shown). Conversely, knockdown of $p27^{Kip1}$ expression resulted in a reduction of HuC/D and β III-tubulin expression, compared to GFP control (Supplementary Fig. 2D,E). Notably, only $p27^{Kip1}$ among the Cip/Kip genes promoted neuronal differentiation, and this activity was independent of its role in cell cycle regulation, since the cell cycle mutant form $p27^{ck-}$ was as efficient as wild-type $p27^{Kip1}$ at inducing expression of neuronal markers (Fig. 3F–H).

To demonstrate that the increased number of differentiated neurons observed upon $p27^{Kip1}$ overexpression re-

sulted from premature differentiation of VZ/SVZ progenitors rather than a change in cell fate (e.g., from glial to neuronal), we measured both neuronal (β III-tubulin⁺) and progenitor (nestin⁺) populations among electroporated (GFP⁺) cells by cultivating E14.5 electroporated cortices as slices for 2 d, followed by acute dissociation and analysis (see Materials and Methods). $p27^{wt}$ overexpression resulted in an increase in β III-tubulin⁺ neurons and a parallel decrease in nestin⁺ progenitors, demonstrating that it accelerates neuronal differentiation (Supplementary Fig. 3). A similar effect was obtained with the cell cycle mutant $p27^{ck-}$. Conversely, $p27^{Kip1}$ knockdown led to a significant reduction of β III-tubulin⁺ neurons and to an increase in nestin⁺ cells (Supplementary Fig. 3). To rule out that differences in radial migration and differentiation involved induction of cell death, we examined apoptotic cells with an antibody to activated caspase-3. The percentage of apoptotic cells among electroporated cells was very low (<1%) and similar with all constructs tested (data not shown).

Taken together, these observations demonstrate that $p27^{Kip1}$ promotes both the differentiation of VZ/SVZ progenitors into neurons and the radial migration of neurons into the CP. These functions are unique to $p27^{Kip1}$ among the Cip1/Kip1 genes and are independent of its cell cycle regulatory function.

p27^{Kip1} is required at different times for radial migration and differentiation

$p27^{Kip1}$ is expressed throughout the developing cerebral cortex, but its effects on neuronal differentiation and migration mostly take place in VZ/SVZ cells, as shown by changes in the fraction of cells migrating out of the VZ/SVZ and in the fraction of VZ/SVZ cells expressing neuronal markers when $p27^{Kip1}$ is up- or down-regulated (Figs. 2, 3). To determine whether $p27^{Kip1}$ acts in VZ/SVZ cells that are still cycling or have exited the cell cycle, we manipulated $p27^{Kip1}$ expression by ex vivo electroporation followed by organotypic slice culture (Hand et al. 2005) and monitored the proliferation status of electroporated cells by continuous BrdU exposure during the culture period (Supplementary Fig. 4A). Cell migration reached similar levels when E14.5 cortices were electroporated ex utero and cultivated in slices for 4 d, or electroporated in utero and harvested after 3 d, both in control conditions and following $p27^{wt}$ or $p27^{ck-}$ overexpression and $p27^{Kip1}$ knockdown (cf. Supplementary Fig. 4B and Fig. 3B). Neuronal differentiation was slightly reduced in the slice culture assay compared with an in utero electroporation experiment in control conditions, but overexpression of $p27^{wt}$ or $p27^{ck-}$ and $p27^{Kip1}$ knockdown affected neuronal differentiation to the same extent in the two assays (Fig. 3F,G; Supplementary Fig. 4C). The slice culture assay is therefore a valid approach to study $p27^{Kip1}$ function in neuronal differentiation and radial migration in the cortex.

When electroporated cortices were cultivated for 4 d in the continuous presence of BrdU, a fraction of GFP⁺ cells remained BrdU-negative, indicating that these cells had

Figure 3. *p27^{Kip1}* overexpression promotes differentiation and radial migration of cortical neurons. (A) Coronal section through an E17.5 mouse brain electroporated in utero with a GFP construct at E14.5. The inset shows GFP fluorescence in the dorso-lateral cortex in the same brain. Three days after electroporation, the progeny of GFP⁺ electroporated cells (green) were distributed in all zones of the developing cortex (marked by TOTO-3 in red) indicating that electroporated cells have undergone radial migration. (B) Distribution of GFP⁺ cells in the different zones of the cortex at E17.5, following in utero electroporation at E14.5 of various bicistronic constructs expressing a Cip/Kip protein and GFP. *p27^{Kip1}* is the only Cip/Kip gene that promotes radial cell migration when overexpressed, and this effect is independent of cell cycle regulation as the cell cycle mutant form *p27^{ck-}* is as efficient as the wild-type version *p27^{wt}*. Wild-type *p21^{Cip1}* and *p57^{Kip2}* (*p21^{wt}* and *p57^{wt}*) and mutant versions that lack cell cycle regulation activity (*p21^{ck-}* and *p57^{ck-}*) lack radial migration activity ($n = 3-5$ brains; $*p < 0.05$; $**p < 0.01$). (C-E) Immunostaining for GFP illustrating the position of cells electroporated with GFP, *p27^{wt}*, or *p27^{ck-}*. (F-H) Differentiation of cortical cells electroporated in utero at E14.5 and analyzed at E17.5 for expression of the neuronal differentiation markers HuC/D (F,H) and βIII-tubulin (G). Overexpression of *p27^{Kip1}* promotes expression of neuronal markers independently of its role in cell cycle regulation, as its action is mimicked by *p27^{ck-}*, while overexpression of wild-type or cell cycle mutant forms of *p21^{Cip1}* or *p57^{Kip2}* does not significantly promote neuronal differentiation. The histograms show the percentage of GFP⁺ Hu⁺ (F) and GFP⁺ βIII-tubulin⁺ (G) double-labeled cells over the total population of GFP⁺ electroporated cells ($n = 3-5$ brains; $**p < 0.01$). (H) Double immunostaining for GFP (green) and HuC/D (red) illustrating the induction of neuronal differentiation by *p27^{wt}* and *p27^{ck-}*. Arrows in insets indicate double-labeled cells. Bars: C-E, 50 μm.

passed the last S phase but still had a ventricular process at the time of electroporation, while the remaining GFP⁺ cells were BrdU⁺ and had therefore passed through S phase at least once during the culture period (Supplementary Fig. 4A). When *p27^{wt}* or *p27^{ck-}*-electroporated cortices were cultivated in the presence of BrdU, there was a highly significant increase of migration among the

BrdU⁻ cell population. *p27^{Kip1}* knockdown reciprocally induced a decrease in migration among BrdU⁻ cells, suggesting that *p27^{Kip1}* exerts its migration activity on VZ cells that have become post-mitotic (Supplementary Fig. 4D,F). In contrast, *p27^{Kip1}* knockdown significantly reduced HuC/D expression only in the cycling (BrdU⁺) cohort of electroporated cells and not in the population of

electroporated cells that were exiting the cell cycle (BrdU⁺) (Supplementary Fig. 4E,F). This suggests that *p27^{Kip1}* is required before or during the last S phase of VZ precursors for their differentiation while it is required after the last S phase for their migration. Thus, *p27^{Kip1}* may regulate cell differentiation and cell migration by different mechanisms, operating at different times in the cell cycle.

p27^{Kip1} regulates Ngn2 expression by stabilizing Ngn2 protein

To further address the possibility that *p27^{Kip1}* independently regulates cell migration and neuronal differentiation in the cortex, we examined the molecular mechanisms underlying these cell cycle-independent functions. The proneural basic helix–loop–helix (bHLH) gene *Ngn2* plays a central role in cortical neurogenesis, specifying cortical progenitors to the neuronal fate, inducing a glutamatergic pyramidal neuron phenotype and promoting the radial migration of cortical neurons (Nieto et al. 2001; Schuurmans et al. 2004; Hand et al. 2005). We thus asked whether *p27^{Kip1}* might exert its cell cycle-independent activities in the cortex by regulating *Ngn2*.

