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Mcl1 regulates the terminal mitosis of neural precursor cells in the mammalian brain through p27^{Kip1}

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SUMMARY

Cortical development requires the precise timing of neural precursor cell (NPC) terminal mitosis. Although cell cycle proteins regulate terminal mitosis, the factors that influence the cell cycle machinery are incompletely understood. Here we show in mice that myeloid cell leukemia 1 (Mcl1), an anti-apoptotic Bcl-2 protein required for the survival of NPCs, also regulates their terminal differentiation through the cell cycle regulator p27^{Kip1}. A BrdU-Ki67 cell profiling assay revealed that *in utero* electroporation of Mcl1 into NPCs in the embryonic neocortex increased NPC cell cycle exit (the leaving fraction). This was further supported by a decrease in proliferating NPCs (Pax6$^+$ radial glial cells and Tbr2$^+$ neural progenitors) and an increase in differentiating cells (Dcx$^+$ neuroblasts and Tbr1$^+$ neurons). Similarly, BrdU birth dating demonstrated that Mcl1 promotes premature NPC terminal mitosis giving rise to neurons of the deeper cortical layers, confirming their earlier birthdate. Changes in Mcl1 expression within NPCs caused concomitant changes in the levels of p27^{Kip1} protein, a key regulator of NPC differentiation. Furthermore, in the absence of p27^{Kip1}, Mcl1 failed to induce NPC cell cycle exit, demonstrating that p27^{Kip1} is required for Mcl1-mediated NPC terminal mitosis. In summary, we have identified a novel physiological role for anti-apoptotic Mcl1 in regulating NPC terminal differentiation.

KEY WORDS: Bcl-2, Mcl-1, Cdkn1b, Neurogenesis, Cell cycle, Mouse

INTRODUCTION

During mammalian nervous system development, neural stem cells give rise to committed progenitor cells that divide sequentially to produce neurons followed by macroglial cells. In the mouse cortex, the neurogenic period extends from embryonic day (E) 11-17 (Angevine and Sidman, 1961). The total number of neural stem cells and progenitor cells, collectively known as neural precursor cells (NPCs), determines the overall number of neurons and ultimately the size of the brain (Joseph and Hermanson, 2010; Mitsuhashi and Takahashi, 2009). As more neurons are generated from committed progenitor cells, the six distinct cortical layers are formed in an inside-out manner (Molyneaux et al., 2007). As a result, the laminar fate of differentiated neurons depends on the timing of their cell cycle exit.

Whether an NPC proliferates or exits the cell cycle is decided during the G1 phase. Cyclin-dependent kinase (CDK) inhibitors (CKIs), and specifically members of the CIP/KIP family p27^{Kip1} (also known as Cdkn1b) and p57^{Kip2} (also known as Cdkn1c), are key regulators of NPC terminal mitosis and differentiation (Caviness et al., 2003; Dyer and Cepko, 2001; Nguyen et al., 2006b; Tury et al., 2011; Ye et al., 2009). Deletion of either *p57^{Kip2}* or *p27^{Kip1}* increases NPC proliferation, expanding the pool of NPCs and resulting in a larger brain (Caviness et al., 2003; Goto et al., 2004; Mairet-Coello et al., 2012; Nguyen et al., 2006a; Tury et al., 2011). By contrast, overexpression of either CKI induces premature NPC terminal mitosis and differentiation, reducing the size of the NPC pool and consequently the size of the brain (Mairet-Coello et al., 2012; Tarui et al., 2005; Tury et al., 2011; Tury et al., 2012). CKI activity is primarily regulated by the cell cycle machinery including CDK/cyclin complexes (Montagnoli et al., 1999), Cdk5 (Zheng et al., 2010) and through the Rb-Skp2 pathway (Ji et al., 2004). However, little is known about regulators of the CKIs that are not part of the cell cycle machinery.

Members of the B-cell lymphoma-2 (Bcl-2) family of proteins, which are key regulators of apoptosis, also affect cell proliferation. Specifically, the anti-apoptotic Bcl-2 protein myeloid cell leukemia 1 (Mcl1) was initially identified from the upregulation of its gene during myeloid leukemia cell differentiation (Kozopas et al., 1993). Although germline deletion of *Mcl1* results in peri-implantation lethality at E3.5, this lethality is attributed to a defect in trophoblast differentiation and not apoptosis, suggesting that Mcl1 has a role in the differentiation process (Rinkenberger et al., 2000). In proliferating cells, Mcl1 protein levels peak in M phase just prior to cell cycle exit (Harley et al., 2010). Overexpression of Mcl1 in cell lines slows progression through the cell cycle, which might be due to its binding to either proliferating cell nuclear antigen (PCNA) (Fujise et al., 2000) and/or the Cdk1-cyclin B1 complex that regulates progression through the G2-M phase transition (Harley et al., 2010; Jamil et al., 2005). However, the precise mechanism by which Mcl1 influences differentiation *in vivo* is unclear.

Mcl1 is best known as a potent survival factor for hematopoietic, hepatocytic and dermal precursor cells (Opferman et al., 2005; Opferman et al., 2003; Sitailo et al., 2009; Vick et al., 2009). In the nervous system, Mcl1 is also required for the survival of embryonic and adult NPCs (Arbour et al., 2008; Malone et al., 2012). Nestin-mediated *Mcl1* conditional knockout mice are embryonic lethal at E16, with extensive apoptosis throughout the developing brain. Apoptosis was observed not only within the nestin$^+$ NPC population but also in doublecortin (Dcx)$^+$ neuroblasts and β-III tubulin$^+$ immature neurons, indicating that Mcl1 is required for survival as NPCs transition from a proliferating precursor cell to a differentiated neuron (Arbour et al., 2008). Furthermore, acute

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targeted deletion of *Mcl1* in adult NPCs *in vivo* resulted in a 3-fold increase in NPC death, demonstrating that Mcl1 is also required for the survival of adult NPCs (Malone et al., 2012). Besides its role as a survival factor for NPCs, whether Mcl1 also has a role in NPC proliferation has yet to be determined.

Here we show that Mcl1 has a unique role in regulating NPC proliferation. *In utero* electroporation of Mcl1 induced premature NPC terminal mitosis and differentiation as demonstrated by a greater 'leaving fraction' and an increase in immature neurons in the cortical plate. Mcl1-mediated changes in NPC proliferation corresponded with changes in the levels of the CKI $p27^{Kip1}$. Furthermore, in $p27^{Kip1}$ loss-of-function assays, the anti-proliferative effect of Mcl1 was abolished. Taken together, these results demonstrate that Mcl1 promotes NPC terminal mitosis and differentiation by regulating $p27^{Kip1}$ activity. Our study is the first to identify a physiological role for Mcl1 in regulating NPC terminal mitosis.

MATERIALS AND METHODS

Mice

Experiments were approved by Memorial University's Animal Care Ethics Committee adhering to the guidelines of the Canadian Council on Animal Care. Floxed *Mcl1* (*Mcl1$^{f/f}$*) (Opferman et al., 2003) mice were crossed with *Nestin-Cre* transgenic mice to generate *Mcl1* conditional knockout (CKO) *Cre$^{+/-}$:Mcl1$^{f/f}$* mice (Arbour et al., 2008) on an FVBN background. Heterozygous (Het; *Cre$^{+/-}$:Mcl1$^{+/f}$*) and wild-type (WT; *Mcl1$^{+/f}$* or *Cre$^{+/-}$*) littermates were used for comparison. *p27^{Kip1}* null mice were purchased from Jackson Laboratories (Fero et al., 1996) and maintained on a C57BL/6 background. Mice were genotyped according to published protocols (Opferman et al., 2003; Arbour et al., 2008; Fero et al., 1996). CD-1 mice were obtained from Charles River Laboratories. The time of plug identification was counted as E0.5. Following euthanasia, brains were dissected, submersion-fixed overnight in 4% paraformaldehyde, and cryoprotected in 22% sucrose prior to freezing.

Plasmids and cell transfection

Expression constructs for Mcl1 (Rinkenberger et al., 2000), mutated Mcl1 (mt Mcl1) (Zhong et al., 2005) and $p27^{Kip1}$ (Addgene, CC#631) (Dyer and Cepko, 2001) were inserted 5′ to the internal ribosome entry sequence (IRES) and enhanced green fluorescent protein (GFP) in the pCIG2 vector (Megason and McMahon, 2002). HEK 293A cells were transfected with Lipofectamine 2000 (Invitrogen), whereas NPCs were transfected using the AMAXA Nucleofection Kit (Lonza, vpg-1004).

Surgical procedures

In utero electroporation was performed on pregnant CD-1 mice at E13 according to published protocols (Langevin et al., 2007; Saito and Nakatsuji, 2001). Embryos were collected at 20 hours, 48 hours and 5 days following *in utero* electroporation, or 2 weeks postnatal. For the bromodeoxyuridine (BrdU)-Ki67 cell cycle profiling assay or BrdU birth dating, pregnant dams received a single intraperitoneal injection (50 μg BrdU/g body weight) at 12 or 24 hours post-electroporation, respectively.

Neural precursor cultures and proliferation assay

The neuroepithelia of E12.5 embryos were dissected and the neurosphere assay was performed as previously described (Reynolds et al., 1992; Tropepe et al., 1999; Vanderluit et al., 2004). Neural precursors were grown under proliferating conditions with the addition of human recombinant fibroblast growth factor 2 (FGF2, F0291, Sigma Aldrich Chemical Company). For the BrdU incorporation assay, cultures received a 1 mM BrdU pulse 2 hours prior to fixation at 24 or 48 hours post-transfection (h.p.t.).

Immunohistochemistry, TUNEL, western blot and flow cytometry

Immunohistochemistry was performed on 14 μm coronal sections of the forebrain with antibodies to Mcl1 (Santa Cruz Biotechnology, SC-819), BrdU (Becton Dickinson, 347580), Ctip2 (Abcam, 18465), Cux1 (Santa Cruz Biotechnology, SC-13024), Dcx (Santa Cruz Biotechnology, SC-8066), Ki67 (BD Biosciences, 550609), Pax6 (Covance, PRB-278P), phospho-histone H3 (PH3; Millipore, 06-570), PCNA (Vector Labs, VP-P980), Tbr1 (Abcam, 31940) and Tbr2 (Abcam, 23345), followed by anti-rabbit IgG or anti-mouse IgG Alexa Fluor 488/594 (Invitrogen).

To identify apoptotic cells, terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) was performed according to published protocols (Simpson et al., 2001).

Western blotting was performed as described (Vanderluit et al., 2004) with antibodies to Mcl1 (Rockland, 600-401-394), $p27^{Kip1}$ (Santa Cruz Biotechnology, SC-528), $p57^{Kip2}$ (Santa Cruz Biotechnology, SC-8298) and actin (Santa Cruz Biotechnology, SC-1616), followed by horseradish peroxidase-tagged anti-mouse IgG or anti-rabbit IgG (Bio-Rad), and developed by chemiluminescence using Western Lightning Plus ECL (PerkinElmer, NEL100) according to the manufacturer's instructions.

