Cell Death and Differentiation (2009) 16, 1256–1265

www.nature.com/cdd

p57Kip2 is a repressor of Mash1 activity and neuronal differentiation in neural stem cells

B Joseph[1,3], ER Andersson[2,3], P Vlachos[1], E Södersten[2], L Liu[2], AI Teixeira[2] and O Hermanson*[,2]

Mammalian central nervous system (CNS) development is a highly organized process involving the precise and coordinated timing of cell-cycle exit, differentiation, survival, and migration. These events require proper expression of pro-neuronal genes but also repression of alternative cell fates and restriction of cell-type-specific gene expression. Here, we show that the cyclin-dependent kinase (CDK) inhibitor p57Kip2 interacted with pro-neuronal basic helix-loop-helix (bHLH) factors such as Mash1, NeuroD, and Nex/Math2. Increased levels of p57Kip2 inhibited Mash1 transcriptional activity independently of CDK interactions and acted as a direct repressor in transcriptional assays. Proliferating telencephalic neural progenitors co-expressed basal levels of Mash1 and p57Kip2, and endogenous p57Kip2 accumulated transiently in the nuclei of neural stem cells (NSCs) during early stages of astrocyte differentiation mediated by ciliary neurotrophic factor (CNTF), independent of cell-cycle exit and at times when Mash1 expression was still prominent. In accordance with these observations, gain- and loss-of-function studies showed that p57Kip2 repressed neuronal differentiation after mitogen withdrawal, but exerted little or no effect on CNTF-mediated astroglial differentiation of NSCs. Our data suggest a novel role for p57Kip2 as a context-dependent repressor of neurogenic transcription factors and telencephalic neuronal differentiation.
Cell Death and Differentiation (2009) **16,** 1256–1265; doi:10.1038/cdd.2009.72; published online 10 July 2009

The development of the mammalian central nervous system (CNS) critically relies on a tight coordination among cell proliferation, survival, migration, and differentiation.[1,2] Proper neuronal differentiation does not only require pro-neuronal genes but also regulated repression of other cell fates, such as glial differentiation.[2] Thus, early neural progenitors preferentially differentiate into neurons and do not respond properly to astrocyte-inducing cytokines, at least in part due to DNA methylation of astrocyte-characteristic genes such as glial fibrillary acidic protein (GFAP).[3,4] In line with these observations, it has been shown that loss of the DNA methyl transferase DNMT1 results in hypomethylation and precocious onset of astrocytic genes at the expense of neurogenesis in early neural precursors.[5] In later progenitors, astrocytic genes become demethylated.[3,4] Transcriptional repression of astrocytic genes and thus the correct numbers of progenitors and neurons at these late events, instead depends on other factors such as the Notch-regulated transcription factor CSL/RBP-Jκ and the co-repressors N-CoR and SMRT.[6,7] In addition, neurogenic members of the basic helix-loop-helix (bHLH) family of transcription factors, such as Mash1 and Neurogenin (Ngn) 2, interferes with astrocyte differentiation in multipotent neural progenitors at least in part by sequestering the co-activators CBP/p300 required for astrocyte differentiation.[8]

Less is understood regarding a corresponding repression of neuronal differentiation during glial differentiation. Hes transcription factors repress neurogenic genes such as Mash1 at a transcriptional level.[9] Indeed, inactivation of multiple Hes genes results in premature onset of neuronal genes and thus reduced number of both glial precursors and neurons.[9] Correct regulation of the spatial and temporal expression and activity of neurogenic and repressive bHLH transcription factors thus appears to be absolutely essential for proper development of the forebrain and correct number of progenitors and terminally differentiated cells.

The regulation of stem cell division and differentiation is influenced by many additional factors, including cell-cycle regulators.[1,10] The Cip/Kip family of cell-cycle inhibitors consists of three factors in mammals, p21Cip1, p27Kip1, and p57Kip2. The three factors are all characterized by their ability to bind and inhibit cyclin-dependent kinases (CDK) thereby inhibiting proliferation, also in neural precursors of the forebrain.[11,12] In addition, CDK-inhibitors (CKIs) have been shown to play active roles in neural differentiation and migration.[10,13,14] The expression of p21Cip1 in the developing telencephalon is relatively low, but it has been reported that p21Cip1 is essential for the maintenance of adult neural progenitors in the forebrain.[15] p27Kip1 has been implicated in the cell-cycle arrest of neural precursors during forebrain development, and also in oligodendrocytic specification and differentiation.[10,16] More recently, it has been demonstrated that p27Kip1 plays an important role in regulating neuronal

[1]Department of Oncology-Pathology, Cancer Centrum Karolinska (CCK), Karolinska Institutet, Stockholm, Sweden and [2]CoE in Developmental Biology for Regenerative Medicine (CEDB/DBRM), Department of Cell and Molecular Biology (CMB) and Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden
*Corresponding author: O Hermanson, CoE in Developmental Biology for Regenerative Medicine (CEDB/DBRM), Department of Neuroscience, Retzius väg 8, A2:2, Karolinska Institutet, SE17177 Stockholm, Sweden. Tel: +46 8 5248 7477; Fax: +46 8 341 960;
E-mail: Ola.Hermanson@ki.se
[3]These authors contributed equally to this work.
Keywords: staurosporine; apoptosis; caspase-9; Apaf-1; protein kinase
Abbreviations: CDK, cyclin-dependent kinase; CNTF, ciliary neurotrophic factor; CNS, central nervous system; bHLH, basic helix-loop-helix; CDK, cyclin-dependent kinases; Ngn, Neurogenin
Received 01.9.08; revised 11.3.09; accepted 15.4.09; Edited by M Blagosklonny; published online 10.7.09

migration in the developing neocortex.[14,17] In addition, p27Kip1 exerts a direct effect on neuronal differentiation by increasing the stability of the neurogenic bHLH factor Ngn2.[14]

The role for p57Kip2 in brain development is less clear, despite the fact that p57Kip2 is the only CKI of the Cip/Kip family that is absolutely required for survival.[18] p57Kip2 has been implicated in the control of neural precursor proliferation at several levels of the developing nervous system,[11,19] and recent *in vivo* studies have established that the correct expression levels of p57Kip2 is required for proper development of the developing spinal cord.[19] In addition, it was recently shown that p57Kip2 in cooperation with p27Kip2 regulates cell migration in the developing neocortex.[17]

p57Kip2 has also been shown to play a direct role in regulating the activity of transcription factors implicated in neuronal differentiation. For example, p57Kip2 interacts directly with the nuclear receptor Nurr1 and promote maturation of dopaminergic neurons in postmitotic precursors.[20] However, during development, p57Kip2 are also expressed in mitotic multipotent neural progenitors in the ventricular and subventricular zones (SVZs) of specific regions of the forebrain, including the telencephalon,[21] and the understanding of the mechanisms underlying the diverse effects of p57Kip2 in different contexts remains limited.

The aim of this study was to investigate putative interactions of p57Kip2 with bHLH factors involved in neural differentiation, and whether p57Kip2 exerted any influence on the differentiation of neural stem cells (NSCs). We found that p57Kip2 interacted robustly with a subset of neurogenic bHLH proteins, including Mash1, NeuroD, and Nex/Math2. p57Kip2 repressed Mash1 transcriptional activity, and the interaction with Mash1 as well as the repression of transcriptional activity turned out to be independent of CDK interactions. Importantly, p57Kip2-mediated repression was not due to sequestering of Mash1 from the promoter and p57Kip2 exerted direct transcriptional repression in a GAL4 assay. Mash1 and p57Kip2 expression patterns overlap in proliferating cells of the developing ventral telencephalon, and Mash1 and p57Kip2 were both found to be expressed in proliferating cultures of telencephalic NSCs. Accordingly, p57Kip2 accumulated at defined Mash1 response elements in regulatory regions of the Dlx1 gene, an important regulator of telencephalic progenitor differentiation. In addition, a nuclear accumulation of p57Kip2 was seen in telencephalic NSCs after 16–24 h treatment with CNTF and astrocytic differentiation, at a time point when Mash1 was still highly expressed in the nuclei of the differentiating progenitors. Loss- and gain-of-function approaches suggested that p57Kip2 exerted a context-dependent repressive effect on neuronal but not astrocyte differentiation after mitogen withdrawal. We propose that p57Kip2 is a repressor of Mash1 activity and a context-dependent repressor of neuronal differentiation, possibly to allow proper glial differentiation.