Like *p27^{Kip1}*, *Ngn2* transcripts and protein are present in a subset of VZ/SVZ cells, and double labeling showed that *p27^{Kip1}* and *Ngn2* proteins are extensively coexpressed in these cells (Fig. 4A). In contrast with *p27^{Kip1}*,

however, *Ngn2* is sharply down-regulated as cells leave the germinal zones and migrate through the IZ (Fig. 4A, insets). To address the possibility that the two factors interact when coexpressed in VZ/SVZ cells, we examined the effect of manipulating *p27^{Kip1}* expression on *Ngn2* mRNA and protein distribution. Overexpression of *p27^{wt}* or *p27^{ck-}* resulted in a marked increase in number of electroporated cells expressing *Ngn2* protein compared with control. Conversely, knockdown of *p27^{Kip1}* expression led to a decrease in the number of *Ngn2*⁺ cells (Fig. 4B,C). Strikingly, the cortical VZ/SVZ of *p27^{Kip1}*-null mutant embryos also showed a reduction in *Ngn2*⁺ cells, while *p27^{CK-}* knock-in embryos have a normal level of *Ngn2* expression compared to wild-type embryos (Fig. 4D). Up-regulation of *Ngn2* expression was also observed when the N-terminal part of *p27^{ck-}* (*p27^{ck-}*-N-term; see Materials and Methods) was overexpressed, while the C-terminal portion had no activity (*p27^{ck-}*-C-term; Fig. 4C). No change in distribution of *Ngn2* transcripts was observed following electroporation of *p27^{wt}*, *p27^{ck-}*, or *p27* siRNA, or in *p27^{Kip1}*-null mutant embryos (not shown), suggesting that *p27^{Kip1}* regulates *Ngn2* at a post-transcriptional level.

The sharp down-regulation of *Ngn2* expression as newborn neurons leave the VZ/SVZ and migrate into the IZ (Fig. 4A; see also Kawaguchi et al. 2004; Hand et al. 2005; Britz et al. 2006) suggested that *Ngn2* protein is rapidly turned over in these cells. One possible mecha-

Figure 4. *p27^{Kip1}* stabilizes *Ngn2* protein in cortical progenitors. (A) Double immunostaining for *p27^{Kip1}* (green) and *Ngn2* (red) in a E15.5 mouse cortex showing coexpression of the two proteins in a subset of SVZ/VZ progenitors. *Insets* show enlargements of regions indicated in boxes. (B) Double immunostainings for electroporated cells overexpressing or down-regulating *p27^{Kip1}* as indicated (GFP in green) and for endogenous *Ngn2* (red). (C) Percentage of VZ cells expressing *Ngn2* protein after electroporation of various forms of *p27^{Kip1}* and siRNA in E15.5 VZ progenitors and 24-h slice culture. Electroporation of *p27* siRNA#1 down-regulates *Ngn2* protein and overexpression of *p27^{wt}*, *p27^{ck-}*, or the N-terminal half of *p27^{ck-}* (*p27^{ck-}*-N-term) up-regulate *Ngn2* protein, while the C-terminal part (*p27^{ck-}*-C-term) has no activity ($n = 3$ –5 slices; $*p < 0.05$, $**p < 0.01$). (D) Percentage of VZ cells expressing *Ngn2* protein in E15.5 cortical progenitors from wild-type, *p27^{-/-}*-null mutant *p27^{ck-}* embryos ($n = 3$ –6 brains; $*p < 0.05$). Panels showing immunostainings for *Ngn2* (green) and TOTO-3 nuclear staining (red) illustrate the reduction in *Ngn2* expression in absence of *p27^{Kip1}* (*p27^{-/-}*) and its restoration to wild-type levels by the cell cycle mutant allele *p27^{CK-}*. (E) *Ngn2* protein degradation analyzed by pulse-chase in rabbit reticulocytes (see Materials and Methods for details). Coexpression of *Ngn2* with *p27^{wt}* and *p27^{ck-}* markedly increased *Ngn2* protein stability. *In vitro* translated *p27^{wt}* and *p27^{ck-}* proteins were stable during the course of the experiment. Three experiments were performed and one representative example is shown.

nism of post-transcriptional regulation could thus be at the level of Ngn2 protein stability. Several bHLH proteins with short half-lives are ubiquitinated and degraded by the 26S proteasome in an ATP-dependent fashion (Abu Hatoum et al. 1998; Sriuranpong et al. 2002). We measured the half-life of Ngn2 using an in vitro degradation assay in rabbit reticulocyte lysate, which is rich in multiple proteases including the 26S proteasome (Hoffman et al. 1992). In this assay, Ngn2 was rapidly degraded, with a half-life of ~30 min, in an ATP-dependent manner, as degradation was blocked in the presence of the nonhydrolysable analog, ATP- γ S (not shown). When in vitro translated p27^{wt} or p27^{ck-} was added to the assay, the half-life of Ngn2 was extended to ~70 min or ~100 min, respectively (Fig. 4E), while p21^{wt} had no effect (not shown). Thus, regulation by p27^{Kip1} of Ngn2 expression in cortical VZ/SVZ likely involves stabilizing Ngn2 protein. This activity lies in the N-terminal part of p27^{Kip1} and is independent of interaction with cyclin/CDK.

p27^{Kip1} promotes cortical neuron differentiation via regulation of Ngn2

The previous results raised the possibility that p27^{Kip1} regulates the differentiation and/or migration of cortical

neurons by regulating Ngn2 expression. To address this possibility, we tested the capacity of Ngn2 to rescue the differentiation and migration defects caused by p27^{Kip1} knockdown. First, we established that Ngn2 regulates neuronal differentiation and radial migration in the cortical slice culture assay. In cortices isolated from Ngn2-null mutant embryos (Ngn2^{null}), the fraction of cells expressing HuC/D 4 d after electroporation at E14.5 with a GFP vector was smaller than in wild-type cortices. Acute deletion of Ngn2 by electroporation of a Cre recombinase vector in the cortex of an embryo homozygous for a floxed allele of Ngn2 (Ngn2^{flxed}) also resulted in a reduction in HuC/D expression. Conversely, overexpression of Ngn2 promoted HuC/D expression in electroporated cells (Fig. 5A). Ngn2 also regulated radial migration in this assay (see also Hand et al. 2005; Ge et al. 2006). A smaller fraction of cortical cells reached the CP after 4 d in Ngn2^{null} cortices or in Ngn2^{flxed} cortices electroporated with Cre than in wild-type cortices, while Ngn2 overexpression resulted in a small but significant increase in the number of cells reaching the CP (Fig. 5B). Thus, Ngn2 is involved in both neuronal differentiation and radial migration of cortical neurons in the slice culture assay.