Fluorescence-activated cell sorting (FACS) was performed on NPCs at 24 h.p.t. using IntraStain reagents (Dako, K2311), incubated with antibodies to $p27^{Kip1}$ or $p57^{Kip2}$ followed by DyLight 649-conjugated IgG (Jackson ImmunoResearch, 111-496-144). NPCs were sorted on a BD FACSCalibur flow cytometer (Becton Dickinson) and gated to 20,000 GFP^+ cells per sample.

Cell counts

GFP^+ cells were counted within comparable fields (444×332 μm) for embryonic brain sections and within 300 μm bins for 2-week postnatal brain sections. The cortical location of GFP^+ cells was assessed based on Cux1 immunohistochemistry. In *Nestin-Cre Mcl1* mice (WT, Het, CKO), $PH3^+$ cells were counted in 350 μm bins, $BrdU^+$ cells in 250 μm bins and $Tbr2^+$ cells in 150 μm bins along the ventricle and extending throughout the entire depth of the cortex. Apoptotic $TUNEL^+$ cells were counted in 200×200 μm bins. All quantifications were based on a minimum of three representative brain sections, 140 μm apart, per mouse. *In vitro* proliferation was determined by counting the percentage of proliferating cells from at least four random fields per treatment. Cell counts were analyzed by *t*-test or one-way ANOVA followed by Tukey's post-hoc test.

RESULTS

Mcl1 protein is tightly regulated in NPCs

To identify the role of Mcl1 in the developing mouse brain, we developed a system that allows stable Mcl1 overexpression in a physiological context. The mammalian expression plasmid pCIG2 (Megason and McMahon, 2002), which contains an IRES followed by GFP, was used to direct expression of GFP alone as control (Ctl), Mcl1 (Rinkenberger et al., 2000) or a mutated Mcl1 (mt Mcl1), which were cloned upstream of the IRES. The mt Mcl1 construct was included because previous studies showed that Mcl1 protein has a half-life of minutes to hours due to its rapid proteasomal degradation following the ubiquitylation of its lysine residues (Adams and Cooper, 2007; Warr et al., 2005; Zhong et al., 2005). In the mt Mcl1 construct the lysines are converted to arginines, rendering the protein proteasome resistant (Zhong et al., 2005).

To verify that the wild-type Mcl1 and mt Mcl1 constructs could upregulate Mcl1 protein expression, plasmids were transfected into E12.5 NPCs *in vitro*. Western analysis at 24 hours post-transfection (h.p.t.) revealed that both plasmids increased Mcl1 protein expression (supplementary material Fig. S1A). Although endogenous Mcl1 protein levels are low in non-transfected and Ctl-transfected NPCs *in vitro*, we have previously shown by *in situ* hybridization that *Mcl1* mRNA is highly expressed in the ventricular zone (VZ), subventricular zone (SVZ) and cortical plate (CP) at E11 and E15 (Arbour et al., 2008). Mcl1 immunohistochemistry on E14 brain sections revealed variable levels of endogenous Mcl1 protein within the NPC population, with the highest expression in the apical NPCs lining the lateral ventricle (supplementary material Fig. S1B), suggesting that Mcl1 protein is tightly regulated within NPCs.

To determine the effect of Mcl1 overexpression on developmental neurogenesis, we used *in utero* electroporation to direct Mcl1 expression in NPCs during early cortical neurogenesis. We electroporated Ctl, Mcl1 or mt Mcl1 plasmids into NPCs lining the lateral ventricles of mice at E13. At 48 hours post-electroporation (h.p.e.), dams were euthanized, embryos collected and Mcl1 immunohistochemistry performed on coronal sections through the developing telencephalon. In Ctl-transfected brains, Mcl1 protein levels were variable within the NPC population, similar to in non-transfected brains. In brains transfected with the wild-type Mcl1 plasmid, elevated levels of Mcl1 protein were only observed in occasional GFP$^+$ NPCs at 48 h.p.e. (Fig. 1) and at 24 h.p.e. (data not shown). By contrast, in mt Mcl1-transfected brains, the majority of GFP$^+$ NPCs had elevated levels of Mcl1 protein. These results suggest that Mcl1 protein levels are tightly regulated in NPCs during embryonic development and, as a result, stable Mcl1 expression can be achieved with the mt Mcl1 plasmid.

In the developing brain, proliferating NPCs are located within the VZ and SVZ and, upon exiting the cell cycle, migrate through the intermediate zone (IZ) and into the CP where they differentiate into neurons. As a result, the location of a cell along the apical-basal axis provides information on its proliferation status (Dehay and Kennedy, 2007; Götz and Huttner, 2005; Takahashi and Liu, 2006). To determine whether Mcl1 affects the proliferation status of NPCs, Ctl, Mcl1 or mt Mcl1 plasmids were electroporated into E13 embryos. At 48 h.p.e. dams were euthanized and embryos collected, and transfected NPCs were found to be located within the dorsomedial cortex, the dorsolateral cortex, or both (Fig. 2A). Independent of the cortical region, a distinct difference in the location of transfected cells along the apical-basal axis was observed depending on which plasmid was electroporated (Fig. 2B-D; supplementary material Fig. S1C). In Ctl- and Mcl1-transfected brains, the distribution of GFP$^+$ cells was primarily within the proliferative zones of either the VZ (26±2% Ctl; 24±2% Mcl1) or the combined SVZ and IZ (52±2% Ctl; 42±4% Mcl1), with less than a third of GFP$^+$ cells in the postmitotic CP (21±3% Ctl; 34±5% Mcl1) (Fig. 2E). By contrast, a striking shift of cells out of the proliferative regions and into the CP was observed in mt Mcl1-transfected brains, with 58±3% in the CP and only 16±2% of GFP$^+$ cells in the VZ and 26±2% in the combined SVZ and IZ. Since no significant difference was observed in the distribution of wild-type Mcl1-transfected cells and Ctl-treated cells, we attributed this to a failure of the wild-type Mcl1 plasmid to maintain elevated levels of Mcl1 protein in all NPCs (Fig. 1). Therefore, to examine the effect of Mcl1 gain-of-function on NPCs, further experimentation was performed with the mt Mcl1 plasmid, as it provided stable expression of Mcl1.

The increased number of Mcl1-transfected NPCs in the CP might be attributed to the anti-apoptotic function of Mcl1 or to an independent effect on terminal mitosis. To assess the anti-apoptotic role of Mcl1, we examined cell death in the developing cortex. Mcl1 has a known role in promoting the survival of NPCs during cortical development. Deletion of *Mcl1* in *Nestin-Cre Mcl1$^{f/f}$* conditional

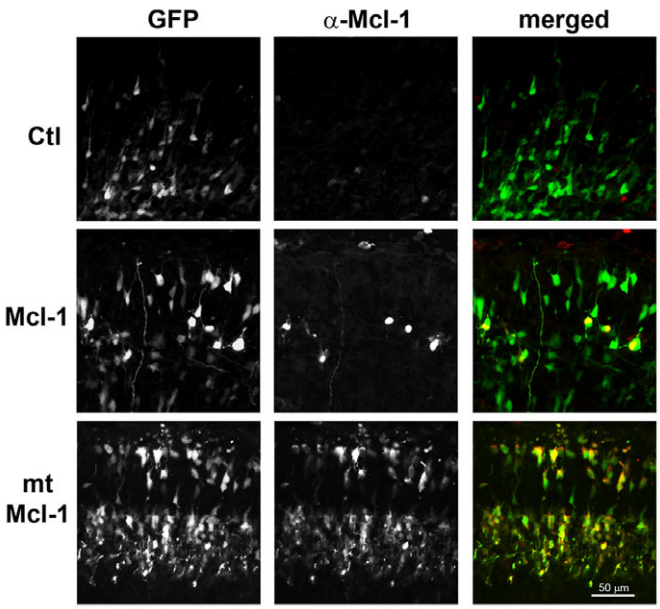

Fig. 1. Mcl1 protein expression is tightly regulated in mouse embryonic NPCs. Mcl1 protein expression 48 hours post-electroporation (h.p.e.) in brains transfected (GFP$^+$) with control (Ctl), Mcl1 or mutated Mcl1 (mt Mcl1) plasmid. In merge: GFP, green; Mcl1, red; both, yellow. Scale bar: 50 μm.

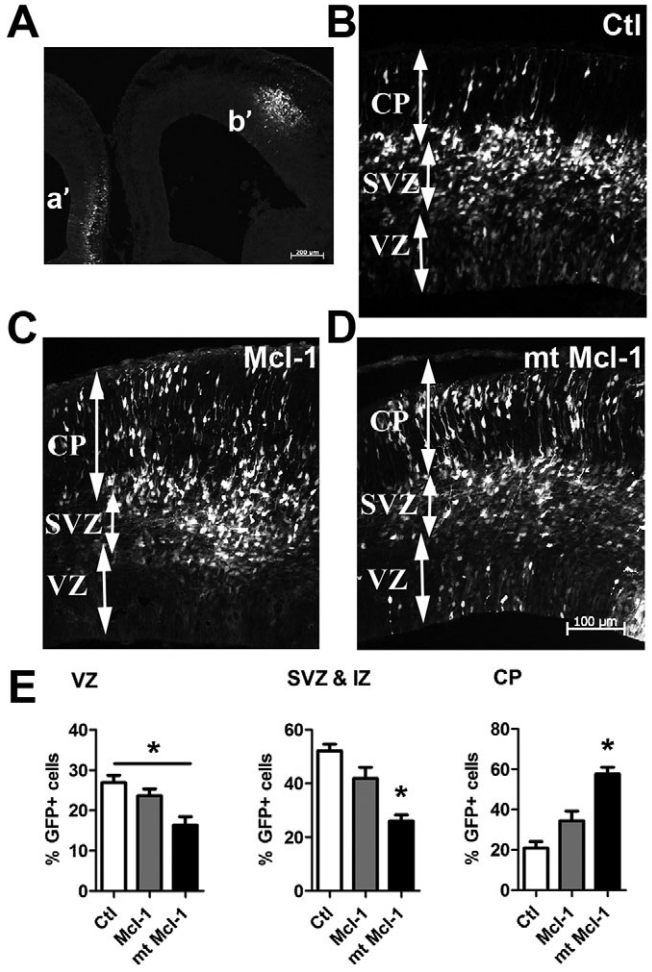

Fig. 2. Mcl1 gain-of-function promotes the migration of NPCs into the cortical plate. (**A**) Transfected (GFP$^+$) NPCs located in the (a′) dorsomedial and the (b′) dorsolateral cortex. Scale bar: 200 μm. (**B-D**) Transfected NPCs in the dorsolateral cortex of (B) Ctl-, (C) Mcl1- and (D) mt Mcl1-electroporated brains at 48 h.p.e. Scale bar: 100 μm. (**E**) Quantification of the percentage of GFP$^+$ cells located in the ventricular zone (VZ), combined subventricular and intermediate zones (SVZ and IZ) and cortical plate (CP). n=5 embryos/treatment. Results are expressed as mean ± s.e.m. One-way analysis of variance (ANOVA) with Tukey's post-hoc test. *P<0.05.

knockout (CKO) embryos results in apoptosis throughout the developing brain (Arbour et al., 2008). To determine the rate of endogenous cell death in wild-type embryos, TUNEL staining was performed on coronal brain sections from six embryos (supplementary material Fig. S1D). The number of apoptotic cells through the cortex from the VZ to the pial surface was quantified and the rate of apoptosis was found to be ~1 apoptotic cell per 64,250 μm^2 (n=6). This low rate of apoptosis is consistent with previous reports (Cai et al., 2002; Haydar et al., 2000; Reznikov and van der Kooy, 1995; Takahashi et al., 1995) and might account for the absence of any double-labeled (GFP$^+$ and TUNEL$^+$) cells in brains transfected with either the Ctl (n=3) or mt Mcl1 (n=3) construct at 24 h.p.e. (supplementary material Fig. S1E,F). Since the rate of endogenous cell death in the cortex is extremely low, it is unlikely that the increased number of cells found in the CP in mt Mcl1-transfected brains at 48 h.p.e. can be explained solely by a rescue from apoptosis. Rather, our data suggest that Mcl1 overexpression might induce NPCs to prematurely differentiate and migrate into the CP.