Results

p57Kip2 interacts with neurogenic bHLH factors including Mash1. Earlier studies on muscle development have shown that p57Kip2 is involved in the differentiation of skeletal muscle and can interact with the myogenic bHLH factor MyoD.[22] We asked whether p57Kip2 could interact in a similar way with neurogenic bHLH factors. HEK293 cells were transfected with p57Kip2 cDNA conjugated with an hemagglutinin (HA)-tag together with various members of different classes of bHLH proteins, and total cellular extracts were immunoprecipitated with p57Kip2 antibodies. The precipitated complexes were then investigated by immunoblotting with antibodies against HA (p57Kip2), myc-tag (myc-Mash1), and Flag (Flag-NeuroD, Flag-Nex/Math2). These experiments revealed that p57Kip2 co-immunoprecipitated robustly with a subset of bHLH factors including Mash1, and also NeuroD and Nex/Math2 (Figure 1a). In contrast, we failed to co-precipitate Ngn1/2/3 or Hes1 with p57Kip2 under similar conditions (data not shown).

To confirm whether p57Kip2 and Mash1 could interact in neural cells, we used the Mash1-expressing neuroblastoma cell line (SH-SY5Y), transfected those cells with HA-p57 and immunoprecipitated the total cell extracts with HA antibody. These experiments demonstrated that endogenous Mash1 co-immunoprecipitated with p57 in neuroectodermal cells (Figure 1b).

p57Kip2 represses Mash1 transcriptional activity and acts as a direct repressor of transcription in a GAL4 assay. To investigate putative effects of p57Kip2 on Mash1 activity, Mash1 was co-expressed with increasing amounts of p57Kip2 in HEK293 cells and the activity of co-transfected reporter genes regulated by Mash1-binding E-box sequences was evaluated after normalization of the activity to a co-transfected pCMX-ß-galactosidase reference plasmid. These experiments demonstrated that p57Kip2 significantly repressed Mash1-dependent transcriptional activation efficiently in a dose-dependent manner (Figure 1c). To evaluate whether this effect was associated with the binding of p57Kip2 to CDKs and regulation of cell-cycle exit, we used a mutant version of p57Kip2 containing four substitutions in the CDK interacting domain ablating the interaction with CDKs[20,23] and performed similar experiments. Importantly, this mutant, p57CKmut, also interacted with Mash1 (Figure 1d) and repressed the Mash1 transcriptional activity to a similar extent as wild-type p57Kip2 (Figure 1e). These results suggested that the effect of p57Kip2 on Mash1 transcriptional activity is independent from binding to CDKs and regulation of cell-cycle exit.

To investigate whether the influence p57Kip2 exerted on Mash1 activity could be direct by acting as a transcriptional repressor, we used a p57Kip2 construct fused to the DNA-binding domain of GAL4 (GAL4-DBD) and studied the effect of this protein on a luciferase reporter construct containing four GAL-binding sites fused to a basal promoter (HSP) and compared with the effects of GAL4-DBD only. The results were compared with the effects by GAL4-DBD and GAL4-p57Kip2 on a construct only containing the basal promoter (HSP luciferase). Whereas the activity of the HSP-luciferase construct was unaffected, GAL4-p57Kip2 potently repressed the GAL4 reporter activity compared with control (Figure 1f). This experiment suggested that the repressive effect of p57Kip2 on Mash1 transcriptional activity could be a direct effect by p57Kip2 acting as a transcriptional repressor.