We next asked whether Ngn2 was mediating the cell

Figure 5. Ngn2 overexpression rescues the neuronal differentiation defect but not the radial migration defect induced by p27^{Kip1} knockdown. (A) Loss of Ngn2 function results in neuronal differentiation defects, as shown by reduced HuC/D expression in cortices of Ngn2 homozygous null mutant embryos (Ngn2^{null}) electroporated with GFP at E14.5 and cultivated as slices for 4 d, and in cortices of embryos homozygous for a floxed allele of Ngn2 (Ngn2^{flxed}) coelectroporated with Cre and GFP. Ngn2 overexpression conversely promotes neuronal differentiation, as shown by increased HuC/D expression in Ngn2 electroporated cells. The defect in HuC/D expression in cells electroporated with p27 siRNA#1 is fully rescued by coelectroporation of Ngn2 ($n = 5-13$ slices; $*p < 0.05$, $**p < 0.01$). Panels showing double immunostainings for GFP (green) and HuC/D (red) illustrate the rescue by Ngn2 of the neuronal differentiation defect induced by p27^{Kip1} knockdown. High magnification pictures show electroporated cortical neurons that have differentiated and remained in the VZ/SVZ compartment. (B) Loss of Ngn2 results in radial migration defects, as shown by electroporation of GFP in E14.5 Ngn2^{null} cortices or of Cre and GFP in E14.5 Ngn2^{flxed} cortices. Ngn2 overexpression results in a small but significant increase in the percentage of electroporated cells reaching the CP in wild-type cortex. However, Ngn2 overexpression does not rescue the migration defect resulting from knockdown of p27^{Kip1} when coelectroporated with p27 siRNA#1 ($n = 3-8$ slices; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Panels illustrate the migration phenotypes resulting from Ngn2 overexpression (Ngn2) and p27^{Kip1} knockdown (p27 siRNA#1) and the lack of rescue when Ngn2 is coelectroporated with p27 siRNA#1. Electroporated cells are labeled for GFP.

cycle-independent functions of $p27^{Kip1}$ in cortical neurons. For this, we examined the capacity of *Ngn2* to rescue $p27^{Kip1}$ knockdown phenotypes. Coexpression of *Ngn2* with a $p27$ siRNA resulted in a large number of cells expressing HuC/D, similar to that observed with *Ngn2* alone and much higher than with $p27$ siRNA alone or a control *GFP* vector (Fig. 5A). This demonstrates that overexpression of *Ngn2* can rescue the neuronal differentiation defect produced by $p27^{Kip1}$ knockdown and suggests that regulation of *Ngn2* is the main mechanism by which $p27^{Kip1}$ promotes neuronal differentiation.

In striking contrast with the differentiation phenotype, the radial migration defect caused by $p27^{Kip1}$ knockdown was not rescued by overexpression of *Ngn2*. Only a few cells expressing $p27$ siRNA reached the CP, and this was not significantly improved when *Ngn2* was coexpressed with $p27$ siRNA, although *Ngn2* alone promoted migration to the CP (Fig. 5B). Therefore, $p27^{Kip1}$ promotes cortical neuron migration by a mechanism that does not involve *Ngn2* and is therefore distinct from the mechanism underlying its differentiation activity.

$p27^{Kip1}$ promotes cortical neuron migration by inhibiting *RhoA*/ROCK activity

Cortical neuron migration has been shown to require inactivation of the GTPase *RhoA* (Kholmanskikh et al. 2003; Hand et al. 2005), which is expressed in the VZ/SVZ (Fig. 6A). Coexpression of a dominant-negative version of *RhoA* (Wennerberg et al. 2003) with $p27$ siRNA completely rescued neuronal migration to the CP (Fig. 6B). Cultivating cortical slices electroporated with $p27$ siRNA#1 in the presence of 10 μ M of CY27632, an inhibitor of the *RhoA* targets ROCK1 and ROCK2, also rescued migration to the CP (Fig. 6B). In contrast, coexpression of *Rac1* with $p27$ siRNA did not rescue the migration of neurons to the CP (Fig. 6B), demonstrating the specificity of dominant-negative *RhoA* in this assay. Together, these results demonstrate that inactivation of the *RhoA*/ROCK1/2 signaling pathway is an important mechanism by which $p27^{Kip1}$ regulates cortical neuron migration.

The N- and C-terminal halves of $p27^{Kip1}$ have different cell cycle-independent activities

The rescue experiments reported above suggested that $p27^{Kip1}$ independently regulates the differentiation and radial migration of cortical neurons. To directly address this possibility, we asked whether these two functions reside in different domains of the $p27^{Kip1}$ protein and could be physically dissociated, by separately testing the activities of the N-terminal and C-terminal halves of $p27^{Kip1}$. Expression of $p27^{Kip1}$ N-term (N-terminal half of $p27^{Kip1}$ containing the cell cycle mutation) (Supplementary Fig. 1) induced expression of *Ngn2* (Fig. 4C) and promoted HuC/D expression as efficiently as full-length $p27^{Kip1}$ (Fig. 7A; Supplementary Fig. 4C) but did not promote migration to the CP (Fig. 7B). In contrast, $p27^{Kip1}$ C-term (C-terminal half of $p27^{Kip1}$) (Supplementary Fig. 1) significantly promoted migration to the CP, less efficiently than full-length $p27^{Kip1}$ but to a level similar to that of dominant-negative *RhoA* alone (Fig. 7B). $p27^{Kip1}$ C-term also induced HuC/D expression, but significantly less efficiently than $p27^{Kip1}$ N-term (Fig. 7A), and had no effect on *Ngn2* expression (Fig. 4C). Together, these results demonstrate that $p27^{Kip1}$ independently promotes neuronal differentiation through a *Ngn2* stabilizing activity residing in its N-terminal half (Figs. 4C, 7A) and radial migration, mostly through its C-terminal half (Fig. 7B), likely by direct inactivation of *RhoA* (Besson et al. 2004).

Discussion

We have addressed in this article the mechanisms that underlie the coordination of multiple cellular events during neurogenesis. We show that $p27^{Kip1}$ regulates neuronal differentiation and neuronal migration in the cerebral cortex independently of its cell cycle regulatory function. Moreover, we show that these activities involve different parts of the protein and distinct downstream pathways. We discuss below these different activities and the role of $p27^{Kip1}$ as a modular protein that coordinately regulates cell cycle exit, differentiation, and migration during neurogenesis.

Figure 6. Blocking *RhoA*-ROCK signaling pathway rescues the radial migration defect induced by $p27^{Kip1}$ knockdown. (A) In situ hybridization of a coronal section through E14.5 telencephalon with a *RhoA* probe showing high levels of *RhoA* transcripts in VZ and SVZ cells. (B) Blocking *RhoA* activity by electroporating a dominant-negative form of *RhoA* (*DNRhoA*) or blocking ROCK1/2 activity by exposing cortical slices to the specific inhibitor CY27632 fully rescues the radial migration defect resulting from electroporation of $p27$ siRNA#1. Overexpression of *Rac1* does not rescue this phenotype ($n = 5$ –13 slices; $*p < 0.05$, $**p < 0.01$). Panels on the right illustrate the rescue of the radial migration defect induced by $p27$ siRNA#1 when *RhoA* signaling is inhibited.