Does Mcl1 promote NPC differentiation?

To determine whether the shift of Mcl1-transfected NPCs into the CP is due to Mcl1 promoting differentiative cell divisions, we used a BrdU-Ki67 cell cycle profiling assay. We examined embryos at 20 h.p.e. because (1) by 48 h.p.e. a significant percentage of mt Mcl1-transfected NPCs were already in the CP and (2) GFP and transgene expression from the pCIG2 plasmid are observed by 9 h.p.e. (Langevin et al., 2007). Pregnant dams received an intraperitoneal injection of BrdU at 12 h.p.e. and embryos were collected at 20 h.p.e. (8-hour BrdU pulse). Since BrdU is cleared from the body within ~2 hours, the BrdU pulse only labels cells in S phase from 12-14 h.p.e. Immunohistochemistry was performed with antibodies to BrdU and Ki67, which labels cells in all phases of the cell cycle (Gerdes et al., 1984). Quantification of transfected cells positive for BrdU revealed no significant differences in the percentage of Ctl-transfected and mt Mcl1-transfected NPCs in S phase from 12-14 h.p.e. (Table 1). However, by 20 h.p.e., significantly fewer mt Mcl1-transfected NPCs were in the cell cycle than Ctl-transfected NPCs, as demonstrated by the total number of Ki67$^+$ cells. Within the 'cycling fraction', there was a significant reduction in BrdU$^+$ Ki67$^+$ cells but not in BrdU$^-$ Ki67$^+$ cells (Table 1). This might be attributed to NPC cell cycles being asynchronous, such that cells are in different phases at the time of electroporation and Mcl1 levels might not be sufficient to promote cell cycle exit in their first division following electroporation. By contrast, quantification of the 'leaving fraction' (BrdU$^+$ Ki67$^-$), comprising cells in S phase at 12 h.p.e. and having exited the cell cycle by 20 h.p.e., revealed that significantly more mt Mcl1-transfected NPCs had exited the cell cycle by 20 h.p.e. (Table 1). In addition, we found a significant increase in the percentage of transfected NPCs in mt Mcl1 brains that were 'unlabeled' (BrdU$^-$ Ki67$^-$), indicating that these cells were likely to be either beyond or prior to S phase at the time that the BrdU pulse was administered and had already exited the cell cycle by 20 h.p.e. (Table 1). Taken together, these results demonstrate that Mcl1 promotes NPC cell cycle exit.

We next examined whether mt Mcl1-transfected NPCs follow a normal pattern of cortical neuron differentiation following cell cycle exit. Cortical glutamatergic neurogenesis can be tracked through the different stages in development according to transcription factor expression. Pax6$^+$ apical radial glial cells (RGCs) in the VZ give rise to Tbr2$^+$ (Eomes – Mouse Genome Informatics) intermediate progenitor cells (IPCs) that are generated in the VZ and migrate to the SVZ. In the SVZ, IPCs divide to expand their population and subsequently give rise to Dcx$^+$ neuroblasts, which exit the cell cycle and migrate into the CP to differentiate into Tbr1$^+$ glutamatergic neurons (Hevner, 2007). Transcription factor expression can assist in determining the stage of neurogenesis, although co-expression of more than one transcription factor occurs during the transition from one stage to the next (Hevner et al., 2006) (supplementary material Fig. S2A). To determine whether Mcl1 affects differentiation, E13 embryos were electroporated with either Ctl or mt Mcl1 plasmids, embryos collected at 20 h.p.e. and brain sections immunostained with antibodies to Pax6, Tbr2 and Dcx to quantify the number of RGCs, IPCs and neuroblasts, respectively. Overexpression of mt Mcl1 resulted in a reduction in proliferating NPCs, with fewer Pax6$^+$ RGCs and Tbr2$^+$ IPCs, whereas there was an increase in Dcx$^+$ neuroblasts (Table 2; supplementary material Fig. S2B-D). By 48 h.p.e., immunohistochemistry for PCNA, which labels cells in all phases of the cell cycle including early G_0 (Barton and Levine, 2008), revealed a 50% reduction in PCNA$^+$ mt Mcl1-transfected NPCs (Table 2; supplementary material Fig. S3A). By contrast, there was a greater than 50% increase in Tbr1$^+$ cells in mt Mcl1-transfected brains, demonstrating that Mcl1 promotes neurogenesis (Table 2; supplementary material Fig. S3B). Taken together, our data show that, by 48 h.p.e., more than 90% of mt Mcl1-transfected NPCs are PCNA$^-$, indicating that the majority have exited the cell cycle within the first few cell divisions and initiated neurogenesis.

To test whether Mcl1 gain-of-function induces NPC terminal mitosis, we performed a BrdU birth dating experiment. Pregnant dams received a single pulse of BrdU at 24 h.p.e. and embryos were collected at 5 days post-electroporation (d.p.e.) (Fig. 3A). Since BrdU labels cells in S phase, those cells in their last mitotic division (i.e. born) at the time of BrdU administration will retain the BrdU label, whereas proliferating cells will dilute the BrdU with each successive division. At 5 d.p.e., GFP$^+$ cells were located in the CP in both Ctl- and mt Mcl1-transfected brains (Fig. 3B). Quantification of the percentage of double-labeled (BrdU$^+$ GFP$^+$) cells in the CP revealed a 2-fold increase in newly born cells in mt Mcl1-transfected brains (13±2%) versus Ctl-transfected littermates (6±1%) (Fig. 3C,D). Mcl1 gain-of-function therefore promotes premature NPC terminal mitosis and differentiation, and this cohort of early newborn neurons survives up to 5 d.p.e.

Table 1. BrdU-Ki67 cell profiling assay of transfected NPCs

			Cycling fractions		Leaving fraction	Unlabeled non-cycling fraction
Treatment	BrdU$^+$ (%)	Ki67$^+$ (%)	BrdU$^+$ Ki67$^+$ (%)	BrdU$^-$ Ki67$^+$ (%)	BrdU$^+$ Ki67$^-$ (%)	BrdU$^-$ Ki67$^-$ (%)
Ctl	34.9±1.4	73.3±1.0	21.9±0.8	51.4±1.4	13.0±0.7	13.7±1.2
mt Mcl1	31.7±0.8	64.1±1.3**	14.8±1.1**	49.3±0.9	16.9±1.4*	19.0±1.7*

n=5 Ctl, n=5 mt Mcl1; *P<0.05, **P<0.001, versus Ctl.

Table 2. Mcl1 promotes premature differentiation

Time point	Antibody	Ctl (%)	mt Mcl1 (%)
20 h.p.e.	Pax6	69.7±4.3	56.5±1.5*
	Tbr2	43.3±2.0	36.3±1.7*
	Dcx	24.7±2.1	39.5±1.4***
48 h.p.e.	PCNA	21.8±2.9	8.5±1.1**
	Tbr1	17.7±3.2	29.4±3.4*

*P<0.05, **P<0.01, ***P<0.001, versus Ctl.

We next investigated whether the premature birth of mt Mcl1-transfected cells affected their laminar destination in the developing cortex. We electroporated E13 embryos and collected the pups at 2 weeks postnatal, when the cortical layers are fully developed and readily identified with immunohistochemical markers (Hevner, 2007; Hevner et al., 2006). Cells born early in the neurogenic period E11-E13 give rise to neurons of the deeper cortical layers V-VI, whereas cells born later from E14-E17 give rise to neurons in the upper cortical layers II-IV (Caviness et al., 2009; Polleux et al., 1997). E14 therefore marks the transition from generating layer V to layer IV neurons (Takahashi et al., 1999). Since we performed *in utero* electroporation at E13 and NPCs begin expressing GFP within 9 hours (Langevin et al., 2007), the first cohort of NPCs to be born would be late on E13 or in the very early hours of E14 and, therefore, these cells can contribute to the deeper layers.

When we examined the location of transfected cells at 2 weeks postnatal, the majority of cells in Ctl brains appeared to be located in the upper layers of the cortex (Fig. 4A). We confirmed this with immunohistochemistry for the homeobox Cut gene product Cux1. Cux1 is initially expressed in IPCs within the VZ and SVZ, and is retained in their neuronal progeny that are destined primarily for cortical layers II-IV, and in a smaller cohort that resides in the deeper cortical layers (Hevner, 2007; Hevner et al., 2006; Kowalczyk et al., 2009; Leone et al., 2008; Nieto et al., 2004). Using Cux1 as a marker for layers II-IV, we found that the majority of Ctl- and mt Mcl1-transfected cells were within these layers. However, a notable difference in the laminar location of transfected NPCs was observed between Ctl- and mt Mcl1-transfected brains, with a proportion of mt Mcl1-transfected cells scattered throughout the deeper cortical layers (Fig. 4A,B). When we quantified the number of transfected (GFP^+) cells in layers II-IV versus V-VI, we found that in Ctl-transfected mice ~98% of all GFP^+ cells were located within layers II-IV, whereas in littermates transfected with mt Mcl1 82% of GFP^+ cells were in layers II-IV with the remaining 18% in the deeper layers V-VI (Fig. 4C). This population of layer V-VI neurons could be indicative of cells with an earlier birthdate and/or with a defect in migration. To determine whether the cells found in the deeper layers in mt Mcl1-transfected brains exhibited characteristics of layer V-VI neurons, we performed immunohistochemistry with markers for these neurons. A proportion of the GFP^+ neurons located in layers V-VI in mt Mcl1-treated brains were $Tbr1^+$, a marker for neurons in layer VI (Fig. 4D), or $Ctip2^+$ (Bcl11b – Mouse Genome Informatics), a marker for neurons in layer V (Leone et al., 2008) (Fig. 4E). Although we cannot rule out a coincident defect in migration, these data demonstrate that mt Mcl1-transfected cells adopt layer-appropriate fates.

Taken together, these results are consistent with and support our 20 h.p.e., 48 h.p.e. and BrdU birth dating results demonstrating that Mcl1 overexpression prompts cells to prematurely differentiate.