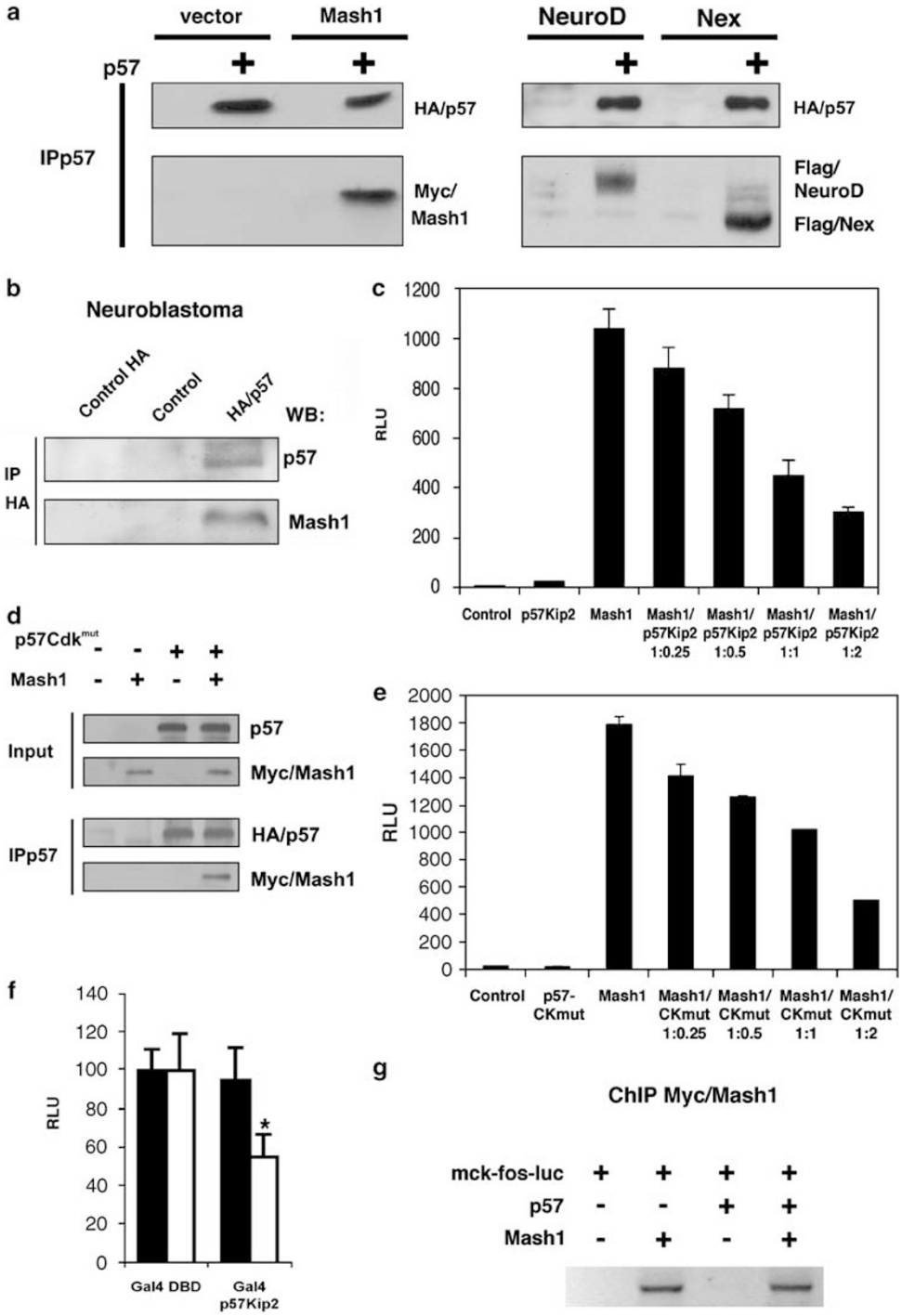

Figure 1 p57Kip2 interacts directly with neurogenic bHLH factors and represses transcription. (**a**) Co-transfections of HA-tagged p57Kip2 and myc-Mash1, Flag-NeuroD, and Flag-Nex/Math2, respectively, were followed by immunoprecipitations with a p57Kip2 antibody. Subsequent immunoblotting with HA, myc, and Flag antibodies revealed that Mash1, NeuroD, and Nex/Math2 co-precipitated with p57Kip2. (**b**) Transfections of HA-tagged p57Kip2 or mock constructs in the neuroblastoma cell line SH-SY5Y, expressing endogenous Mash1, were followed by immunoprecipitations with an HA antibody. Subsequent immunoblotting revealed that endogenous Mash1 co-immunoprecipitated with p57Kip2. (**c**) A transfected TK-luciferase reporter regulated by an E-box was activated by Mash1 co-transfection, whereas co-transfections of increasing amounts of p57Kip2 repressed the Mash1-induced reporter activity in a level-dependent manner. (**d**) Immunoblots of immunoprecipitations of HEK293 cell extracts with a p57 antibody after co-transfections of myc-Mash1 and a cDNA encoding p57Kip2 harboring four-point mutations interrupting interactions with CDK, p57CKmut, demonstrating that the Mash1 interaction was not dependent on the CDK interaction. (**e**) p57CKmut showed similar effects in the luciferase assay as wild-type p57Kip2. (**f**) Full-length p57Kip2 fused to the DNA-binding domain of GAL protein specifically repressed reporter activity, indicating that p57Kip2 can act directly as a transcriptional repressor. Black bars represent HSP-luciferase (control), white bars represent MH100-HSP-luciferase (with 4 GAL-binding sites). (**g**) PCR results after chromatin immunoprecipitation (ChIP) experiments of HeLa cell extracts with a myc antibody after transfections of E-box containing luciferase reporter gene (mck-fos-luc), and HA-p57Kip2 and/or myc-Mash1 cDNA. Error bars in **c**, **e**, and **f**: S.E.M. ($^*P < 0.05$)

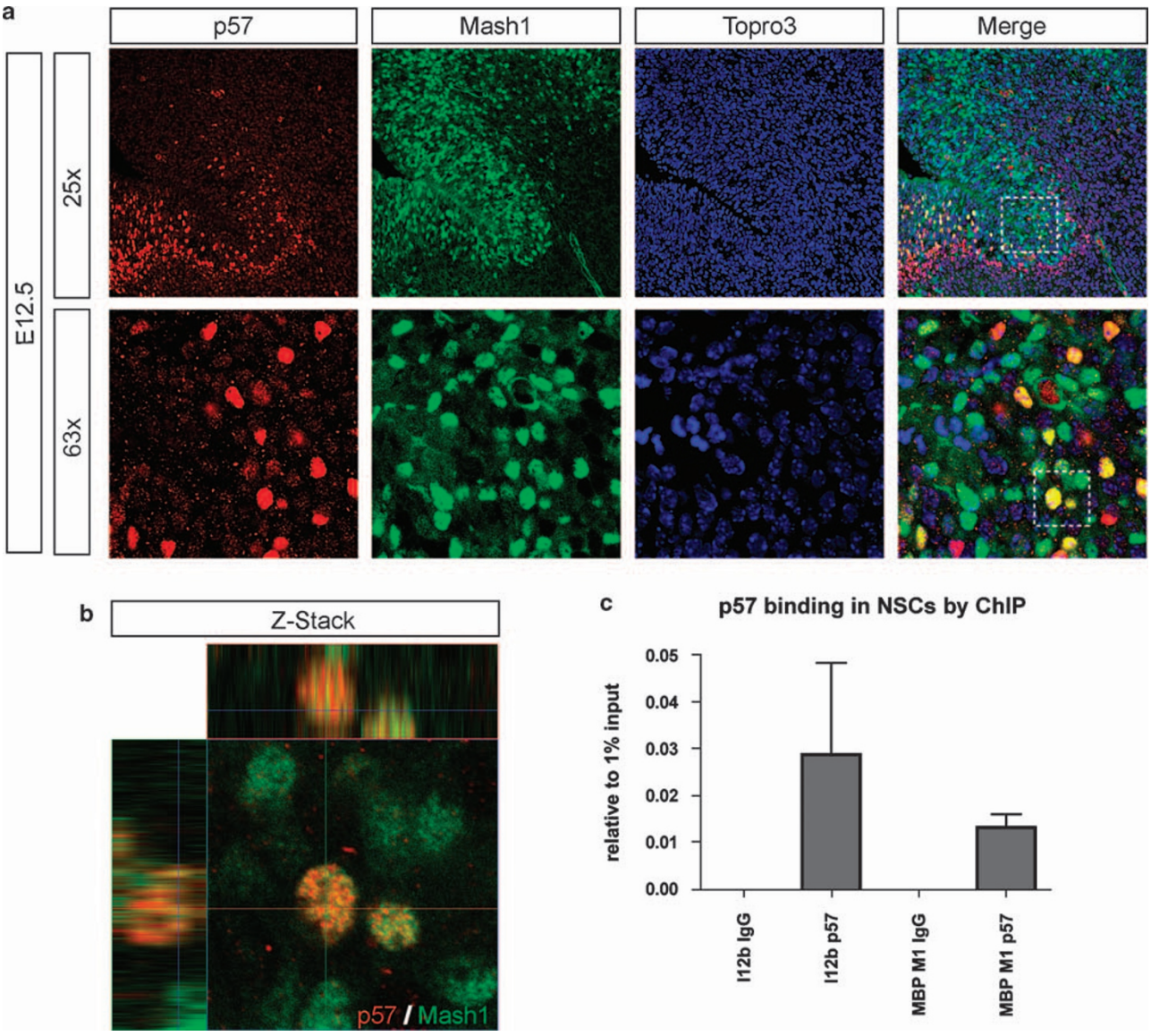

Figure 2 A subset of Mash1-positive neural progenitors in the ventral telencephalon co-express nuclear p57Kip2 and p57Kip2 is enriched at defined Mash1-binding sites in neural stem cells. (**a**) High and low magnification confocal imaging micrographs depicting transverse sections of mouse brain from embryonic day (E) 12.5 after immunohistochemistry experiments using antibodies against p57Kip2 and Mash1, respectively. Topro3 staining was used to demonstrate nuclei. Red labeling: p57Kip2 immunoreactivity; green labeling: Mash1 immunoreactivity; blue labeling: Topro3 staining. (**b**) High magnification confocal imaging revealed nuclear double labeling of p57Kip2 and Mash1 in the ventral telencephalon. (**c**) Quantitative PCR results after chromatin immunoprecipitation experiments of neural stem cell extracts using a p57Kip2 antibody demonstrating an accumulation of p57Kip2 on an earlier described Mash1-binding site in the regulatory region I12b of the Dlx1/2 gene

This is to our knowledge the first demonstration of a CKI acting directly as a transcriptional repressor.

Although these data demonstrate that p57Kip2 can act as a transcriptional regulator, it was still possible that the decrease in Mash1 activity was due to p57Kip2-mediated sequestering of Mash1 from the promoter. We therefore performed chromatin immunoprecipitation (ChIP) experiments to investigate the presence of Mash1 on the reporter construct in the absence and presence of p57Kip2. Mash1 was found to occupy the E-box sites in the promoter both during basal conditions as well as when co-transfected with p57Kip2 (Figure 1g), suggesting that the repressive effects of p57Kip2 on Mash1 transcriptional activity is not likely to be the result of sequestering Mash1 away from the promoter.

p57Kip2 is co-expressed with Mash1 and p57Kip2 is transiently accumulated in NSC nuclei during CNTF-mediated astrocytic differentiation. We next investigated the expression pattern of p57Kip2 in the developing mouse telencephalon by immunohistochemistry. At embryonic day (E) 12.5–13, a critical time point in telencephalic development, we found prominent p57Kip2 expression in the SVZ of the ventral telencephalon including the medial ganglionic eminence and the developing preoptic area (Figure 2a). Cells of the medial ganglionic eminence SVZ have been shown to contribute to the developing striatum and cortical GABAergic interneurons.[24] Notably, the p57Kip2 labeling was found to be predominantly nuclear (Figure 2a). As Mash1 at this time of development is

expressed in the SVZ of the ventral telencephalon, we performed double-labeling immunohistochemistry and confocal imaging that revealed p57Kip2 co-expression with Mash1 in the nuclei of a subset of telencephalic progenitors in the SVZ (Figure 2a and b).