Figure 7. The N- and C-terminal halves of p27^{Kip1} harbor different activities. (A) The N-terminal half of p27^{ck-} (p27^{ck-N-term}) efficiently promotes expression of the neuronal marker HuC/D, while the C-terminal half (p27^{C-term}) only has a moderate differentiation activity. (B) p27^{C-term} promotes cell migration to the CP as efficiently as DNRhoA, while p27^{ck-N-term} has no migration activity. (C) Summary scheme illustrating the modular nature of the p27^{Kip1} molecule and its role in coordinating multiple cellular pathways during neurogenesis. p27^{Kip1} N-term harbors the cell cycle regulation function and the neuronal differentiation promoting activity, involving stabilization of Ngn2, while p27^{Kip1} C-term harbors the radial migration promoting activity, involving inactivation of RhoA. p27^{Kip1} and Ngn2 form a regulatory loop that coordinates cell cycle exit with differentiation and radial migration of cortical neurons. Black arrows represent nontranscriptional interactions, and white arrows represent transcriptional interactions.

Regulation of neuronal differentiation

We show here that p27^{Kip1} promotes the neuronal differentiation of cortical progenitors and that this activity is mediated in large part by stabilization of Neurogenin2 protein. Overexpression of wild-type or a cell cycle mutant version of p27^{Kip1} induced prematurely the neuronal differentiation markers β III-tubulin and HuC/D in VZ/SVZ cells. Reciprocally, our analysis of p27^{Kip1}-null mutant cortices revealed a defect in expression of these markers by VZ/SVZ cells, and a similar phenotype was observed following p27^{Kip1} down-regulation by siRNA electroporation. Coexpression of Ngn2 with a p27 siRNA more than compensated this defect and neuronal markers were up-regulated to the same level as with Ngn2 alone, demonstrating that Ngn2 acts genetically downstream of p27^{Kip1} and that p27^{Kip1} regulates neuronal differentiation primarily through regulation of Ngn2 expression. Indeed, overexpression and knockdown experiments showed that p27^{Kip1} regulates Ngn2 expression in cortical progenitors and acts primarily by stabilizing Ngn2 protein. Xenopus p27^{Xic1} has been shown to stabi-

lize the Neurogenin protein X-NGNR1 (Vernon et al. 2003), indicating that p27^{Kip1} has an evolutionary conserved role in coupling neuronal differentiation with cell cycle exit by independently regulating cyclin/CDK activity and Neurogenin protein stability. We can envisage different mechanisms by which p27^{Kip1} stabilizes Neurogenin2. p27^{Kip1} has been shown to interact with F-box proteins, the substrate-specific components of E3 ubiquitin ligases (Laman et al. 2005). It may thus directly interfere with the activity of an E3 ubiquitin ligase that targets Ngn2 for degradation. Alternatively, p27^{Kip1} might bind Ngn2 and protect it from degradation, as demonstrated for the stabilization of MyoD by p57^{Kip2} (Reynaud et al. 2000). Besides stabilizing Ngn2, p27^{Kip1} may also interact with other factors involved in neuronal differentiation. Indeed, regulation of white blood cell differentiation by p21^{Cip1} involves interaction with multiple regulatory proteins, including the transcription factors Stat3 and C/EBP α and the cofactor p300 (Steinman 2002).

Is stability of Ngn2 a limiting factor for the differentiation of VZ/SVZ cells? Genetic experiments have established that Ngn2 activates a neuronal differentiation program when expressed in neural progenitors (Bertrand et al. 2002). Detailed studies of Ngn2 protein distribution in the cortex have shown that Ngn2 protein expression is induced in VZ progenitors after their last mitosis, that it is maintained in SVZ cells and then down-regulated in newborn neurons as they migrate through the IZ (Kawaguchi et al. 2004; Hand et al. 2005; Britz et al. 2006). Stabilization of Ngn2 by p27^{Kip1} could result in accelerated accumulation of Ngn2 protein in VZ cells and/or in delayed downregulation of Ngn2 in the SVZ or IZ. Whether p27^{Kip1} activity depends mostly on accelerating the accumulation or delaying the degradation of Ngn2 depends on when exactly Ngn2 activates the downstream differentiation program, which is not known. Ngn2 is thought to promote neuronal differentiation by activating a cascade of transcriptional regulators, including bHLH genes such as *NeuroD1*, *Math3/NeuroM*, and *Math2/Nex*, and T-box genes such as *Tbr2* and *Tbr1* (Bertrand et al. 2002; Schuurmans et al. 2004; Englund et al. 2005). However, which among these genes are direct targets of Ngn2 and what is their precise timing of expression in cortical neurons remains to be determined.

Regulation of radial migration

p27^{Kip1} has been shown to regulate the migration of different cell types in vitro, but its role in cell migration in vivo, let alone in the cortex, has not yet been established. We have demonstrated that p27^{Kip1} is an important player in the control of cortical neuron migration. We show for the first time that p27^{Kip1}-deficient embryos present defects in the radial migration of cortical neurons. This phenotype is not due to defects in cell cycle exit since the cortex of a mouse homozygous for the knock-in cell cycle mutant allele p27^{CK-} (Besson et al. 2006) is not affected. No defects related to abnormal cortical neuronal migration have previously been reported in adult p27^{Kip1}-deficient mice. Whether the reduced mi-

gration that we observed at E17.5 is a transient defect that is eventually compensated remains to be addressed. Supporting an important role of *p27^{Kip1}* in migration, overexpression of the wild-type or the cell cycle mutant versions of *p27^{Kip1}* efficiently promoted the migration of cortical neurons to the cortical plate, while *p27^{Kip1}* down-regulation largely inhibited this migration. Since *Ngn2* has also been shown recently to regulate cortical neuron migration (Hand et al. 2005), we anticipated that *Ngn2* would mediate both the differentiation and migration activities of *p27^{Kip1}*. However, coexpression of *Ngn2* and a *p27* siRNA did not ameliorate the migration defect caused by *p27^{Kip1}* knockdown, indicating that the migration activity of *p27^{Kip1}* involves a different pathway. In support of this idea, *p27^{Kip1}* migration activity resides entirely in its C-terminal portion, whereas its differentiation activity resides mostly in its N-terminal part.