Does Mcl1 regulate NPC proliferation *in vitro*?

Our *in vivo* Mcl1 gain-of-function results demonstrate that Mcl1 induces NPC terminal mitosis and differentiation. However, because the cortical environment in the E13 cortex promotes neurogenesis (McConnell and Kaznowski, 1991), we questioned whether Mcl1 could induce NPCs to differentiate independent of environmental cues. To address this question, we used an *in vitro* assay in which we could manipulate the external environment. To maintain NPCs in a proliferative state, we added fibroblast growth factor 2 (FGF2), a

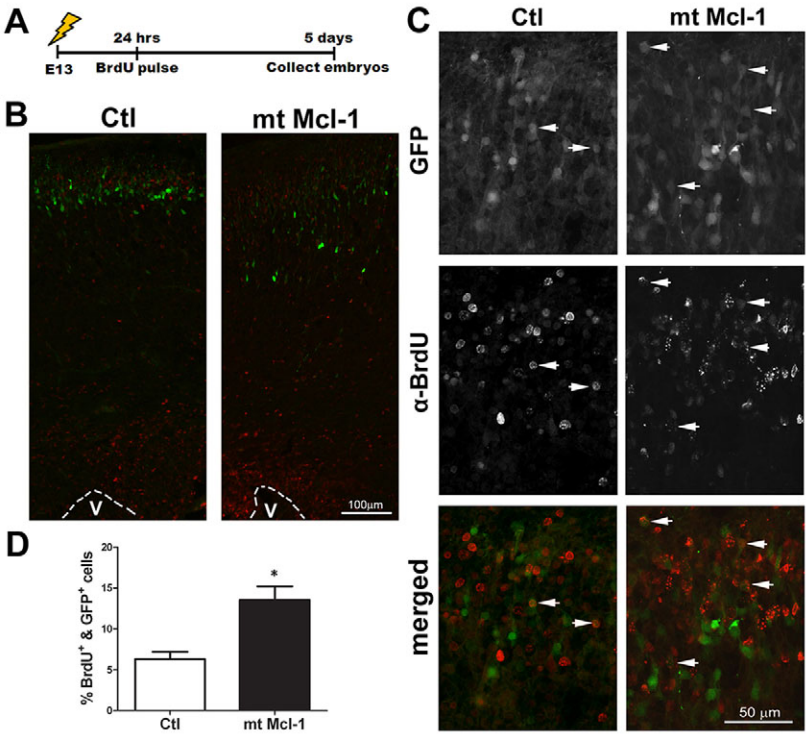

Fig. 3. Mcl1 promotes premature NPC terminal mitosis. (**A**) Timeline of the BrdU birth dating experiment. (**B**,**C**) Low (B) and high (C) magnification photomicrographs of anti-BrdU immunostained sections of mt Mcl1-transfected versus Ctl-transfected brains. Arrows indicate double-labeled [$BrdU^+$ (red) GFP^+ (green)] cells. V, ventricle. Scale bars: 100 μm (B); 50 μm (C). (**D**) Quantification of the percentage of $BrdU^+$ GFP^+ double-labeled cells in the CP. n=10 embryos/treatment. Results are expressed as mean ± s.e.m. Means were analysed by t-test. *P<0.01.

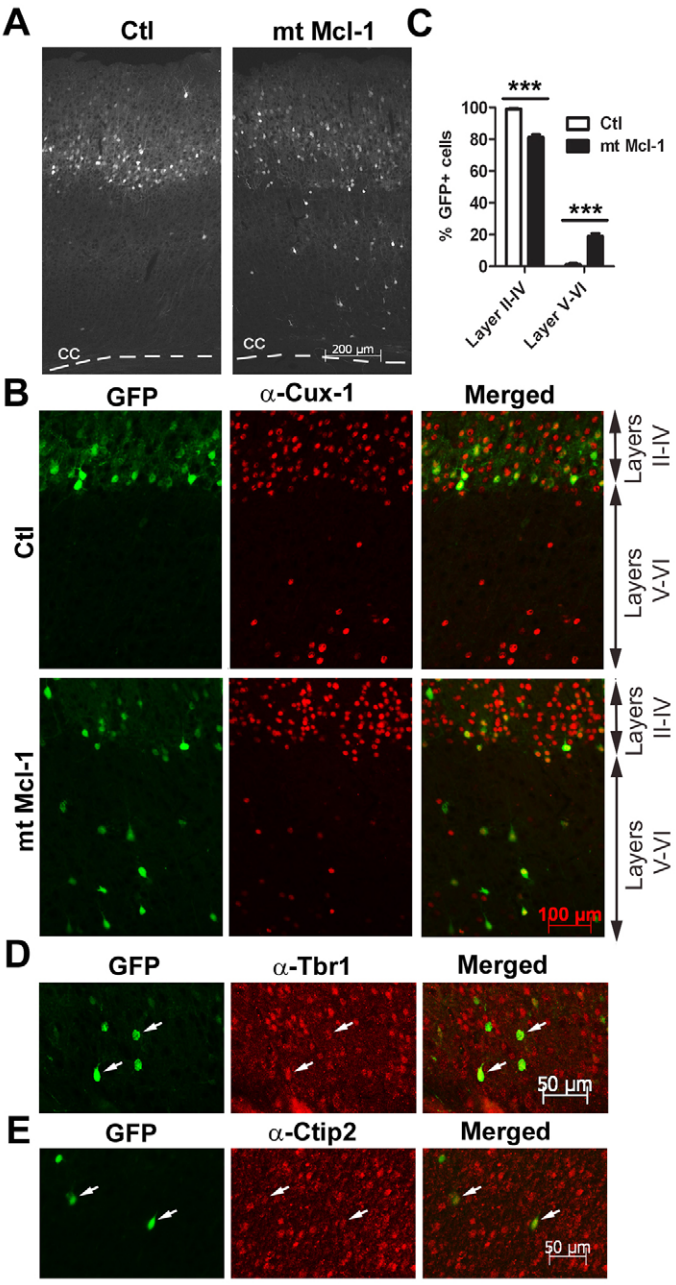

Fig. 4. Mcl1 alters the laminar fate of NPCs in the mouse embryonic brain. (**A**) The location of GFP$^+$ cells in Ctl- and mt Mcl1-electroporated brains from 2-week-old mice. CC, corpus callosum. Scale bar: 200 μm. (**B**) Layer II-IV-specific Cux1 immunohistochemistry. Scale bar: 100 μm. (**C**) Quantification of the percentage of GFP$^+$ cells in layers II-IV and layers V-VI. *n*=10 embryos/treatment. Means were analysed by two-way ANOVA and Bonferroni post hoc. ***P<0.001. Error bars indicate s.e.m. (**D**,**E**) mt Mcl1-transfected brain sections immunostained with antibodies to (D) Tbr1 and (E) Ctip2. Arrows indicate double-labeled cells. Scale bars: 50 μm.

trophic factor that promotes NPC proliferation (Vaccarino et al., 1999), to the media. NPCs were transfected with Ctl or mt Mcl1 plasmids and proliferation was assessed by applying a BrdU pulse 2 hours prior to fixation. Quantification revealed significantly fewer (*n*=4 embryos/treatment/time point, P<0.05) BrdU$^+$ cells at both 24 and 48 h.p.t. in mt Mcl1-transfected cultures (12.9±1.3% and 14.2±2.4%) than in Ctl-transfected cultures (38.0±6.1% and 31.4±1.2%). This reduction in proliferation in mt Mcl1-transfected NPCs demonstrates that Mcl1 gain-of-function reduces NPC proliferation in a cell-autonomous manner.

Does Mcl1 loss-of-function affect NPC proliferation and differentiation?

Nestin-mediated deletion of *Mcl1* in CKO embryos results in complete loss of Mcl1 protein expression in the NPC population and their progeny (supplementary material Fig. S4A). To determine whether Mcl1 loss-of-function affects NPC proliferation, we used an *in vivo* 4-hour BrdU pulse assay followed by immunohistochemistry with antibodies to phospho-histone H3 (PH3), a marker of cells in M phase, and to Tbr2. No significant differences were found in the number of BrdU$^+$ or PH3$^+$ proliferating cells or Tbr2$^+$ IPCs between *Mcl1* wild-type (WT), heterozygous (Het) and CKO embryos (supplementary material Fig. S4B-D).

Because *Mcl1* CKO embryos exhibit higher levels of NPC apoptosis (Arbour et al., 2008), we examined whether a greater dependence on Mcl1 for survival could obscure the effect of Mcl1 loss-of-function on NPC proliferation. We performed TUNEL staining on *Mcl1* WT, Het and CKO brains and quantified the number of apoptotic cells. The majority of apoptotic cells were located within the proliferative regions of the VZ and SVZ, consistent with the location of the NPC population (supplementary material Fig. S4E). There were significantly more TUNEL$^+$ cells in both *Mcl1* Het and CKO embryos than in WT littermate controls (supplementary material Fig. S4F).

We next examined whether NPCs, which have neural stem cell-like characteristics (multipotent and self-renewing), are affected by Mcl1 loss-of-function using the neurosphere assay. *Mcl1* CKO and Het cultures produced significantly fewer primary neurospheres (supplementary material Fig. S4G). Neurospheres from all three genotypes were multipotent and had the capacity for self-renewal (data not shown); however, in both *Mcl1* CKO and Het cultures, higher rates of cell death were observed (supplementary material Fig. S4H).

Taken together, our results suggest that the strong requirement for Mcl1 as a survival factor for NPCs might conceal its effect on proliferation.

Do changes in Mcl1 expression affect p27^{Kip1}?

Since our *in vivo* and *in vitro* results demonstrate that Mcl1 regulates NPC terminal mitosis, we investigated the molecular mechanism through which this occurs. In the neocortex, the CKIs p27^{Kip1} and p57^{Kip2} play fundamental roles in NPC terminal mitosis and differentiation (Caviness et al., 2003; Dyer and Cepko, 2001; Nguyen et al., 2006a; Nguyen et al., 2006b; Tury et al., 2011; Ye et al., 2009). p57^{Kip2} is highly expressed in Pax6$^+$ RGCs and regulates the terminal mitosis and differentiation of early born neurons, which form the deeper cortical layers V-VI (Mairet-Coello et al., 2012; Tury et al., 2011; Tury et al., 2012; Ye et al., 2009). By contrast, p27^{Kip1} is more highly expressed in IPCs and regulates the terminal mitosis and differentiation of neurons primarily destined for cortical layers II-V (Goto et al., 2004; Mairet-Coello et al., 2012; Mitsuhashi et al., 2001; Tarui et al., 2005). Since gain-of-function of either p57^{Kip2} or p27^{Kip1} results in premature NPC terminal mitosis, we questioned whether the expression of either CKI was affected by changes in Mcl1 expression.