To investigate a putative role for p57Kip2 in neural differentiation, we pursued a series of experiments investigating the expression of p57Kip2 in fibroblast growth factor-2 (FGF2) expanded embryonic telencephalic multipotent NSCs isolated from primary cortices. One passage after primary isolation, these NSCs constitute a homogenous progenitor population positive for stem cell markers such as nestin and negative for markers of terminal differentiation (Supplementary Figure 1A–D). The telencephalic NSCs can rapidly and efficiently be differentiated into neurons, astrocytes, and smooth muscle-like cells after various treatments as assessed by morphology and cell fate markers.[6,7,25,26] For example, treatment of NSCs with PDGF, BDNF, or valproic acid results in increased neuronal differentiation and TuJ1 antibody staining, CNTF, LIF, CT1, or BMP4 treatment induce astrocytic differentiation and increased GFAP staining, and treatment with fetal bovine serum or BMP4 yields increased mesenchymal differentiation and increased numbers of cells expressing smooth muscle actin (SMA) (Supplementary Figure 1F–H).

We first aimed at investigating whether p57Kip2 was associated with the role for Mash1 in NSCs. We, therefore, performed ChIP experiments using the p57Kip2 antibody and cell extracts from NSC, and assessed the enrichment of p57Kip2 at a well-characterized Mash1 response element in the regulatory region of the Dlx1/2 gene.[27] We found a significant accumulation of p57Kip2 at the I12b intergenic regulatory region, around three-fold compared with the presumably less relevant and thus control M1 region of the MBP gene (Figure 2c), suggesting that endogenous p57Kip2 is enriched at Mash1-binding sites in NSCs.

We next aimed at assessing the expression and subnuclear localization of p57Kip2 in FGF2-expanded NSCs during proliferating conditions. Immunocytochemistry experiments revealed basal p57Kip2 immunoreactivity both in the nucleus and the cytoplasm in FGF2-treated NSCs (Figure 3a, left panels). Immunoblotting of FGF2-expanded NSCs confirmed basal expression of p57Kip2 in FGF2-expanded NSCs (Supplementary Figure 1I). We next investigated the expression of p57Kip2 during differentiating conditions. Treatment with ciliary neurotrophic factor (CNTF) induces a rapid (<48 h) and efficient ($>90\%$) differentiation of NSCs into GFAP-positive astrocytes.[6,25] CNTF treatment of NSCs induced a clear increase in nuclear staining of p57Kip2 in a large number of cells (Figure 3a, right panels). Immunoblotting showed no increase in total p57Kip2 protein levels but an induction of GFAP protein and a decrease in N-CoR, a known repressor of astrocytic differentiation (Supplementary Figure 1I).[6] Time-course experiments revealed that the increase in nuclear p57Kip2 started at around 8 h, peaked after around 20 h, subsequently decreased and returned to control levels 72–96 h after the initiation of astrocyte differentiation by CNTF (Figure 4a and b). This nuclear accumulation of p57Kip2 was abolished by pre-treatment with the PI3K/Akt kinase inhibitor LY294002 at 10 μM but unaffected

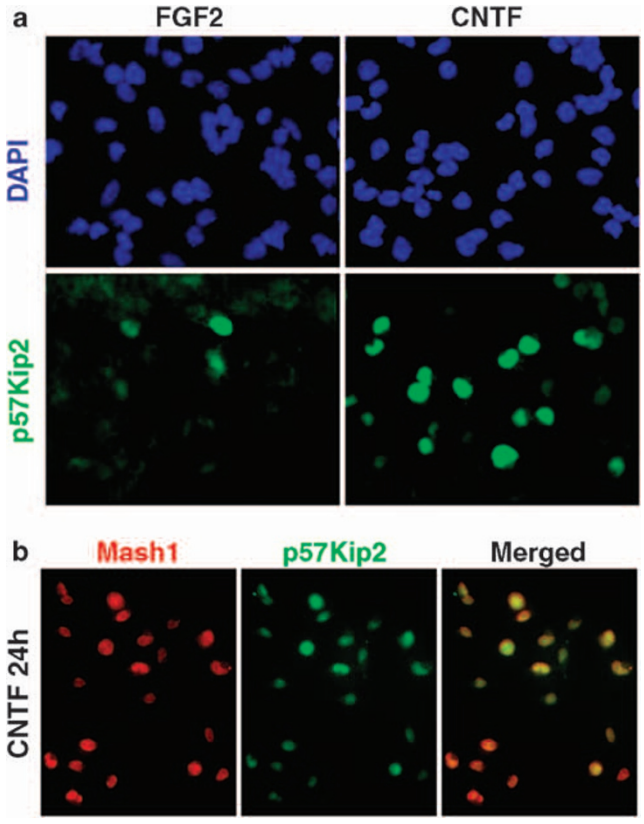

Figure 3 p57Kip2 displays a nuclear accumulation during CNTF-mediated astrocyte differentiation and co-localize with Mash1 immunoreactivity in the nucleus. (**a**) Micrographs demonstrating cell nuclei (DAPI) and p57Kip2 immunostaining in FGF2- *versus* CNTF-treated NSCs 24 h after initiation of CNTF stimulation. (**b**) Micrographs depicting immunostaining for Mash1 and p57Kip2 in NSCs demonstrating nuclear co-localization 24 h after initiation of CNTF treatment

by pre-treatment with the MEK inhibitor PD98059 at 25 μM (Supplementary Figure 1J). *In silico* investigation of putative phosphorylation sites of the p57Kip2 protein (Scansite) at high or medium stringency failed to reveal any putative Akt kinase motifs, but identified a direct GSK3 kinase site (data not shown). GSK3 kinase is a major target for Akt kinase in NSCs,[6] suggesting the possibility that the regulation by the PI3K/Akt kinase signaling pathway is indirect. We and others have shown earlier that the subcellular localization of p57Kip2 is regulated and that this protein can shuttle between the nucleus and the cytoplasm.[28,29] Taken together, these observations suggest that the increase in nuclear immunoreactivity was primarily the result of a PI3K/Akt kinase-dependent accumulation of p57Kip2 protein in the nucleus.

FGF2 treatment has been shown to influence telencephalic NSCs by regulating an increase in the expression of genes usually highly expressed in the ventral telencephalon, such as Mash1.[30] It has, however, also been shown that Mash1 can rapidly be degraded during BMP-mediated astroglial differentiation of forebrain progenitors.[31] We, therefore, assessed whether nuclear Mash1 expression was affected by the CNTF-mediated astrocytic differentiation. Immunocytochemistry experiments showed that at the peak of nuclear p57Kip2 labeling (16–24 h after CNTF stimulation), Mash1 expression