Regulation of fibroblast migration by *p27^{Kip1}* involves inactivation of the small GTPase RhoA (Besson et al. 2004). We thus examined whether the same mechanism was operating in the cortex and found that indeed expression of a dominant-negative form of *RhoA* (*DNRhoA*) or exposure to a pharmacological inhibitor of the RhoA effectors ROCK1/2 efficiently rescued the migration defect resulting from *p27^{Kip1}* knockdown. Regulation of cell migration by inhibition of RhoA activity is therefore likely to be a general function of *p27^{Kip1}* in various tissues. A recent study has identified the actin-binding protein cofilin as a phosphorylation substrate of RhoA and proposed that derepression of cofilin underlies *p27^{Kip1}* activity in cortical neuron migration (Kawauchi et al. 2006). The lack of rescue of the *p27^{Kip1}* migration phenotype by *Ngn2* may come as a surprise since RhoA down-regulation has also been proposed as a mechanism by which *Ngn2* promotes cortical neuron migration, as shown by repression of *RhoA* transcription by *Ngn2* (Ge et al. 2006) and by the rescue of migration defects in *Ngn2* mutant cortex by *DNRhoA* (Hand et al. 2005). However, the repression of *RhoA* by *Ngn2* was not very robust, and the rescue of the *Ngn2* mutant migration defect by *DNRhoA* was only partial. Therefore, down-regulation of *RhoA* is unlikely to be the main mechanism whereby *Ngn2* promotes cortical neuron migration, and *Ngn2* has indeed been shown to regulate other migration genes such as *Dcx* and *p35* (Ge et al. 2006). This likely explains why *Ngn2* overexpression does not repress *RhoA* to a sufficient extent to rescue the *p27^{Kip1}* migration phenotype. The complete rescue of this defect by *DNRhoA* indicates that inactivation of RhoA/ROCK1/2 signaling is the major mechanism by which *p27^{Kip1}* promotes cortical neuron migration. However, since *p27^{Kip1}* also regulates *Ngn2*, which, in turn, regulates multiple migration genes (Ge et al. 2006; our unpublished data), *p27^{Kip1}* is likely to activate cortical neuron migration via multiple pathways (Fig. 7C).

p27^{Kip1} as a modular protein coupling multiple pathways

The tight coupling of multiple cellular processes is a striking feature of the neurogenesis program. Although

progress has been made in characterizing the molecular pathways that regulate individual cellular events during neurogenesis, including cell cycle control (Ohnuma and Harris 2003), neuronal differentiation (Guillemot et al. 2006), and particularly neuronal migration (Bielas et al. 2004), how these various processes are coordinately regulated in a coherent developmental program remains poorly understood. Here, we provide evidence that *p27^{Kip1}* plays an important role in the coordination of multiple aspects of neurogenesis in the cortex. The role of *p27^{Kip1}* in controlling the timing of cell cycle exit of cortical progenitors is well established (Goto et al. 2004; Lukaszewicz et al. 2005; Tarui et al. 2005). We show in this article that *p27^{Kip1}* also promotes the differentiation and migration of newborn cortical neurons, independently of this cell cycle function. Importantly, the multifunctionality of *p27^{Kip1}* relies on the existence of different domains in the molecule that independently regulate distinct pathways. The C-terminal half of *p27^{Kip1}*, which has been shown to directly interact with RhoA and inhibit its activation (Besson et al. 2004), harbors the migration-promoting activity of *p27^{Kip1}* and functions in isolation from the N-terminal half, although not as well as the full-length molecule. The N-terminal half, which also contains the cyclin/CDK-interacting domain of *p27^{Kip1}*, promotes *Ngn2* protein expression and neuronal differentiation as efficiently as wild-type *p27^{Kip1}*. Although the C-terminal half does not influence *Ngn2* expression, it has a moderate differentiation-promoting activity on its own, suggesting the existence of additional pathways regulating neuronal differentiation downstream of *p27^{Kip1}*.

Interestingly, *p27^{Kip1}* protein is present in both cytoplasm and nucleus, in different ratios at different stages of differentiation (Fig. 1), and it likely regulates different processes in different cellular compartments, interacting with cyclin/CDKs and stabilizing *Ngn2* in the nucleus while interacting with RhoA in the cytoplasm. Moreover, the different activities of *p27^{Kip1}* may be segregated not only spatially but also temporally. The regulation of cell cycle exit and the promotion of *Ngn2* stability and differentiation take place in the VZ/SVZ before the last division of progenitors and during the following G1 phase, while its migration promoting activity may take place in the IZ, when neurons are motile. *p27^{Kip1}* expression remains high in neurons that have reached their final position in the cortical plate, suggesting that *p27^{Kip1}* may yet have additional functions at later stages.

Besides *p27^{Kip1}*, *Ngn2* also plays an important role in coordinating neurogenesis in the cortex, by independently regulating neuronal differentiation, specification of multiple aspects of cortical neuron identity, and neuronal migration (Bertrand et al. 2002; Schuurmans et al. 2004; Hand et al. 2005). Experiments in cultured cells have provided evidence that neural bHLH genes including *Neurogenins* induce expression of *p27^{Kip1}* and promote cell cycle exit (Farah et al. 2000), and we have shown that *p27^{Kip1}* regulates *Ngn2* expression in cortical progenitors, suggesting that these two molecules are engaged in a regulatory loop. Thus, a regulatory network

involving *p27^{Kip1}*, *Ngn2*, and perhaps other multifunctional proteins (Ferguson et al. 2005) coordinates the various pathways that contribute to the neurogenic program in the cortex.

Materials and methods

Animals

Mice were housed, bred, and treated according to the guidelines approved by the Home Office under the Animals (Scientific procedures) Act 1986. The generation and genotyping of transgenic mice have been reported previously: *Ngn2^{KILacZ}* (Nieto et al. 2001), *Ngn2^{K1loxNgn2}* (Hand et al. 2005), *p27^{CK-}* (Besson et al. 2006), and *p27⁻* (Fero et al. 1996).

RNA in situ hybridization and immunohistochemistry

Embryonic brains were dissected in 1× phosphate buffered saline (PBS) and fixed for 45 min (for immunohistochemistry) or overnight (for RNA in situ hybridization) in 4% paraformaldehyde (PFA)/1× PBS at 4°C. Cultivated brain slices were fixed in 4% PFA/1× PBS for 30 min. Fixed samples were cryoprotected overnight in 20% sucrose/1× PBS at 4°C and mounted in OCT Compound (VWR) and sectioned coronally (10 μm) with a cryostat (CM3050S, Leica).

Nonradioactive RNA in situ hybridization was performed as described previously (Cau et al. 1997) using antisense RNA probes for *p21^{Cip1}* (gift from Dr. Jay Cross, University of Calgary, Alberta, Canada), *p27^{Kip1}* and *p57^{Kip2}* (gift from Dr. A. Mallamaci, San Raffaele Scientific Institute, Milano, Italy), *RhoA* (gift from Dr. Y.E. Sun, University of California at Los Angeles, CA), and *Ngn2* (Gradwohl et al. 1996). For immunohistochemistry, cryostat sections were washed three times in PBST (PBS, 0.1% Triton X-100), and blocked at room temperature for 1 h in PBST supplemented with 10% goat serum (Vector Laboratories). Primary antibodies were incubated overnight at 4°C; rat anti-BrdU (Oxford Biotechnology; 1:20), rabbit anti-BLBP (gift from Dr. M. Götz, Forschungszentrum für Umwelt und Gesundheit, Neuherberg, Germany; 1:1000), rabbit anti-GFP (Molecular Probes; 1:2000), chicken anti-GFP (Chemicon; 1:500), mouse anti-*nestin* (Rat 401, Developmental Hybridoma Bank; 1:10), mouse anti-*Ngn2* (1:20, gift from Dr. David Anderson, California Institute of Technology, Pasadena, CA), mouse anti-βIII-tubulin (Tuj1, Covance; 1:1000), mouse anti-HuC/D (Molecular Probes; 1:100), rabbit anti-*p21^{Cip1}* (C-19, Santa Cruz Biotechnology; 1:400), mouse anti-*p27^{Kip1}* (BD Biosciences; 1:200), rabbit anti-*p27^{Kip1}* N terminus (N-20, Santa Cruz Biotechnology; 1:100), rabbit anti-*p27^{Kip1}* C terminus (Lab vision; 1:100), rabbit anti-*p57^{Kip2}* (C-20, Santa Cruz Biotechnology; 1:100), and rabbit anti-activated caspase-3 (R&D system; 1:1000). Slides for BrdU detection were pretreated with 2N HCl for 30 min. After washing, slides were incubated for 1 h at room temperature with the appropriate secondary antibodies: goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 568, goat anti-chicken Alexa Fluor 488, goat anti-rabbit Alexa Fluor 488, goat anti-rabbit Alexa Fluor 568 (Molecular Probes; 1:1000), and Cy5 conjugated goat anti-rat (Jackson ImmunoResearch; 1:500). For in vivo BrdU incorporation, pregnant females were injected intraperitoneally at E14.5 with 100 mg BrdU/kg body mass (Boehringer Mannheim #280879) and embryos were harvested at E17.5. For counting, cells were counterstained with TOTO-3 (Molecular Probes; 1:1000) during secondary antibody incubation. Sections were washed three times in PBST and coverslipped using Aqua Polymount (Polysciences, Inc.). Images