Overexpression of mt Mcl1 had no effect on p57^{Kip2} protein levels in NPCs (Fig. 5A). Similarly, neither overexpression of wild-type Mcl1 nor mt Mcl1 had an effect on the number of cells

expressing p57^{Kip2} compared with Ctl-transfected NPCs as determined by FACS (Fig. 5B). By contrast, overexpression of mt Mcl1 resulted in a greater than 3-fold increase in p27^{Kip1} protein levels (Fig. 5C). In addition, overexpression of either wild-type Mcl1 or mt Mcl1 resulted in a significant increase in the number of NPCs expressing p27^{Kip1}, indicating that this is a direct result of Mcl1 gain-of-function and is not due to non-specific effects of the mt Mcl1 construct (Fig. 5D; supplementary material Fig. S5). Conversely, we found a greater than 50% decrease in p27^{Kip1} expression in NPCs from *Mcl1* CKO embryos compared with control littermates (Fig. 5E). Taken together, these results demonstrate that changes in Mcl1 expression specifically affect the levels of p27^{Kip1} protein in NPCs.

Since Tarui et al. (Tarui et al., 2005) have shown that p27^{Kip1} overexpression promotes premature NPC differentiation, we questioned whether the effect of p27^{Kip1} on NPC proliferation is comparable to that of Mcl1. The p27^{Kip1} construct (Dyer and Cepko, 2001) was cloned into the pCIG2 plasmid and tested for protein expression in HEK 293A cells (supplementary material Fig. S6A). To determine whether p27^{Kip1} promotes NPC differentiation in a similar fashion to Mcl1, we electroporated p27^{Kip1} or GFP (Ctl) plasmids into E13 embryos and examined the location of transfected NPCs at 48 h.p.e. In Ctl-transfected brains, cells were primarily located in the proliferative zones in the VZ (33±4%) and in the combined SVZ and IZ (44±4%), with less than a third (24±4%) in the CP (supplementary material Fig. S6B). By contrast, in p27^{Kip1}-transfected brains, the majority of cells (52±3%) were located in the CP, with 18±1% in the VZ and 30±2% in the combined SVZ and IZ.

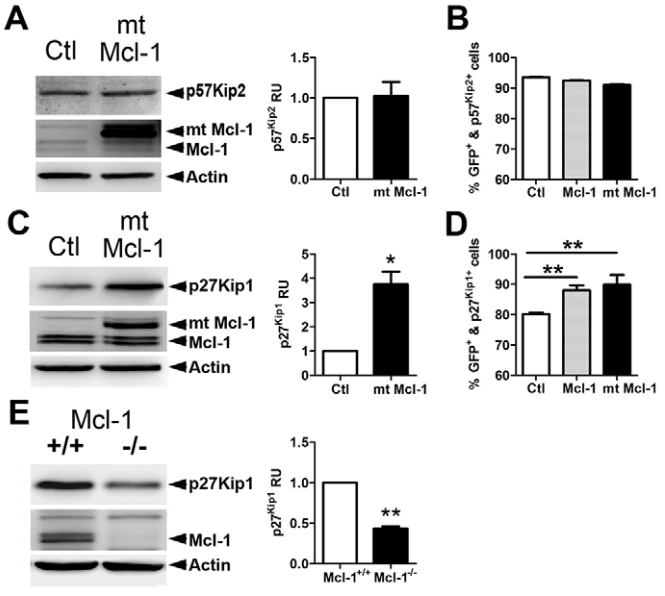

Fig. 5. Mcl1 upregulates p27^{Kip1} in NPCs. (**A**) Western analysis of NPCs transfected with Ctl or mt Mcl1 plasmids revealed no significant difference in p57^{Kip2} protein levels. (**B**) FACS analysis of p57^{Kip2} protein from Ctl-, Mcl1- and mt Mcl1-transfected NPCs. *n*=3/treatment. (**C**) Western analysis revealed a greater than threefold increase in p27^{Kip1} protein expression in mt Mcl1-transfected NPCs. (**D**) FACS analysis of p27^{Kip1} protein from Ctl-, Mcl1- and mt Mcl1-transfected NPCs. *n*=3/treatment. (**E**) Western analysis revealed a 50% reduction in p27^{Kip1} protein expression in NPCs from *Mcl1* CKO (–/–) embryos compared with control littermates. Blots are representative of three separate experiments. Actin provides a loading control. Means were analysed by *t*-test or one-way ANOVA and Tukey's post-hoc test. *P<0.05, **P<0.01. Error bars indicate s.e.m.

This is similar to the shift we observed in mt Mcl1-electroporated brains (Fig. 2). Furthermore, Tbr1 immunohistochemistry revealed a 2-fold increase in Tbr1^{+} cells in p27^{Kip1}-transfected (26±4%) versus littermate Ctl-transfected (11±4%) embryos at 48 h.p.e. (supplementary material Fig. S6C). These results are consistent with previous studies showing that p27^{Kip1} gain-of-function promotes premature neuronal differentiation (Nguyen et al., 2006a; Tarui et al., 2005) and are comparable to our Mcl1 gain-of-function results (Fig. 2, Table 2).

To determine whether Mcl1 and p27^{Kip1} affect NPC proliferation in a comparable manner, we used our *in vitro* proliferation assay. As previously, NPCs were transfected with Ctl, mt Mcl1 or p27^{Kip1} plasmids and cultured under proliferating conditions by adding FGF2 to the culture media. PCNA immunocytochemistry was performed to quantify the number of proliferating cells; because PCNA is expressed in all phases of the cell cycle, we could identify a greater percentage of the proliferating NPC population. A significant reduction in proliferating cells in both mt Mcl1- and p27^{Kip1}-transfected cultures was found at each time point studied (Fig. 6). No significant differences were observed between the mt Mcl1- and p27^{Kip1}-treated cultures. This shows that both Mcl1 and p27^{Kip1} gain-of-function negatively regulate NPC proliferation in the same temporal pattern.

Mcl1 promotes NPC terminal mitosis through p27^{Kip1} activity

Since both *in vivo* and *in vitro* results demonstrate that Mcl1 and p27^{Kip1} promote NPC terminal mitosis and that changes in Mcl1 protein affect p27^{Kip1} protein levels, we examined whether p27^{Kip1} is required for Mcl1-mediated NPC cell cycle exit. We cultured NPCs from E13 *p27^{Kip1}* null (*p27$^{Kip1-/-}$*), heterozygous (*p27$^{Kip1+/-}$*) and wild-type (WT) littermates, then transfected them with Ctl or mt Mcl1 plasmids. As before, to maintain NPCs in a proliferative state we added FGF2 to the culture media. BrdU was added to the cultures 2 hours prior to fixation to label proliferating cells.

Overexpression of mt Mcl1 in NPC cultures from WT embryos resulted in a significant reduction in BrdU^{+} cells at both 24 and 48 h.p.t. (Fig. 7A,B). In both *p27$^{Kip1-/-}$* and *p27$^{Kip1+/-}$* cultures, NPCs had an enhanced proliferative rate, consistent with previous studies (Goto et al., 2004; Mitsuhashi et al., 2001; Tarui et al., 2005). Overexpression of mt Mcl1 in *p27$^{Kip1+/-}$* cultures significantly

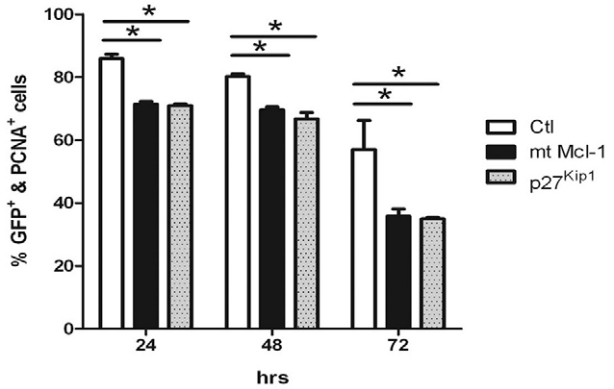

Fig. 6. p27^{Kip1} reduces NPC proliferation *in vitro* similar to Mcl1. E12.5 NPCs were transfected with Ctl, mt Mcl1 or p27^{Kip1} plasmids and cultured in proliferating conditions. Quantification of the percentage of proliferating (PCNA^{+} GFP^{+}) cells at 24, 48 and 72 hours post-transfection (h.p.t.). *n*=3 embryos/treatment. Means were analysed by two-way ANOVA and Bonferroni post-hoc test. *P<0.05. Error bars indicate s.e.m.

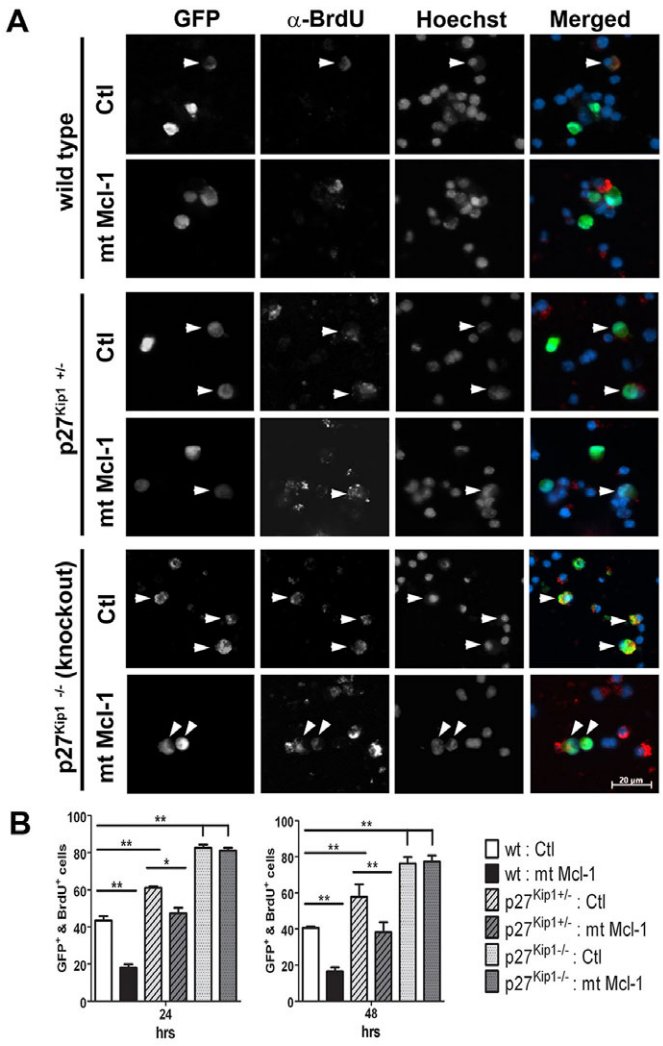

Fig. 7. Mcl1 promotes NPC cell cycle exit through p27^{Kip1} activity. (**A**) GFP$^+$ and BrdU$^+$ cells at 48 h.p.t. of Ctl and mt Mcl1 plasmids into E13 NPCs from wild-type, *p27$^{Kip1+/-}$* and *p27$^{Kip1-/-}$* mouse embryos. Arrowheads indicate double-labeled cells. In merge: GFP, green; BrdU, red; Hoechst, blue. Scale bar: 20 μm. (**B**) Quantification of the percentage of double-labeled cells at 24 and 48 h.p.t. n=5 wild type (wt), n=3 *p27$^{Kip1+/-}$*, n=4 *p27$^{Kip1-/-}$*. Means were analysed by two-way ANOVA and Bonferroni post-hoc test. *P<0.05, **P<0.01. Error bars indicate s.e.m.

reduced proliferation at both 24 and 48 h.p.t. compared with Ctl-transfected *p27$^{Kip1+/-}$* cultures. By contrast, overexpression of mt Mcl1 in *p27$^{Kip1-/-}$* cultures had no effect on NPC proliferation at either 24 or 48 h.p.t. Despite an increased rate of proliferation in both *p27$^{Kip1-/-}$* and *p27$^{Kip1+/-}$* cultures, Mcl1 was capable of negatively regulating NPC proliferation only in cultures containing p27^{Kip1}. These results demonstrate that p27^{Kip1} is required for Mcl1-mediated NPC terminal mitosis.