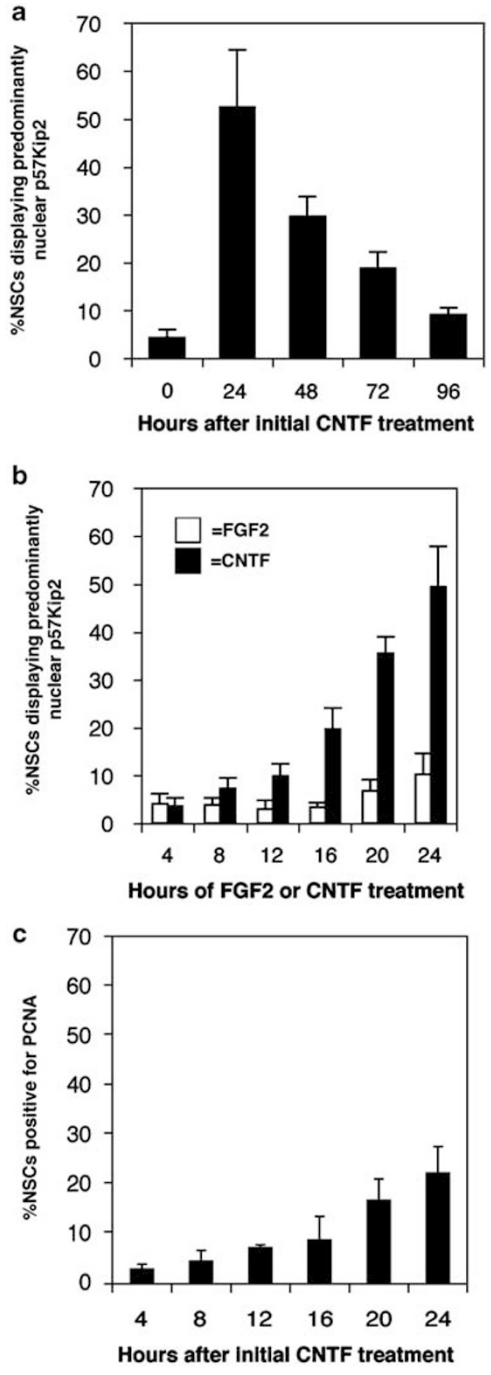

Figure 4 The nuclear accumulation of p57Kip2 during astrocyte differentiation is transient, peaks at 20–24 h, and is not associated with an increase in cell-cycle exit. (**a** and **b**) Quantifications of NSCs that displayed predominantly nuclear p57Kip2 staining at different time points during CNTF-mediated astrocyte differentiation. Long time course experiments demonstrating a peak after 24 h (**a**). Short term course experiments confirming the peak of NSCS that displayed strong nuclear p57Kip2 staining at 20–24 h of CNTF-treatment in comparison with FGF2-treated cells (**b**). (**c**) Quantifications of numbers of CNTF-stimulated NSCs displaying PCNA immunostaining at 4–24 h after initiation of treatment. All experiments were repeated three times independently. Error bars: S.E.M. (**a** and **b**), S.D. (**c**)

was still prominent in the nuclei and co-localized to a large extent with p57Kip2 immunoreactivity in the nucleus (Figure 3b), whereas the Mash1 expression at later time points (>48 h) was below detection levels (data not shown).

We next investigated whether a change in proliferative state of the cells after CNTF treatment was associated with the increase in nuclear p57Kip2 staining by using time-lapse microscopy. Bright-field micrographs were taken of NSCs every 30 min for 48 h after FGF2 or CNTF administration. These experiments demonstrated that there was no significant increase in cell-cycle exit at the times when p57Kip2 nuclear expression was most prominent after CNTF treatment (Supplementary Video 1 and 2). Immunocytochemical detection of the proliferative marker PCNA at 4-h intervals further supported this observation as there was no significant decrease but rather an increase in PCNA staining in CNTF-stimulated NSCs after 16–24 h compared with earlier time points (Figure 4c). These experiments indicate that the increase in nuclear p57Kip2 was not primarily associated with cell-cycle exit.

Overexpression of p57Kip2 or p57CKmut inhibits neuronal differentiation of NSCs. To investigate a putative role for p57Kip2 in neural differentiation, we transfected NSCs with wild-type p57Kip2 and the p57CKmut that does not interact with CDKs, and subsequently treated the cells with FGF2 for 12 h followed by CNTF stimulation or withdrawal of FGF2. FGF2 withdrawal results in a differentiation of NSCs into roughly 30% neuronal cells and 30% astrocytes after 120 h and is to be considered as permissive conditions. NSCs treated with CNTF for 24 h after transfection with p57Kip2 and p57CKmut plasmids did not show any significant difference in the expression of GFAP compared with mock-transfected NSCs (Supplementary Figure 2B). In contrast, NSCs that had been transfected with wild-type p57Kip2 in the presence of FGF2 and subsequent withdrawal of FGF2 for 120 h, showed a clear (46.3%, $P<0.05$) decrease in the number of cells that stained positive for the neuronal marker TuJ1, and little change in the number of GFAP-positive cells (Figure 5a). Cells transfected with p57CKmut showed a similar decrease (42.2%, $P<0.05$) in neuronal marker expression indicating that the effect on neuronal differentiation was uncoupled from putative effects on proliferation (Figure 5a).

Reduced levels of endogenous p57Kip2 result in an increase in expression of neuronal-specific antigens. To evaluate the loss of p57Kip2 function, we overexpressed antisense oligonucleotides directed against p57Kip2 together with an EGFP vector that labeled the transfected cells. These antisense constructs effectively and selectively reduce p57Kip2 expression compared with control cultures (Supplementary Figure 2A and 3). NSCs treated with CNTF for 48 h after transfection with antisense or sense oligonucleotides showed no significant difference in the number of GFAP-expressing cells (93.7 *versus* 91.2%). In contrast, in conditions of growth factor withdrawal for 120 h after transfection, there was a clear increase (62.6%, $P<0.05$) in the proportion of cells that stained positive for TuJ1 and a reduction of GFAP expression (30.7%, $P<0.05$) in cells that

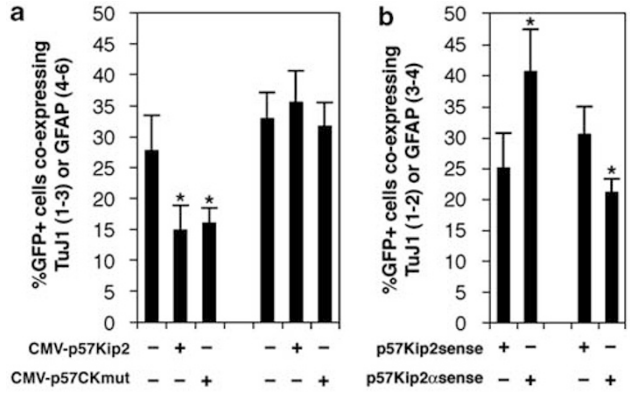

Figure 5 p57Kip2 inhibits neuronal differentiation of NSCs after mitogen withdrawal. (**a**) Quantifications of transfected NSCs identified by GFP expression that were positive for staining after incubation with TuJ1 antibody (bars 1–3) or GFAP antibody (bars 4–6) indicating neurons and astrocytes, respectively, after 120 h of growth factor (FGF2) withdrawal. The transfected constructs are indicated. (**b**) Quantifications of TuJ1- or GFAP-positive cells transfected with p57Kip2 sense or p57Kip2 antisense oligonucleotides and counted 120 h after FGF2 withdrawal. All experiments were repeated five times independently. Error bars: S.E.M. (*$P<0.05$)

had received p57Kip2 antisense *versus* sense oligonucleotides, respectively (Figure 5b). It should be noted that like in the overexpression experiments above, the transfection efficiency of NSCs was low compared with cell lines (see Materials and Methods), and the possibility that the transfected cells thus represented a subset of NSCs that theoretically could be more committed cells should be taken into account. In addition, a significant proportion of cells that received p57Kip2 antisense did indeed still express GFAP and were TuJ1 negative, suggesting that the increase in the number of TuJ1 cells was due to a selective induction of TuJ1 expression in a subset of the differentiating progenitors.

Discussion

It is known that certain CKIs play important roles in neural development as well as in neuro- and gliogenesis in addition to regulation of cell-cycle exit, but the molecular mechanisms underlying the diverse effects have not been extensively investigated. We show that p57Kip2 acts as a transcriptional repressor, a repressor of Mash1 activity and exerts a context-dependent inhibition of neuronal differentiation, independent of the interaction with CDKs and cell-cycle exit. Our results expand the understanding of CKI function in neural differentiation and strengthen the hypothesis that CKIs provide a direct molecular link between cell-cycle exit and differentiation, in addition to previous reports on roles in neural migration.

Independent observations from other laboratories have shown a transient expression of p57Kip2 in the mouse and chick spinal cord and also a higher number of neurons after loss of function experiments.[19] Although these experiments clearly indicate that higher number of neurons observed after loss of p57Kip2 is due to aberrant regulation of progenitor cell-cycle exit and timing, additional mechanisms should also be considered. In our *in vitro* assays, we found no association of the increase in nuclear p57Kip2 staining with cell-cycle exit or CDK-interacting function. It is, therefore, plausible that p57Kip2 plays multiple spatial and temporal context-dependent roles in neural differentiation and proliferation.

p57Kip2 has been shown earlier to exert important roles in neuronal migration but less so in neuronal differentiation of the dorsal pallium.[17] There are several differences between this study and the present. Most importantly, Itoh *et al*.[17] focused at roles for p57Kip2 and other CKIs in the dorsal telencephalon and neocortex, whereas in this study, we used an *in vitro* system of telencephalic neural progenitors that include both dorsal and ventral progenitors and is known to be 'ventralized' in character by the FGF2-mediated expansion of the stem cell cultures.[30] As the expression of CKIs and neurogenic factors such as bHLH transcription factors show spatiotemporal variations during telencephalic development, conditional gene deletion strategies will be required to elucidate these questions in more detail.