were acquired using a laser scanning confocal microscope (Radiance 2100, Bio-Rad).

Plasmids and siRNA

Plasmid DNA were prepared using a Plasmid Maxi Kit (Qiagen). pCDNA-RhoA19N and pCDNA-Rac1 were purchased from University of Missouri-Rolla cDNA Resource Center; pCS2-*p21^{wt}* and pCS2-*p21^{N50S}* were gifts from S. Ohnuma (University of Cambridge, UK); pQCXIP27^{wt}, pCDNAp27^{wt}, pCDNAp27^{ck-}, and pCDNA-p27C-term were constructed by A. Besson (Besson et al. 2004); pCDNA-HA-p57^{wt} and pCDNA-HA-p57^{ck-} were provided by Y. Xiong (University of North Carolina at Chapel Hill, NC) (Watanabe et al. 1998); and p27^{ck-}-N-term cDNA (amino acids 1–86) was generated by PCR using pCDNA3.1 p27^{ck-} (Besson et al. 2004). All full-length cDNA were subcloned into a pCAGGS-IRES-GFP vector generously provided by J. Briscoe (National Institute for Medical Research, London, UK), where a [cDNA]-nlsIRES-EGFP cassette is under the control of an CMV-enhancer and a chicken β-actin promoter. All constructs were verified by sequencing.

The following siRNAs were purchased from Ambion: 5'-GCU UGCCCCGAGUUCUACUAtt-3' (sense strand) and 5'-UAG UAGAACUCGGGCAAGCtg-3' (antisense strand) for p27^{Kip1} siRNA#1 (predesigned siRNA no. 118712 directed against the exon 1 of mouse and human p27^{Kip1}); 5'-GGUAAUUUUCAAG AUUACGtt-3' (sense strand) and 5'-CGUAAUCUUGAAAA AUACCTg-3' (antisense strand) for p27^{Kip1} siRNA#2 (predesigned siRNA no. 161159 directed against the exon 2 of mouse p27^{Kip1}).

The extent of p27^{Kip1} knockdown elicited by both siRNA was compared with that of a control siRNA (Silencer Negative Control#1 siRNA no. 4611) with no significant homology with known gene sequences in rodent and human.

In utero electroporation, ex vivo electroporation, and dissociated cell culture

In utero electroporation was performed as described previously (Saito and Nakatsuji 2001) with minor modifications (see Supplemental Material). Ex vivo electroporation was performed on injected mouse embryos' heads by using electrical settings similar to those applied for in vivo electroporation (see Supplemental Material). Following electroporation, brains were dissected in L15 (Invitrogen) and transferred into liquid 3% low melting agarose (38°C; Sigma) and incubated on ice for 1 h. Embedded brains were cut coronally (250 μm) with a vibratome (VT1000S, Leica), and slices were transferred onto sterilized culture plate inserts (0.4-μm pore size; Millicell-CM, Millipore) and cultivated in semidry condition in wells containing Neurobasal medium supplemented with B27 (1%), N2 (1%), glutamine (1%), penicillin/streptomycin (1%), fungizone (0.1%; Invitrogen), and ciprofloxacin (5 μg/mL; MP Biomedicals). Slices were cultivated for up to 4 d, with half the culture medium renewed every day. For identification of cells cycling at the time of electroporation, slices were incubated with 10 μM BrdU (Sigma) until the end of the culture.

For dissociated cell cultures, slices were cultivated 2 d following electroporation, the VZ/SVZ was microdissected under a GFP binocular to identify the electroporated regions, and tissues were pooled and incubated with 0.05% trypsin (Invitrogen) for 15 min followed by trituration in DMEM FCS 10% with fire-polished Pasteur pipettes. Fifty microliters of dissociated cells in suspension (3×10^6 /mL) were seeded for 30 min on poly-D-lysine-coated coverslips in 24-well plates, then covered with

450 μ L medium and further incubated for 30 min before being fixed with 4% PFA at 4°C for 15 min.

P19 cell culture and Western blot

Freshly dissociated P19 cells (ATCC) were seeded at 2×10^5 cells/well in 6-well plates and cultivated overnight in DMEM supplemented with glutamine (1%), FCS (10%), and penicillin-streptomycin (1%; Invitrogen). Efficiency of p27^{Kip1} knockdown with siRNAs was tested on exogenous p27^{Kip1} by transiently cotransfecting P19 cells with 2 μ g of pQCXIPp27^{wt}, or 2 μ g of a control pSC2 plasmid, and 100 nM of siRNA using Lipofectamine 2000 (Invitrogen). After 30 h, proteins were extracted with NP40 lysis buffer, denatured with SDS lysis buffer, fractionated on a 12% SDS-PAGE gel, and transferred to a nitrocellulose membrane nylon (HybondECL, Amersham Biosciences) for immunoblotting. Primary antibodies were rabbit anti-p27^{Kip1} (Lab vision; 1:1000) and mouse anti-actin (C2, Santa Cruz Biotechnology; 1:1000) and secondary antibodies were goat anti-rabbit IgG (H+L) HRP conjugate and goat anti mouse IgG (H+L) HRP conjugated (Bio-Rad; 1:5000). Signal was revealed using ECL Western blotting detection reagents according to the manufacturer's instructions (Amersham Biosciences).

Protein degradation analysis in rabbit reticulocyte lysates

Ngn2, p27^{wt}, p27^{ck}, and p21^{wt} proteins were individually transcribed and translated using a TNT Coupled Reticulocyte Lysate system and following the manufacturer's recommendations (Promega). Post-translation, 2 μ L of RNase A (10 mg/mL) were added to each reaction and incubated for 30 min at 37°C, 15 μ L of in vitro transcribed and translated protein were then incubated with 2.5 μ L 1 M Tris-Cl (pH 7.5), 2 μ L 5 M NaCl, 3 μ L 0.1 M DTT, and 2 μ L 0.1 M ATP or ATP γ S in 100 μ L total volume at 37°C. Ten microliters of each reaction were then removed each hour between 0 and 5 h, mixed with 5 μ L of 4X-SDS-PAGE sample buffer, and incubated at 95°C for 5 min. Samples were loaded on 10% SDS-PAGE gels, subjected to fluorography using En³Hance (Dupont), followed by autoradiography.