DISCUSSION

In this study we investigated the role of Mcl1 during neurogenesis in the developing mammalian brain. We show that besides its pro-survival role in NPCs, Mcl1 also promotes NPC terminal mitosis and differentiation. Furthermore, we demonstrate that the mechanism by which Mcl1 promotes terminal differentiation is by regulating p27^{Kip1} expression. Taken together, our results show that Mcl1 performs two distinct functions during mammalian neurogenesis by promoting both NPC survival and terminal differentiation.

Mcl1 induces NPC differentiative cell division

Brain size is determined by the number of proliferating NPCs that divide and differentiate to form the neurons and macroglial cells of the brain. The size of the NPC pool is regulated by proliferative divisions that expand the pool and differentiative divisions that reduce the pool. Our data show that overexpression of Mcl1 results in an increase in differentiative divisions at the expense of proliferative divisions. Our BrdU-Ki67 cell cycle profiling assay revealed an increase in cells that had exited the cell cycle (the leaving fraction) by 20 h.p.e. and a corresponding decrease in proliferating cells. Consistent with these results, we observed a reduction in Pax6$^+$ RGCs and Tbr2$^+$ IPCs and a concomitant increase in Dcx$^+$ neuroblasts in Mcl1-transfected brains. Since the cell cycle duration at E13-E14 is ~15 hours (Takahashi et al., 1993), the initial transfected RGC and IPC populations would have divided only once by 20 h.p.e. and by producing more Dcx$^+$ neuroblasts have favored terminal differentiative divisions rather than proliferative divisions. BrdU birth dating data further support this, with greater numbers of neurons born at 24 h.p.e. in mt Mcl1-transfected brains. By 48 h.p.e., only 8% of mt Mcl1-transfected NPCs were still in the cell cycle and PCNA$^+$. Therefore, our data suggest that it is the phase of the cell cycle during which *Mcl1* transgene expression begins that determines whether cells exit the cell cycle in the current or in the subsequent cycle. Collectively, our results demonstrate that Mcl1 functions to promote NPC terminal mitosis and differentiation in a cell-autonomous manner.

Mcl1 promotes premature cell cycle exit through p27^{Kip1}

We show that Mcl1 promotes terminal mitosis by regulating the levels of p27^{Kip1} protein. Overexpression of wild-type Mcl1 or mt Mcl1 increased p27^{Kip1} in NPCs, whereas decreased levels of p27^{Kip1} were observed in *Mcl1* CKO NPCs, demonstrating that changes in Mcl1 protein result in concomitant changes in p27^{Kip1} protein. Consistent with previous studies, overexpression of p27^{Kip1} resulted in premature NPC terminal mitosis and differentiation both *in vitro* and *in vivo*. In addition, we found that Mcl1 overexpression promoted NPC terminal mitosis and differentiation in a similar temporal pattern as p27^{Kip1} overexpression (Fig. 2B-E; Fig. 6; supplementary material Fig. S6B). Finally, we show that p27^{Kip1} is required for Mcl1-mediated NPC terminal mitosis.

P27^{Kip1} is a key regulator of terminal mitosis in NPCs. Conditional deletion of *p27^{Kip1}* in NPCs in the developing brain causes hyperplasia as a result of NPCs undergoing additional rounds of proliferation (Caviness et al., 2003; Goto et al., 2004; Nguyen et al., 2006a). By contrast, inducible overexpression of p27^{Kip1} in NPCs during neurogenesis results in premature terminal mitosis and differentiation (Nguyen et al., 2006a; Tarui et al., 2005). The accumulation of p27^{Kip1} protein with each round of division of oligodendrocyte precursor cells has been suggested to act as a crucial component of the cell-intrinsic timing mechanism for terminal mitosis (Casaccia-Bonnefil et al., 1997; Durand et al., 1998). Similarly, elevated levels of p27^{Kip1} in NPCs during G1 increases the probability of cell cycle exit and thereby the 'leaving fraction' (Mitsuhashi et al., 2001; Mitsuhashi and Takahashi, 2009; Tarui et al., 2005). Since p27^{Kip1} is a key regulator of terminal mitosis in NPCs and our data show that (1) changes in Mcl1 protein levels within the cell cause concomitant changes in p27^{Kip1} protein

and (2) an increase in Mcl1 results in premature NPC terminal mitosis, Mcl1 may act as a regulator of the intrinsic timing mechanism in NPCs. Endogenous Mcl1 protein is known to fluctuate during the cell cycle, specifically peaking during M phase (Harley et al., 2010); this increase in Mcl1 protein might stabilize $p27^{Kip1}$ protein, leading to an accumulation of $p27^{Kip1}$ in G1 and, subsequently, NPC terminal mitosis.

$P27^{Kip1}$ is a labile protein that is regulated by phosphorylation, which either stabilizes the protein or targets it for proteasomal destruction. Phosphorylation of $p27^{Kip1}$ by G1 cyclin/CDK complexes results in its subsequent ubiquitylation and proteasomal degradation (Montagnoli et al., 1999; Vlach et al., 1997). By contrast, phosphorylation by the atypical kinase Cdk5 stabilizes $p27^{Kip1}$ protein (Kawauchi et al., 2006). In the nervous system, the increase in $p27^{Kip1}$ resulting from Cdk5-mediated phosphorylation in postmitotic neurons prevents their re-entry into the cell cycle (Cicero and Herrup, 2005) and in NPCs promotes cell cycle exit and differentiation (Zheng et al., 2010). Whether Mcl1 directly or indirectly regulates $p27^{Kip1}$ protein levels remains to be determined. However, a link between the anti-apoptotic Bcl-2 family members Bcl2 and Bcl-xL (Bcl2l1 – Mouse Genome Informatics) with $p27^{Kip1}$ has been reported in quiescent cells. Forced expression of Bcl2 or Bcl-xL in quiescent fibroblasts or HL60 promyelocytic leukemia cells inhibited cell cycle re-entry through upregulation of $p27^{Kip1}$ (Greider et al., 2002; Vairo et al., 2000). In the nervous system, Bcl2 and Bcl-xL are expressed in postmitotic neurons, where they play crucial anti-apoptotic roles (Krajewski et al., 1997). However, a physiological role for either in preventing neurons from re-entering the cell cycle has yet to be demonstrated *in vivo*. By contrast, in this study we show that, besides its anti-apoptotic role in NPCs during brain development, Mcl1 promotes terminal mitosis through a mechanism that requires $p27^{Kip1}$ activity.

In summary, we have shown that Mcl1 has dual anti-apoptotic and pro-differentiation roles during neuronal differentiation. Mcl1 induces premature NPC terminal mitosis and differentiation and this is mediated through the CKI $p27^{Kip1}$. As with its pro-survival role, Mcl1 regulates NPC terminal mitosis in a cell-autonomous manner. These results provide new insight into the physiological role of the interaction of Bcl-2 proteins with the cell cycle machinery in NPCs and their impact on neurogenesis and mammalian brain development.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
S.M.M.H., A.D.S., A.M.P., J.X., M.F. and K.D. performed experiments. L.M.L., C.S. and J.L.V. assisted with *in utero* electroporation experiments. J.T.O. provided Mcl1f/f mice. C.S. and J.T.O. provided critical input. S.M.M.H. and J.L.V. designed the experiments and wrote the paper.

Supplementary material
Supplementary material available online at
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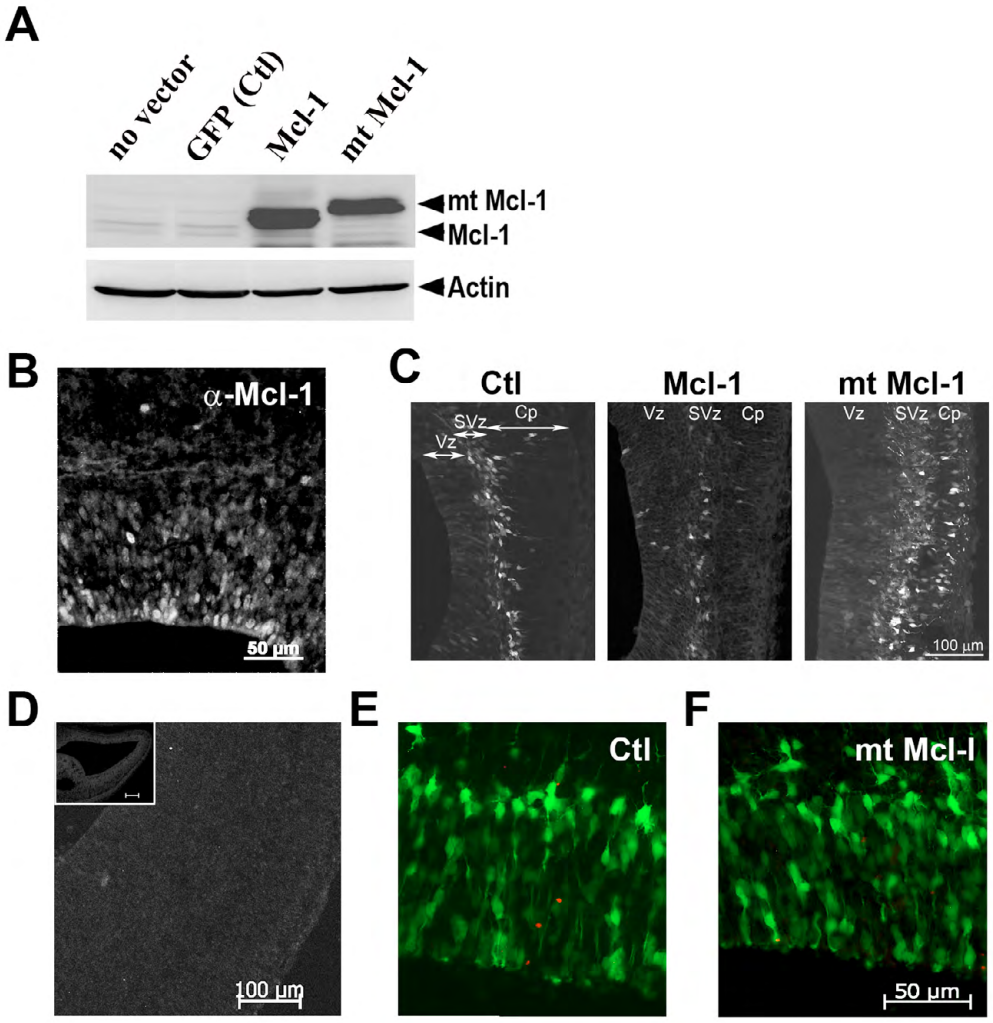

Fig. S1. Mcl1 is expressed in embryonic NPCs during cortical neurogenesis. (**A**) Mcl1 protein expression in NPCs 24 hours following *in vitro* transfection with Ctl, Mcl1 and mt Mcl1 constructs. Non-transfected cell lysates (no vector) were also loaded as controls. (**B**) Endogenous Mcl1 expression in an E14 mouse brain. (**C**) Representative high-magnification photomicrographs of transfected cells in the dorsomedial cortex in Ctl-, Mcl1- and mt Mcl1-electroporated brains. (**D**) TUNEL staining of an E14 non-transfected brain. Inset shows low magnification of the cortical hemisphere. (**E**,**F**) TUNEL staining of (E) Ctl- and (F) mt Mcl1-transfected brains at 24 h.p.e. Green, GFP^+ cells; red, $TUNEL^+$ cells.