The CKI p27Xic1 has been shown earlier to promote glial differentiation in the Xenopus retina in a Notch-dependent manner,[13] and recent studies have reported clear associations between Notch signaling and p57Kip2 expression in different systems.[32,33] Because of the essential role for Notch signaling in stem cell maintenance and glial differentiation, we tested whether p57Kip2 could interact directly with the Notch signaling transcription factor CSL/RBP-Jκ, but we failed to detect an interaction between these two factors in immunoprecipitation experiments (BJ, unpublished observations). We further did not find any interaction between p57Kip2 or Hes proteins. Yet our results provide an indirect association of p57Kip2 to Notch signaling as Mash1 is a second-order target of Notch signaling via Hes1. Context-dependent differences in the function of p57Kip2 and other CKIs may further be explained by the variations in levels,[34] spatial and temporal expression patterns, as well as the subcellular localization of p57Kip2;[29] present study).

Ventral telencephalon is known to give rise to the early waves of oligodendrocytes.[35] As the expression of p57Kip2 was found to be high in the ventral telencephalon (Figure 2a), it is tempting to speculate whether a potential role for p57Kip2 could be to repress neuronal differentiation to secure proper oligodendrocyte proliferation, specification, and/or differentiation. An expression-level-dependent role for p57Kip2 in Notch-regulated oligodendrocyte specification in the ventral spinal cord has been suggested from elegant experiments in zebrafish,[34] and it was recently shown that proper levels of p57Kip2 is critical for correct proliferation as well as differentiation of primary oligodendrocyte progenitor cells as well as Schwann cells.[36,37] Furthermore, the timing and levels of Mash1 and Dlx1/2 expression, as well as Notch signaling, have been shown to be critical parameters to secure the proper proliferation, differentiation, and migration of ventral telencephalic progenitors.[24,38] It is at present difficult experimentally to yield efficient oligodendrocyte production from embryonic NSCs hampering such investigations in NSCs, but future studies should address this hypothesis in greater detail. Our present findings indicate that p57Kip2 can interact with a subset of neurogenic bHLH factors, such as Mash1, and thereby contribute to the balanced regulation of neurogenesis in the ventral telencephalon (Figure 6). We speculate that this regulation occurs in a subset of glial progenitors.

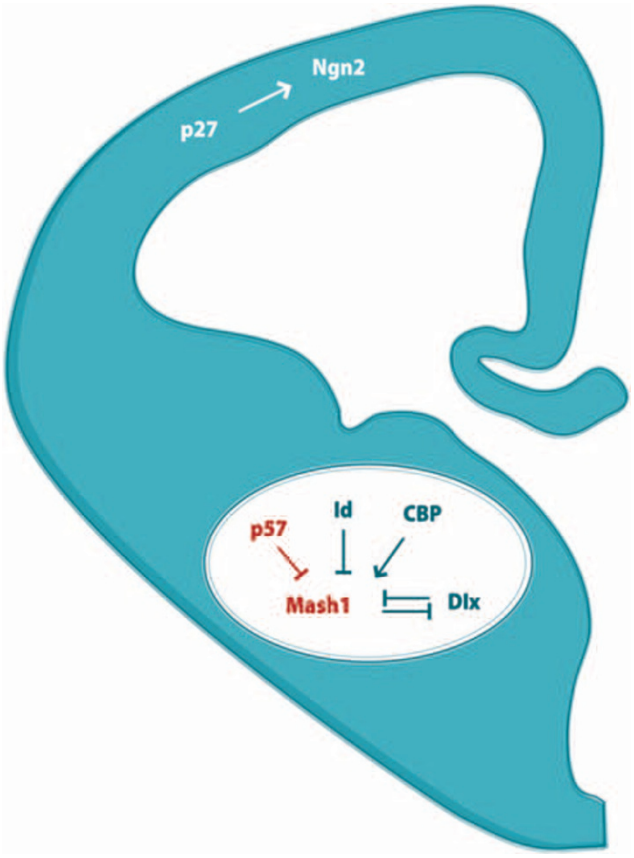

Figure 6 A representation of the left side of an embryonic rodent forebrain suggesting a putative integration of p57Kip2 as a Mash1 interacting protein with earlier data on telencephalic progenitor differentiation. It has been shown earlier that the balance of the expression of Mash1 and Dlx genes, such as Dlx1/2, is a critical parameter for differentiation of neural progenitors in the ventral telencephalon[2,24] and that Mash1 can directly regulate the Dlx1/2 gene through binding to the I12b enhancer[27] suggesting that p57Kip2 may play a specific role in the generation of cortical GABAergic interneurons and/or the developing striatum. The coactivator CBP has been shown to be critical for Mash1 activity, whereas the Id family of bHLH-interacting proteins repress the activity of bHLH transcription factors.[39] Earlier studies have shown that p27Kip2 and Neurogenin2 (Ngn2) interacts in the dorsal pallium and developing neocortex[14,17] suggesting in addition to the temporal aspects, a spatial component in the expression and function of different CKIs

Materials and Methods

Cell cultures. Human neuroblastoma SH-SY5Y cell line (ATCC CRL-2266), human cervical carcinoma HeLa cell line (ATCC CCL-2), and human embryonic kidney HEK-293 (ATCC CRL-1573) were cultured as described.[20,29] Isolation and culture of multipotent neural progenitors, referred to as NSCs, were performed essentially as described earlier.[6,7,25,26] In brief, cortices from rats at embryonic day 15 were dissected and mechanically dispersed in a modified serum-free, HEPES-free N2-supplemented DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA). All animal experiments were done in accordance with the approval of the local ethics committee (Stockholms Norra Djurförsöksetiskanämnd). The primary cells were plated on dishes (35, 60, 100 mm) coated with poly-L-ornithine and fibronectin (Sigma, St Louis, MO, USA). The cells were treated with human recombinant basic FGF2 at 10 ng/ml every 24 h and the N2 medium was replaced every 48 h. All treatments and transfections were performed after the first passage when $>99\%$ of the cells displayed nestin expression and typical NSC morphology and $<1\%$ of the cells expressed neuronal and glial differentiation markers accompanied with very low levels of mRNA levels for terminal markers (see Results). To achieve astrocytic differentiation, the cells received CNTF (R&D Systems, Minneapolis, MN, USA) at 10 ng/ml for 48 h. When used, LY294002 (10 μM) or PD98059 (25 μM; both Cell Signaling Technology, Beverly, MA, USA) was added 45 min before the CNTF. All results are based on at least three independent experiments.

Immunohistochemistry and antibodies. All animal experiments were done in accordance with the approval of the local ethics committee (Stockholms Norra Djurförsöksetiskanämnd). Time mated C57BL6 mice from Scanbur, Sweden, were used for immunohistochemistry experiments. Noon of day of plug was taken as embryonic day (E) 0.5. Embryos were dissected out at E12.5, decapitated and fixed in 4% paraformaldehyde for 2 h at 4°C, and then washed in phosphate buffered saline (PBS). Embryos were transferred to 30% sucrose, rocked overnight, and then embedded in OCT (TissueTek) and frozen on dry ice. Serial coronal 14 mm sections were collected on a cryostate. Sections were first immersed in PBS for 5 min, and then blocked with 5% goat serum (GS) in PBS with 0.1% Triton-X (PBST) for 1 h. Primary antibodies were diluted in 5% GS/PBST overnight at 4°C. Slides were then washed in PBS, 3 × 15 min, and secondary antibodies, diluted in 5% GS/PBST, were applied for 1 h at room temperature (RT). The slides were then washed again, 3 × 15 min, counterstained with Topro-3 (Invitrogen) and washed twice more. Slides were mounted in glycerol/PBS 9 : 1. The following antibodies were used: rabbit anti-p57Kip2 (1 : 50, Santa Cruz, Santa Cruz, CA, USA), mouse anti-Mash1 (1 : 500, BD Pharmingen, San Diego, CA, USA), secondary antibodies from Molecular Probes (Leiden, Netherlands) (see further below). All images were acquired on a Zeiss LSM 510 confocal (Zeiss, Germany) with Zeiss software.