Cell counting and statistics

Different subregions of the cerebral cortex were identified based on cell density and visualized with TOTO-3 iodide nuclear staining (Molecular Probes) and neuronal marker (β III-tubulin) expression, the VZ/SVZ with high cell density and faint β III-tubulin staining, IZ with low cell density and strong β III-tubulin staining, and CP with high cell density and strong β III-tubulin staining. In all experiments, brains or slices from at least three independent experiments were processed for each experimental condition, except for p27^{-/-} brains ($n = 2$). For each sample, two or three adjacent sections were analyzed by confocal microscopy and 40 $\times$ magnified fields zoomed 1.5 $\times$ were acquired. Results are indicated as mean $\pm$ SEM. A statistical analysis was performed using either unpaired two-tailed Student's *t*-test between control and experimental condition, or one-way ANOVA (ANOVA-1) followed by a Dunnett's post hoc test for multiple comparisons (GraphPad Prism software, version 3.03).

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References

- Abu Hatoum, O., Gross-Mesilaty, S., Breitschopf, K., Hoffman, A., Gonen, H., Ciechanover, A., and Bengal, E. 1998. Degradation of myogenic transcription factor MyoD by the ubiquitin pathway in vivo and in vitro: Regulation by specific DNA binding. *Mol. Cell. Biol.* **18**: 5670–5677.
- Bertrand, N., Castro, D.S., and Guillemot, F. 2002. Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**: 517–530.
- Besson, A., Gurian-West, M., Schmidt, A., Hall, A., and Roberts, J.M. 2004. p27Kip1 modulates cell migration through the regulation of RhoA activation. *Genes & Dev.* **18**: 862–876.
- Besson, A., Gurian-West, M., Chen, X., Kelly-Spratt, K.S., Kemp, C.J., and Roberts, J.M. 2006. A pathway in quiescent cells that controls p27Kip1 stability, subcellular localization, and tumor suppression. *Genes & Dev.* **20**: 47–64.
- Bielas, S., Higginbotham, H., Koizumi, H., Tanaka, T., and Gleeson, J.G. 2004. Cortical neuronal migration mutants suggest separate but intersecting pathways. *Annu. Rev. Cell Dev. Biol.* **20**: 593–618.
- Britz, O., Mattar, P., Nguyen, L., Langevin, L.-M., Zimmer, C., Alam, S., Guillemot, F., and Schuurmans, C. 2006. A role for proneural genes in the maturation of cortical progenitor cells. *Cereb. Cortex* (in press).
- Carruthers, S., Mason, J., and Papalopulu, N. 2003. Depletion of the cell-cycle inhibitor p27(Xic1) impairs neuronal differentiation and increases the number of ElrC(+) progenitor cells in *Xenopus tropicalis*. *Mech. Dev.* **120**: 607–616.
- Casini, T. and Pelicci, P.G. 1999. A function of p21 during promyelocytic leukemia cell differentiation independent of CDK inhibition and cell cycle arrest. *Oncogene* **18**: 3235–3243.
- Cau, E., Gradwohl, G., Fode, C., and Guillemot, F. 1997. Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* **124**: 1611–1621.
- Caviness Jr., V.S., Goto, T., Tarui, T., Takahashi, T., Bhidé, P.G., and Nowakowski, R.S. 2003. Cell output, cell cycle duration and neuronal specification: A model of integrated mechanisms of the neocortical proliferative process. *Cereb. Cortex* **13**: 592–598.
- Diez-Juan, A. and Andres, V. 2003. Coordinate control of proliferation and migration by the p27Kip1/cyclin-dependent kinase/retinoblastoma pathway in vascular smooth muscle cells and fibroblasts. *Circ. Res.* **92**: 402–410.
- Doetsch, F., Verdugo, J.M., Caille, I., Alvarez-Buylla, A., Chao, M.V., and Casaccia-Bonnel, P. 2002. Lack of the cell-cycle inhibitor p27Kip1 results in selective increase of transit-amplifying cells for adult neurogenesis. *J. Neurosci.* **22**: 2255–2264.
- Durand, B., Gao, F.B., and Raff, M. 1997. Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation. *EMBO J.* **16**: 306–317.