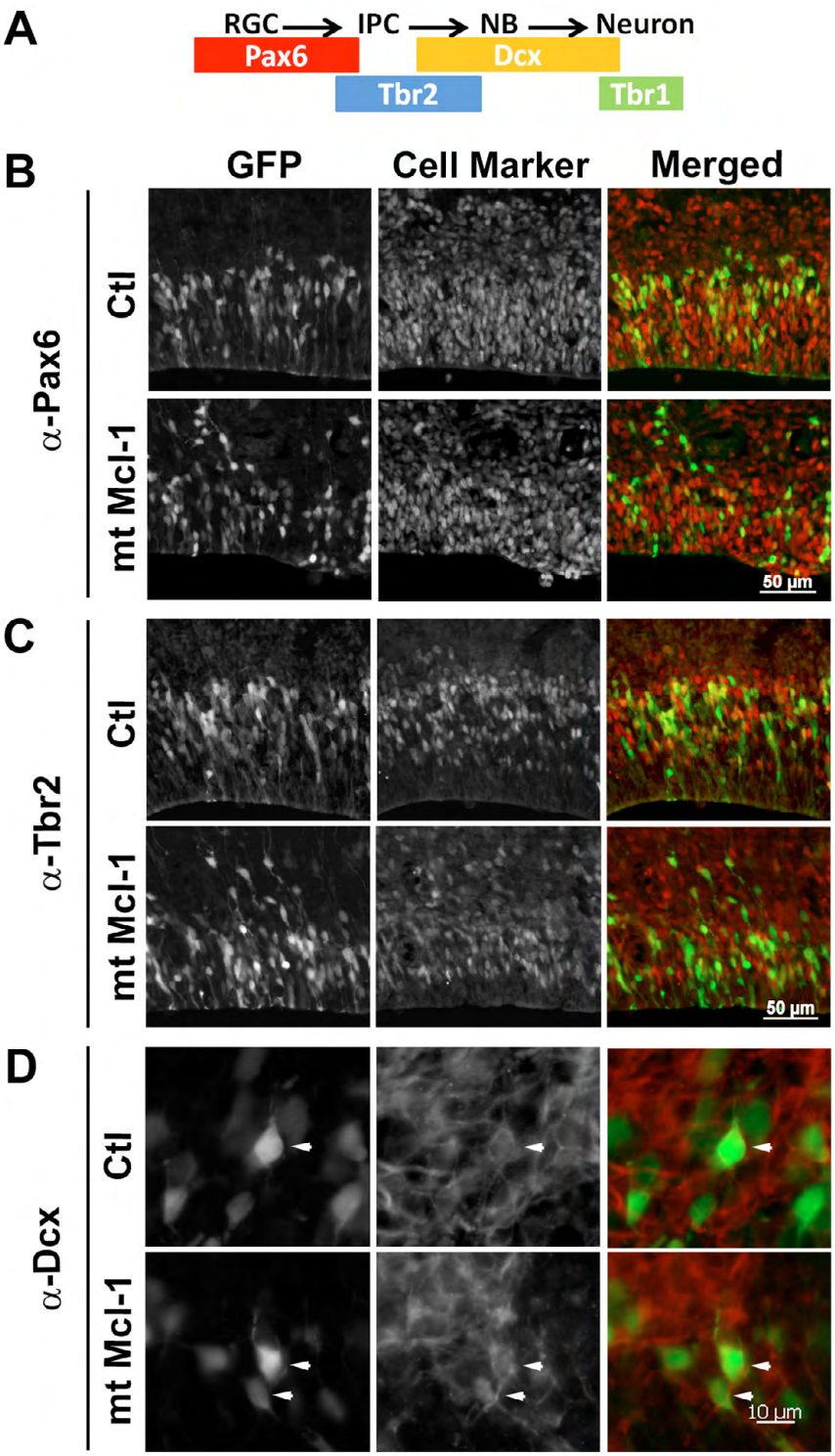

Fig. S2. Mcl1 promotes premature NPC differentiation in the embryonic brain. (**A**) Schematic showing the overlap in transcription factor expression during the transition from radial glial cell (RGC) to intermediate progenitor cell (IPC) to neuroblast (NB) to neuron (N). (**B-D**) E13 mouse embryos were *in utero* electroporated and collected at 20 h.p.e. and brain sections immunostained with antibodies to (B) Pax6, (C) Tbr2 and (D) Dcx. Note that higher magnification was used to observe GFP^+ Dcx^+ double-labeled cells. Green, GFP^+ cells; red, $Pax6^+$, $Tbr2^+$ or Dcx^+ cells.

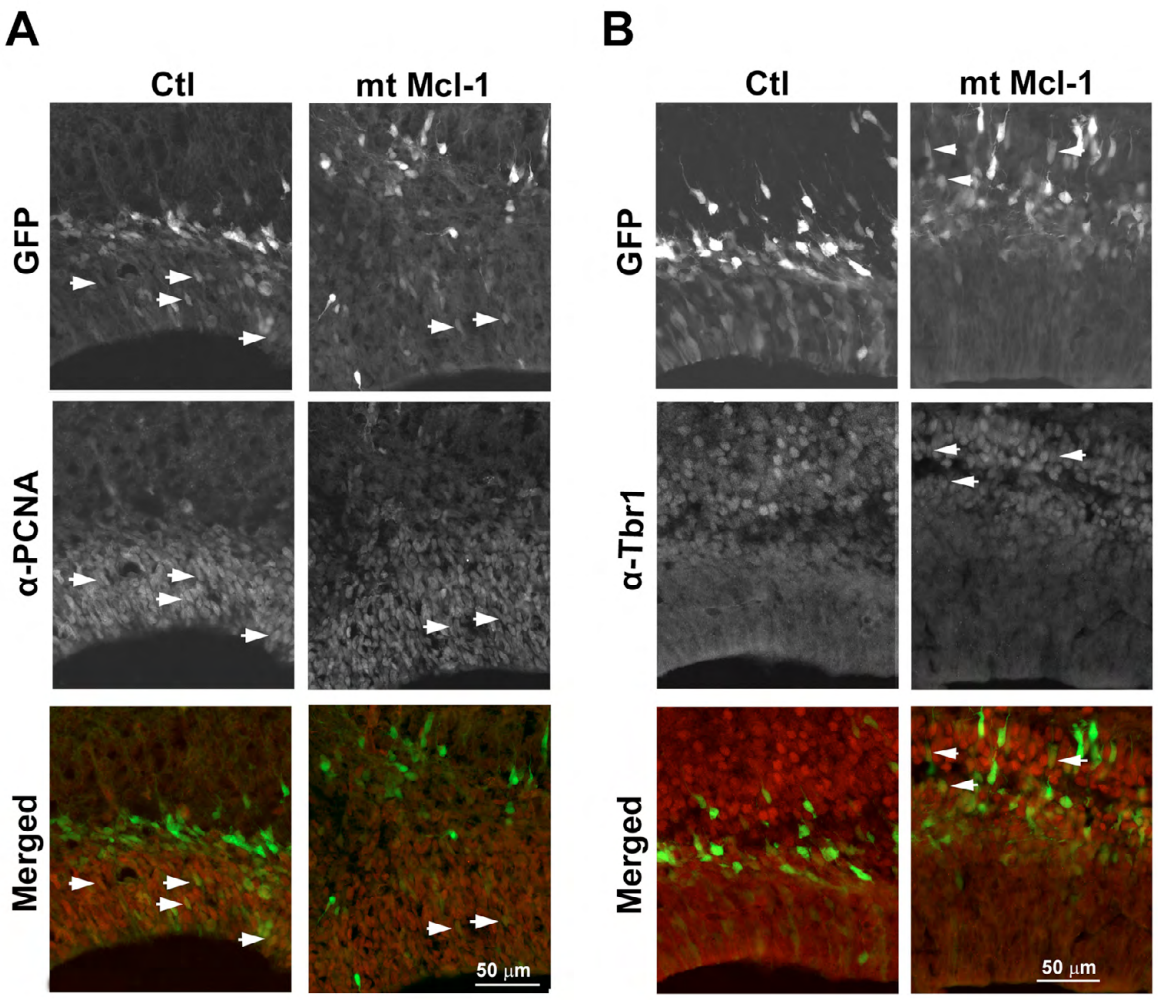

Fig. S3. Mcl1 promotes premature NPC terminal mitosis in the embryonic brain. E13 mouse embryos were *in utero* electroporated and collected at 48 h.p.e. and immunostained with antibodies to (**A**) Pcna and (**B**) Tbr1. Green, GFP^+ cells; red, $PCNA^+$ or $Tbr1^+$ cells.