Immunocytochemistry and antibodies. For immunocytochemistry, cells were fixed in the plates with formalin for 20 min at RT, rinsed three times in PBS, and incubated in primary antibodies O/N at 4°C under agitation. The cells were then rinsed six times in PBS before incubation in secondary antibody for 1 h at RT in the dark under agitation. After three additional rinses, the plates were coverslipped with Vectashield with DAPI. Primary antibodies: goat anti-p57Kip2, rabbit anti-PCNA (1 : 100–1000; Santa Cruz Biotechnology), mouse anti-p57Kip2, mouse anti-nestin, mouse anti-Mash1 (1 : 100–1000; Pharmingen, San Diego, CA, USA), mouse anti-Tuj1 (1 : 500; Covance), mouse anti-MAP2 (1 : 500; Sigma), rabbit anti-GFAP (1 : 500; DAKO), rabbit anti-SMA (1 : 1000, Sigma), rabbit anti-Actin (1 : 2000; Sigma), chicken anti-GFP (1 : 1000; Chemicon, Temecula, CA, USA). Secondary antibodies for immunocytochemistry were purchased from Molecular Probes and included donkey-anti-mouse, donkey-anti-goat, donkey-anti-rabbit, and donkey-anti-chicken conjugated to Alexa 488 and Alexa 594 and used at 1 : 500. Secondary antibodies for immunoblotting were purchased from Chemicon and included peroxidase-conjugated rabbit-anti-goat and rabbit-anti-mouse used at 1 : 2000.

Cell extract preparations, immunoprecipitations and immunoblotting. For whole cell lysates, cells from 10 cm dishes were scraped and lysed in NETN buffer with 1 × protease inhibitor cocktail (Complete, Roche) for 15 min followed by centrifugation at 4°C for 20 min. For immunoprecipitation, protein Sepharose-precleared nuclear extracts were incubated with the indicated antibody in nuclear extract buffer overnight at 4°C. Immunocomplexes bound to protein A- or G-Sepharose were collected by centrifugation and washed in radioimmunoprecipitation assay buffer. For immunoblot analysis, nuclear-cell extracts or immunoprecipitates were resolved on SDS–PAGE and blotted; protein pieces were detected with anti-p57Kip2 (Santa Cruz Biotechnology), anti-Mash1 (Pharmingen), anti-Flag (Sigma), or anti-HA (Roche Diagnostics, Stockholm, Sweden) antibodies.

DNA delivery and constructs. SH-SY5Y cells and HEK293 cells were cultured and transfected as described elsewhere.[20] Transient transfections of NSCs were performed essentially according to the supplier's recommendations (Effectene, Qiagen, Courtaboeuf, France). Antisense and eGFP were in addition delivered by nucleofection using a Nucleofector device and the Rat NSC Nucleofector Kit according to the supplier's protocol (Amaxa, Lonza, Switzerland). Transfections efficiencies of NSCs ranged between 5 and 20%. Expression constructs used in this study: pCMV-HAp57Kip2 and pCMV-HA-p57CKmut were gifts of Dr. SA Leibovitch (Centre National de la Recherche Scientifique, Villejuif, France) and Dr. Y Xiong (University of North Carolina, Chapel Hill), respectively. pCMX-asp57Kip2 was obtained by inserting in antisense orientation the NcoI–HindIII fragment from pEX10X-p57Kip2 (gift from Dr. J Massagué, Sloan–Kettering Cancer Center, New York, USA), into expression plasmid pCMX.[20] Gal4DBD-p57Kip2 encodes full-length cDNA sequence of the mouse p57Kip2 in frame with the yeast Gal4 DNA-binding domain. pCDNA3.1-Hes1 was a gift from Dr. K Jepsen, University of California, San Diego, USA. pCMV-MASH1 and pCMV-Ngn 1–3 were

gifts from Drs. J Frisén and J Ericson, Karolinska Institutet, Sweden. pCMV-NeuroD and pCMV-Nex were gifts from Dr. MH Schwab, Max Planck Institute, Göttingen, Germany.

Reporter gene assays. The E-box reporter plasmid containing region of the MCK enhancer coupled to luciferase, MCK-fos-luc was a gift from Dr. M Sigvardsson. Dr. T Perlmann provided us with plasmids encoding ß-galactosidase, HSP-luc and MH100-HSP-luc. Transfections were performed in 24-well plates with Lipofectamine 2000, according to the manufacturer's protocol. For promoter-luciferase constructs, each well was transfected with 100 ng of reporter plasmid (MCK-fos-luc), 300 ng of expression vectors (pCMV-HA-p57Kip2; pCMV-HA-p57CKmut; pCMV-MASH1) and 100 ng of pCMX-ß-gal reference plasmid containing a bacterial ß-galactosidase gene. For Gal4-RE-luciferase constructs, each well was transfected with 100 ng reporter plasmid (MH100-HSP-luc; HSP-luc), 200 ng expression vector (Gal4DBD; Gal4DBD-p57Kip2), and 100 ng pCMX-ß-gal reference plasmid. The same amount of DNA was added to each well. Cells were harvested 24 h after transfection, lysed and extracts were assayed for luciferase and ß-galactosidase activities in a microplate luminometer/photometer reader (Orion Microplate Luminometer; Berthold detection systems). Relative light units were computed after normalization to ß-galactosidase activities. Values shown are representatives of at least three independent experiments, made in triplicates, with error bars representing S.E.M.

ChIP and qPCR. HeLa cells were transfected with plasmids encoding mck-fos-luc, Mash1 and p57. ChIP were carried out using ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's protocol and using c-myc antibody (Santa cruz sc-7189). PCR were performed using primers for the mck-fos-luc promoter (forward: RVprimer3 5′-ctagcaaaataggctgtccc-3′, reverse: GLprimer2 5′-ctttatgtttttggcgtcttcca-3′). For NSCs, ChIP-IT Express (Active Motif, Carlsbad, CA, USA) was used according to the supplier's recommendations. Protein content of cells was cross-linked using 1 mM dithiobissuccinimidyl-propionate for 30 min, followed by fixation with 1% formaldehyde for 10 min. Cells were rinsed twice with cold PBS, collected by scraping and pelleted at 2000 r.p.m. for 4 min at 4°C. Frozen pelleted cells were resuspended in lysis buffer, centrifuged for 10 min at 4°C and the pelleted nuclei was resuspended in shearing buffer. Chromatin was then sonicated using a Bioruptor 200 (Diagenode, Sparta, NJ, USA), high frequency, 0.5 min/0.5 min, for 12 min. Sonicated chromatin was analyzed in a 2% agarose gel, to confirm optimal chromatin shearing efficiency. Input was collected for further analysis. A measure of 5 μg of chromatin was incubated for 1 h at 4°C with protein G magnetic beads and then immunoprecipitated overnight at 4°C with anti-p57Kip2, along with Histone H3 (Abcam, ab1791, positive control, 2 μg) and rabbit IgG (Active motif, negative control, 2 μg). After three washes with ChIP buffer 1 and two washes with ChIP buffer 2, the IP DNA was decross-linked and resuspended in a final volume of 130 μl. Purified DNA and 1% input were analyzed by qPCR, using five-fold dilutions of the input for standard curves and triplicates per sample. As the standard curve method was used, potential differences in primer efficiency were taken in account and occupancy in different regulatory regions could be compared for a specific antibody. Primers used: I12b enhancer: for 3′-ggcccatcaaacacaacata-5′ rev 3′-gattccctgggcgaaaaa-5′; MBP, M1 3′-ttcaagaccccaggaagaaa-5′, rev 3′- ttctttgggtctgctgtgtg-5′.

Acknowledgements. We thank Drs. SA Leibovitch, Y Xiong, J Massagué, MH Schwab, K Jepsen, M Sigvardsson, J Frisén, and J Ericson for various constructs and reagents, and Dr. D Castro for valuable comments. Supported by grants from the Swedish Childhood Cancer Foundation (BCF), the Magn. Bergvall Foundation, the Lars Hierta Foundation, the Jeansson Foundation, the Åhlén Foundation, the Swedish Foundation for Strategic Research (SSF), the K&A Wallenberg Foundation (OH), the Karolinska Institutet Foundations (KI Cancer), the Swedish Research Council, the Åke Wiberg Foundation, the Swedish Medical Society, and the Swedish Cancer Society (BJ and OH).