- Elledge, S.J. and Harper, J.W. 1994. Cdk inhibitors: On the threshold of checkpoints and development. *Curr. Opin. Cell Biol.* **6**: 847–852.
- Englund, C., Fink, A., Lau, C., Pham, D., Daza, R.A., Bulfone, A., Kowalczyk, T., and Hevner, R.F. 2005. Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J. Neurosci.* **25**: 247–251.
- Farah, M.H., Olson, J.M., Sucic, H.B., Hume, R.I., Tapscott, S.J., and Turner, D.L. 2000. Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development* **127**: 693–702.
- Ferguson, K.L., McClellan, K.A., Vanderluit, J.L., McIntosh, W.C., Schuurmans, C., Polleux, F., and Slack, R.S. 2005. A cell-autonomous requirement for the cell cycle regulatory protein, Rb, in neuronal migration. *EMBO J.* **24**: 4381–4391.
- Fero, M.L., Rivkin, M., Tasch, M., Porter, P., Carow, C.E., Firpo, E., Polyak, K., Tsai, L.H., Broudy, V., Perlmutter, R.M., et al. 1996. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* **85**: 733–744.
- Ge, W., He, F., Kim, K.J., Bianchi, B., Coskun, V., Nguyen, L., Wu, X., Zhao, J., Heng, J.L., Martinowich, K., et al. 2006. Coupling of cell migration with neurogenesis by proneural bHLH factors. *Proc. Natl. Acad. Sci.* **103**: 1319–1324.
- Goto, T., Mitsuhashi, T., and Takahashi, T. 2004. Altered patterns of neuron production in the p27 knockout mouse. *Dev. Neurosci.* **26**: 208–217.
- Gradwohl, G., Fode, C., and Guillemot, F. 1996. Restricted expression of a novel murine atonal-related bHLH protein in undifferentiated neural precursors. *Dev. Biol.* **180**: 227–241.
- Guillemot, F., Molnar, Z., Tarabykin, V., and Stoykova, A. 2006. Molecular mechanisms of cortical differentiation. *Eur. J. Neurosci.* **23**: 857–868.
- Hand, R., Bortone, D., Mattar, P., Nguyen, L., Heng, I.-T.K., Guerrier, S., Boutt, E., Peters, E., Barnes, A.P., Parras, C., et al. 2005. Phosphorylation of Neurogenin2 specifies the migration properties and the dendritic morphology of pyramidal neurons in the neocortex. *Neuron* **48**: 45–62.
- Hoffman, L., Pratt, G., and Rechsteiner, M. 1992. Multiple forms of the 20 S multicatalytic and the 26 S ubiquitin/ATP-dependent proteases from rabbit reticulocyte lysate. *J. Biol. Chem.* **267**: 22362–22368.
- Kawaguchi, A., Ogawa, M., Saito, K., Matsuzaki, F., Okano, H., and Miyata, T. 2004. Differential expression of Pax6 and Ngn2 between pair-generated cortical neurons. *J. Neurosci. Res.* **78**: 784–795.
- Kawauchi, T., Chihama, K., Nabeshima, Y., and Hoshino, M. 2006. Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration. *Nat. Cell Biol.* **8**: 17–26.
- Kholmanskikh, S.S., Dobrin, J.S., Wynshaw-Boris, A., Letourneau, P.C., and Ross, M.E. 2003. Disregulated RhoGTPases and actin cytoskeleton contribute to the migration defect in Lis1-deficient neurons. *J. Neurosci.* **23**: 8673–8681.
- Kiyokawa, H., Kineman, R.D., Manova-Todorova, K.O., Soares, V.C., Hoffman, E.S., Ono, M., Khanam, D., Hayday, A.C., Frohman, L.A., and Koff, A. 1996. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* **85**: 721–732.
- Laman, H., Funes, J.M., Ye, H., Henderson, S., Galinanes-Garcia, L., Hara, E., Knowles, P., McDonald, N., and Boshoff, C. 2005. Transforming activity of Fbxo7 is mediated specifically through regulation of cyclin D/cdk6. *EMBO J.* **24**: 3104–3116.
- Lee, E.Y., Chang, C.Y., Hu, N., Wang, Y.C., Lai, C.C., Herrup, K., Lee, W.H., and Bradley, A. 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis [see comments]. *Nature* **359**: 288–294.
- Lukaszewicz, A., Savatier, P., Cortay, V., Giroud, P., Huisoud, C., Berland, M., Kennedy, H., and Dehay, C. 2005. G1 phase regulation, area-specific cell cycle control, and cytoarchitectonics in the primate cortex. *Neuron* **47**: 353–364.
- Luo, L. 2000. Rho GTPases in neuronal morphogenesis. *Nat. Rev. Neurosci.* **1**: 173–180.
- McAllister, S.S., Becker-Hapak, M., Pintucci, G., Pagano, M., and Dowdy, S.F. 2003. Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions. *Mol. Cell. Biol.* **23**: 216–228.
- Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., and Loh, D.Y. 1996. Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**: 707–720.
- Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. 2001. Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. *Neuron* **29**: 401–413.
- Ohnuma, S. and Harris, W.A. 2003. Neurogenesis and the cell cycle. *Neuron* **40**: 199–208.
- Ohnuma, S., Philpott, A., Wang, K., Holt, C.E., and Harris, W.A. 1999. p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in *Xenopus* retina. *Cell* **99**: 499–510.
- Reynaud, E.G., Leibovitch, M.P., Tintignac, L.A., Pospel, K., Guillier, M., and Leibovitch, S.A. 2000. Stabilization of MyoD by direct binding to p57(Kip2). *J. Biol. Chem.* **275**: 18767–18776.
- Saito, T. and Nakatsuji, N. 2001. Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. *Dev. Biol.* **240**: 237–246.
- Schuurmans, C., Armant, O., Nieto, M., Stenman, J.M., Britz, O., Klenin, N., Seibt, J., Brown, C., Tang, H., Cunningham, J.M., et al. 2004. Sequential phases of neocortical fate specification involve Neurogenin-dependent and -independent pathways. *EMBO J.* **23**: 2892–2902.
- Sherr, C.J. and Roberts, J.M. 1999. CDK inhibitors: Positive and negative regulators of G1-phase progression. *Genes & Dev.* **13**: 1501–1512.
- Sidman, R.L. and Rakic, P. 1973. Neuronal migration, with special reference to developing human brain: A review. *Brain Res.* **62**: 1–35.
- Sriuranpong, V., Borges, M.W., Strock, C.L., Nakakura, E.K., Watkins, D.N., Blaumueller, C.M., Nelkin, B.D., and Ball, D.W. 2002. Notch signaling induces rapid degradation of achaete-scute homolog 1. *Mol. Cell. Biol.* **22**: 3129–3139.
- Steinman, R.A. 2002. Cell cycle regulators and hematopoiesis. *Oncogene* **21**: 3403–3413.
- Sun, J., Marx, S.O., Chen, H.J., Poon, M., Marks, A.R., and Rabani, L.E. 2001. Role for p27(Kip1) in vascular smooth muscle cell migration. *Circulation* **103**: 2967–2972.
- Tanaka, H., Yamashita, T., Asada, M., Mizutani, S., Yoshikawa, H., and Tohyama, M. 2002. Cytoplasmic p21(Cip1/WAF1) regulates neurite remodeling by inhibiting Rho-kinase activity. *J. Cell Biol.* **158**: 321–329.
- Tarui, T., Takahashi, T., Nowakowski, R.S., Hayes, N.L., Bhidé, P.G., and Caviness, V.S. 2005. Overexpression of p27 Kip 1, probability of cell cycle exit, and laminar destination of neocortical neurons. *Cereb. Cortex* **15**: 1343–1355.
- Vernon, A.E. and Philpott, A. 2003. A single cdk inhibitor, p27Xic1, functions beyond cell cycle regulation to promote muscle differentiation in *Xenopus*. *Development* **130**: 71–83.
- Vernon, A.E., Devine, C., and Philpott, A. 2003. The cdk inhibi-

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- tor p27Xic1 is required for differentiation of primary neurons in *Xenopus*. *Development* **130**: 85–92.
- Watanabe, H., Pan, Z.Q., Schreiber-Agus, N., DePinho, R.A., Hurwitz, J., and Xiong, Y. 1998. Suppression of cell transformation by the cyclin-dependent kinase inhibitor p57KIP2 requires binding to proliferating cell nuclear antigen. *Proc. Natl. Acad. Sci.* **95**: 1392–1397.
- Welcker, M., Lukas, J., Strauss, M., and Bartek, J. 1998. p21WAF1/CIP1 mutants deficient in inhibiting cyclin-dependent kinases (CDKs) can promote assembly of active cyclin D/CDK4(6) complexes in human tumor cells. *Cancer Res.* **58**: 5053–5056.
- Wennerberg, K., Forget, M.A., Ellerbroek, S.M., Arthur, W.T., Burridge, K., Settleman, J., Der, C.J., and Hansen, S.H. 2003. Rnd proteins function as RhoA antagonists by activating p190 RhoGAP. *Curr. Biol.* **13**: 1106–1115.
- Zezula, J., Casaccia-Bonofil, P., Ezhevsky, S.A., Osterhout, D.J., Levine, J.M., Dowdy, S.F., Chao, M.V., and Koff, A. 2001. p21cip1 is required for the differentiation of oligodendrocytes independently of cell cycle withdrawal. *EMBO Rep.* **2**: 27–34.
- Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J.W., and Elledge, S.J. 1999. p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes & Dev.* **13**: 213–224.
- Zindy, F., Cunningham, J.J., Sherr, C.J., Jorgal, S., Smeyne, R.J., and Roussel, M.F. 1999. Postnatal neuronal proliferation in mice lacking Ink4d and Kip1 inhibitors of cyclin-dependent kinases. *Proc. Natl. Acad. Sci.* **96**: 13462–13467.

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