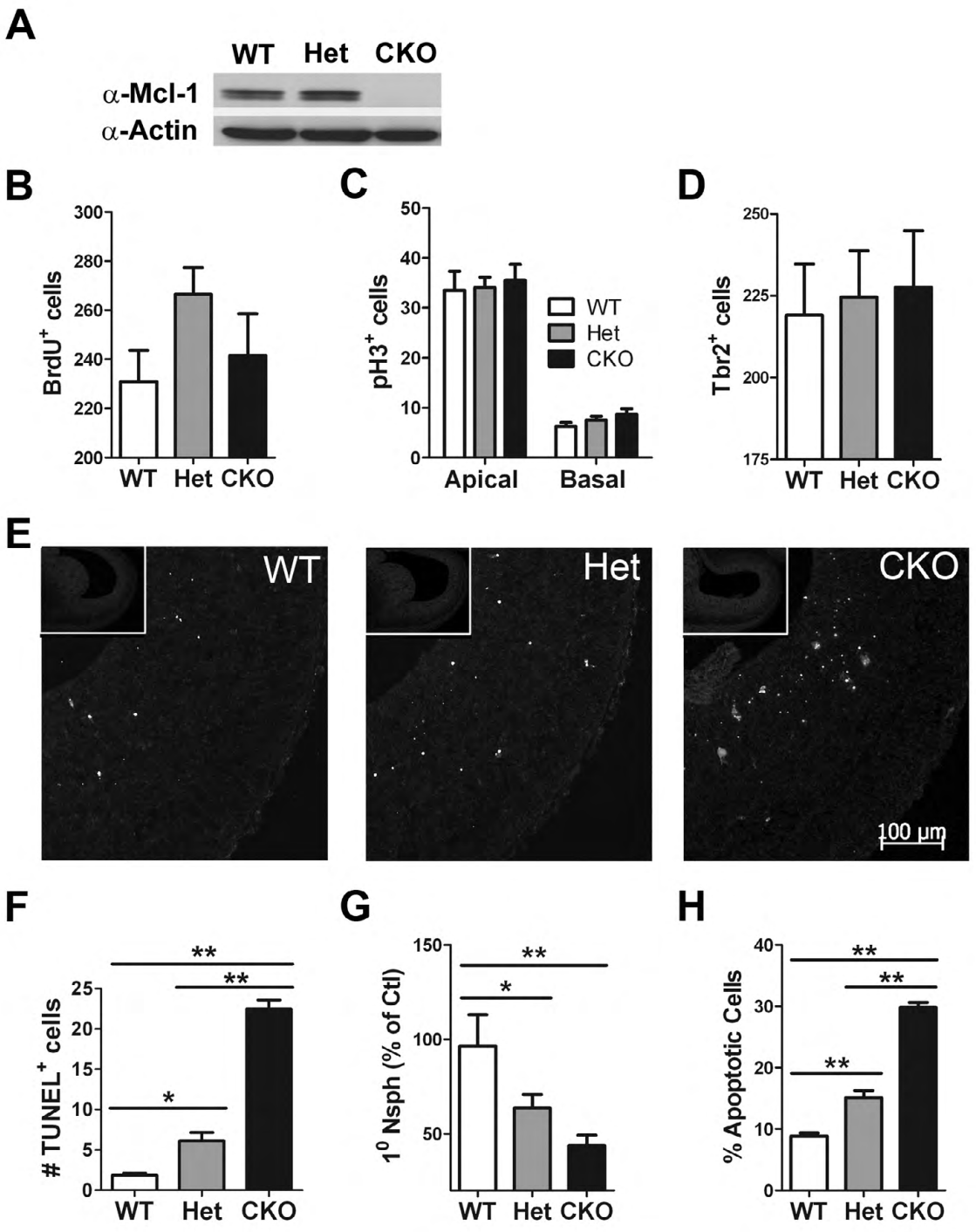

Fig. S4. Mcl1 loss-of-function *in vivo* results in apoptosis. (**A**) Mcl1 protein expression in NPCs from Mcl1 wild-type (WT), heterozygous (Het) and conditional knockout (CKO) embryos. Actin was used as a loading control. (**B**) Quantification of $BrdU^+$ cells following a 4-hour BrdU pulse in Mcl1 WT, Het and CKO embryos at E14 (*n*=4 embryos/genotype). (**C**,**D**) Quantification of (C) $PH3^+$ cells, a marker of cells in M phase, and of (D) $Tbr2^+$ cells in the cortices of E14 Mcl1 WT, Het and CKO littermates. (*n*=5 embryos/genotype). (**E**) TUNEL staining of E14 Mcl1 WT, Het and CKO embryos. Cortical hemispheres are shown at low magnification in insets. (**F**) Quantification of TUNEL-positive cells revealed significantly higher levels of apoptosis in Mcl1 CKO and Het embryos. (**G**) The neurosphere assay was performed with NPCs cultured from E12 Mcl1 WT, Het and CKO NPCs and after 7 days *in vitro* the number of primary neurospheres was quantified. *n*=13 WT, *n*=23 Het, *n*=24 CKO. (**H**) Quantification of apoptotic cells in E12 Mcl1 WT, Het and CKO NPC cultures after 3 days *in vitro*. *n*=5 embryos/genotype. Means were analysed by one-way ANOVA and Tukey's post hoc test (B,D,F-H) or two-way ANOVA (C). **P*<0.05, ***P*<0.01. Error bars indicate s.e.m.

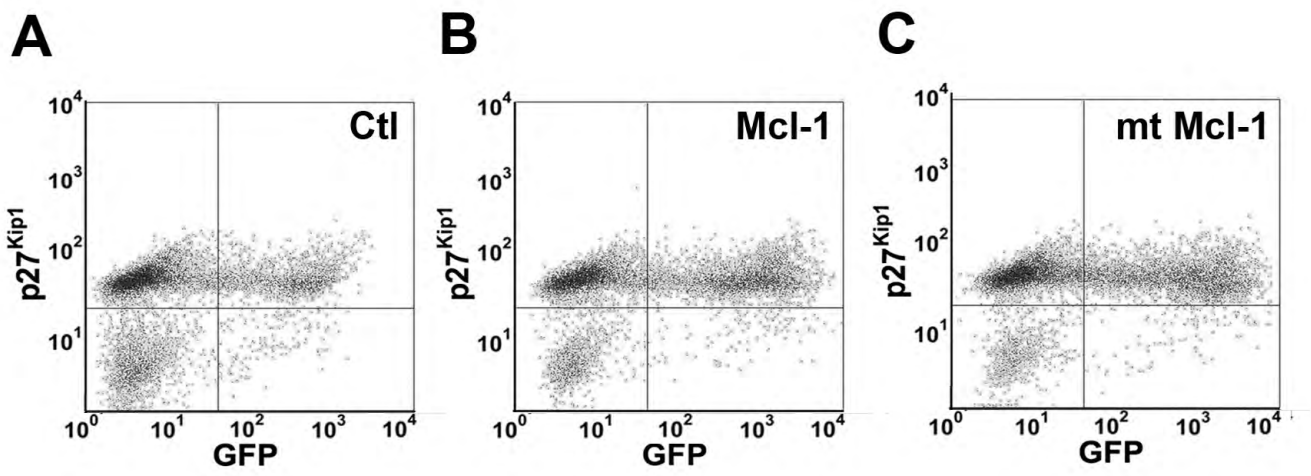

Fig. S5. Mcl1 upregulates p27^{Kip1} in NPCs. FACS analysis was performed 24 h.p.t. on NPCs transfected with (**A**) Ctl, (**B**) Mcl1 and (**C**) mt Mcl1 plasmids. Analysis was performed on 20,000 gated GFP^{+} cells. *n*=3/treatment.

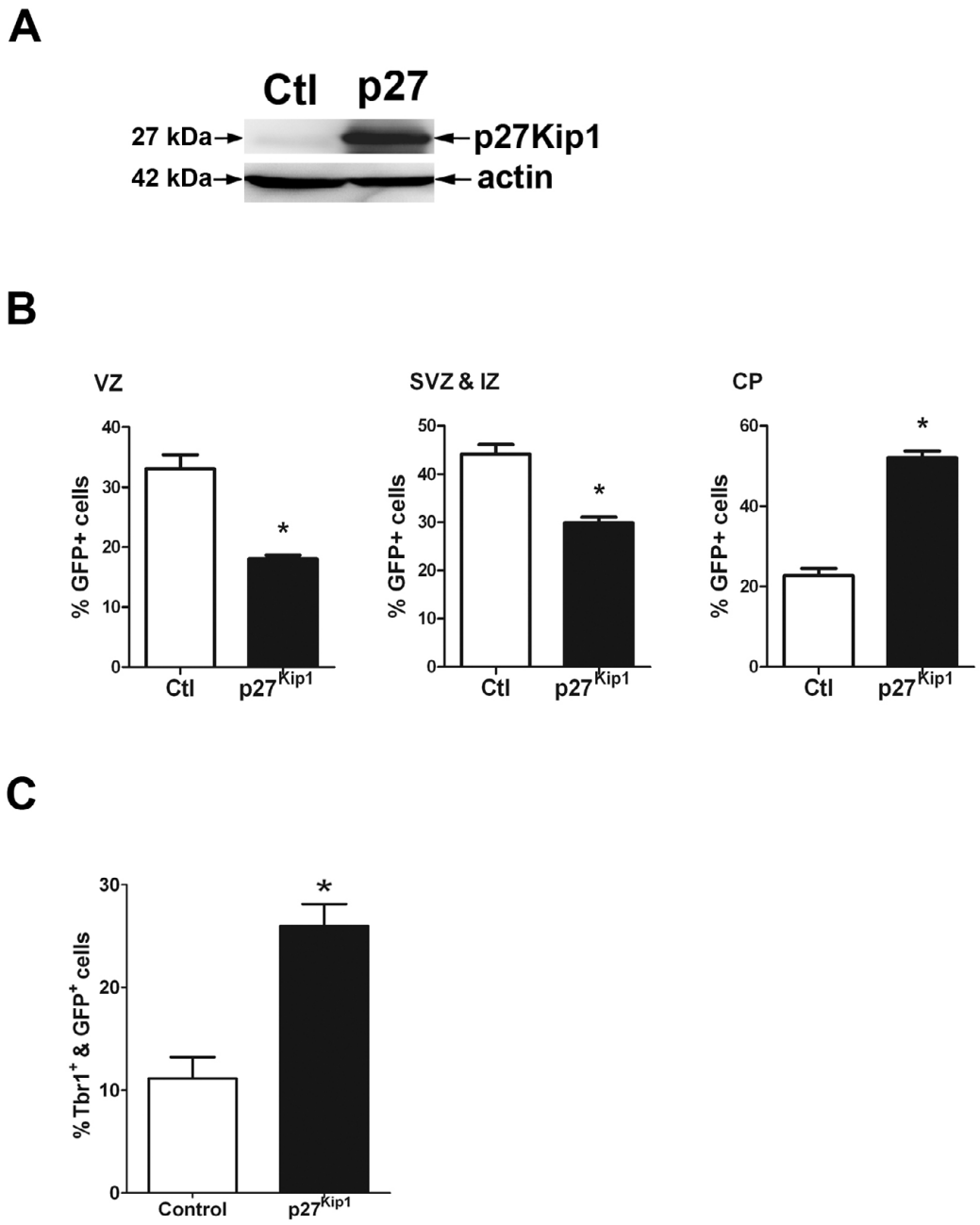

Fig. S6. p27^{Kip1} promotes NPC terminal mitosis similar to Mcl1. (**A**) Western blot analysis of p27^{Kip1} expression in HEK 293A cells at 48 h.p.t. with pCIG2 empty vector (control, Ctl) and pCIG2-p27^{Kip1} constructs. (**B**) Quantification of the percentage of GFP^{+} cells located in the VZ, SVZ and CP at 48 h.p.e. in Ctl- and p27^{Kip1}-electroporated brains. *n*=3 embryos/treatment. (**C**) Quantification of the percentage of Tbr1^{+} GFP^{+} double-labeled neurons at 48 h.p.e. in Ctl- and p27^{Kip1}-electroporated brains. *n*=3 embryos/treatment. Means were analysed by *t*-test. **P*<0.05. Error bars indicate s.e.m.