1. Ohnuma S, Harris WA. Neurogenesis and the cell cycle. *Neuron* 2003; **40**: 199–208.
2. Guillemot F, Molnar Z, Tarabykin V, Stoykova A. Molecular mechanisms of cortical differentiation. *Eur J Neurosci* 2006; **23**: 857–868.
3. Molne M, Studer L, Tabar V, Ting YT, Eiden MV, McKay RD. Early cortical precursors do not undergo LIF-mediated astrocytic differentiation. *J Neurosci Res* 2000; **59**: 301–311.
4. Takizawa T, Nakashima K, Namihira M, Ochiai W, Uemura A, Yanagisawa M *et al.* DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. *Dev Cell* 2001; **1**: 749–758.
5. Fan G, Martinowich K, Chin MH, He F, Fouse SD, Hutnick L *et al.* DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. *Development* 2005; **132**: 3345–3356.
6. Hermanson O, Jepsen K, Rosenfeld MG. N-CoR controls differentiation of neural stem cells into astrocytes. *Nature* 2002; **419**: 934–939.
7. Jepsen K, Solum D, Zhou T, McEvilly RJ, Kim HJ, Glass CK *et al.* SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. *Nature* 2007; **450**: 415–419.
8. Sun Y, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hua X *et al.* Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* 2001; **104**: 365–376.
9. Kageyama R, Ohtsuka T, Hatakeyama J, Ohsawa R. Roles of bHLH genes in neural stem cell differentiation. *Exp Cell Res* 2005; **306**: 343–348.
10. Molofsky AV, Pardal R, Morrison SJ. Diverse mechanisms regulate stem cell self-renewal. *Curr Opin Cell Biol* 2004; **16**: 700–707.
11. Carey RG, Li B, DiCicco-Bloom E. Pituitary adenylate cyclase activating polypeptide anti-mitogenic signaling in cerebral cortical progenitors is regulated by p57Kip2-dependent CDK2 activity. *J Neurosci* 2002; **22**: 1583–1591.
12. Seoane J, Le HV, Shen L, Anderson SA, Massague J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* 2004; **117**: 211–223.
13. Ohnuma S, Philpott A, Wang K, Holt CE, Harris WA. p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. *Cell* 1999; **99**: 499–510.
14. Nguyen L, Besson A, Heng JI, Schuurmans C, Teboul L, Parras C *et al.* p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. *Genes Dev* 2006; **20**: 1511–1524.
15. Kippin TE, Martens DJ, van der Kooy D. p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. *Genes Dev* 2005; **19**: 756–767.
16. Durand B, Gao FB, Raff M. Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation. *EMBO J* 1997; **16**: 306–317.
17. Itoh Y, Masuyama N, Nakayama K, Nakayama KI, Gotoh Y. The cyclin-dependent kinase inhibitors p57 and p27 regulate neuronal migration in the developing mouse neocortex. *J Biol Chem* 2007; **282**: 390–396.
18. Yan Y, Frisen J, Lee MH, Massague J, Barbacid M. Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev* 1997; **11**: 973–983.
19. Gui H, Li S, Matise MP. A cell-autonomous requirement for Cip/Kip cyclin-kinase inhibitors in regulating neuronal cell cycle exit but not differentiation in the developing spinal cord. *Dev Biol* 2007; **301**: 14–26.
20. Joseph B, Wallen-Mackenzie A, Benoit G, Murata T, Joodmardi E, Okret S *et al.* p57(Kip2) cooperates with Nurr1 in developing dopamine cells. *Proc Natl Acad Sci USA* 2003; **100**: 15619–15624.
21. van Lookeren Campagne M, Gill R. Tumor-suppressor p53 is expressed in proliferating and newly formed neurons of the embryonic and postnatal rat brain: comparison with expression of the cell cycle regulators p21Waf1/Cip1, p27Kip1, p57Kip2, p16Ink4a, cyclin G1, and the proto-oncogene Bax. *J Comp Neurol* 1998; **397**: 181–198.
22. Reynaud EG, Leibovitch MP, Tintignac LA, Pelpel K, Guillier M, Leibovitch SA. Stabilization of MyoD by direct binding to p57(Kip2). *J Biol Chem* 2000; **275**: 18767–18776.
23. Watanabe H, Pan ZQ, Schreiber-Agus N, DePinho RA, Hurwitz J, Xiong Y. Suppression of cell transformation by the cyclin-dependent kinase inhibitor p57KIP2 requires binding to proliferating cell nuclear antigen. *Proc Natl Acad Sci USA* 1998; **95**: 1392–1397.
24. Yun K, Fischman S, Johnson J, Hrabe de Angelis M, Weinmaster G, Rubenstein JL. Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. *Development* 2002; **129**: 5029–5040.
25. Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev* 1996; **10**: 3129–3140.
26. Andersson T, Sodersten E, Duckworth JK, Cascante A, Fritz N, Sacchetti P *et al.* CXXC5 is a novel BMP4-regulated modulator of Wnt signaling in neural stem cells. *J Biol Chem* 2009; **284**: 3672–3681.
27. Poitras L, Ghanem N, Hatch G, Ekker M. The proneural determinant MASH1 regulates forebrain Dlx1/2 expression through the I12b intergenic enhancer. *Development* 2007; **134**: 1755–1765.
28. Yokoo T, Toyoshima H, Miura M, Wang Y, Iida KT, Suzuki H *et al.* p57Kip2 regulates actin dynamics by binding and translocating LIM-kinase 1 to the nucleus. *J Biol Chem* 2003; **278**: 52919–52923.
29. Vlachos P, Nyman U, Hajji N, Joseph B. The cell cycle inhibitor p57(Kip2) promotes cell death via the mitochondrial apoptotic pathway. *Cell Death Differ* 2007; **14**: 1497–1507.
30. Hack MA, Sugimori M, Lundberg C, Nakafuku M, Gotz M. Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. *Mol Cell Neurosci* 2004; **25**: 664–678.
31. Shou J, Rim PC, Calof AL. BMPs inhibit neurogenesis by a mechanism involving degradation of a transcription factor. *Nat Neurosci* 1999; **2**: 339–345.
32. Jia J, Lin M, Zhang L, York JP, Zhang P. The notch signaling pathway controls the size of the ocular lens by directly suppressing p57Kip2 expression. *Mol Cell Biol* 2007; **27**: 7236–7247.

33. Riccio O, van Gijn ME, Bezdek AC, Pellegrinet L, van Es JH, Zimber-Strobl U *et al.* Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2. *EMBO Rep* 2008; **9**: 377–383.
34. Park HC, Boyce J, Shin J, Appel B. Oligodendrocyte specification in zebrafish requires notch-regulated cyclin-dependent kinase inhibitor function. *J Neurosci* 2005; **25**: 6836–6844.
35. Kessaris N, Fogarty M, Iannarelli P, Grist M, Wegner M, Richardson WD. Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat Neurosci* 2006; **9**: 173–179.
36. Dugas JC, Ibrahim A, Barres BA. A crucial role for p57(Kip2) in the intracellular timer that controls oligodendrocyte differentiation. *J Neurosci* 2007; **27**: 6185–6196.
37. Heinen A, Kremer D, Gottle P, Kruse F, Hasse B, Lehmann H *et al.* The cyclin-dependent kinase inhibitor p57kip2 is a negative regulator of Schwann cell differentiation and *in vitro* myelination. *Proc Natl Acad Sci USA* 2008; **105**: 8748–8753.
38. Petryniak MA, Potter GB, Rowitch DH, Rubenstein JL. Dlx1 and Dlx2 control neuronal versus oligodendroglial cell fate acquisition in the developing forebrain. *Neuron* 2007; **55**: 417–433.
39. Miller FD, Gauthier AS. Timing is everything: making neurons versus glia in the developing cortex. *Neuron* 2007; **54**: 357–369.

Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)