Id4 regulates neural progenitor proliferation and differentiation in vivo

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Summary

The mechanisms that determine whether a precursor cell re-enters the cell cycle or exits and differentiates are crucial in determining the types and numbers of cells that constitute a particular organ. Here, we report that *Id4* is required for normal brain size, and regulates lateral expansion of the proliferative zone in the developing cortex and hippocampus. In its absence, proliferation of stem cells in the ventricular zone (VZ) is compromised. In early cortical progenitors, Id4 is required for the normal G1-S transition. By contrast, at later ages, ectopically positioned proliferating cells are found in the mantle zone of the $Id4^{-/-}$ cortex. These observations, together with evidence for the premature differentiation of early cortical stem cells, indicate that *Id4* has a unique and complex function in regulating neural stem cell proliferation and differentiation.

Key words: *Id4*, *Id2*, Cortex, Hippocampus, G1-S, Cell cycle, Neurogenesis, CNS development

Introduction

The increased cortical surface area of the human brain is associated with its ability to perform higher order cognitive functions, and hence understanding the molecular mechanisms that regulate cortical progenitor proliferation and differentiation is of great importance. The mammalian telencephalon develops from the rostral-most region of the neural plate. The dorsal part of the telencephalon gives rise to the hippocampus, neocortex, lateral cortex, and parts of the amygdala, along with other smaller structures. These different structures arise from distinguishable progenitor regions in the ventricular zone (VZ) called the medial pallium (MP), dorsal pallium (DP), lateral pallium (LP) and ventral pallium (VP), which are identifiable by combinatorial patterns of homeobox gene expression (Puelles et al., 2000; Yun et al., 2001). From within each zone, there is a sequential generation of neurons and glia in the telencephalon, such that neurogenesis precedes gliogenesis. Most neurons are generated from the proliferative zone between E11.5 and E17.5, whereas glial cells are generated during early postnatal days (P0-P20) (Sauvageot and Stiles, 2002).

During early telencephalic development, there is an exponential expansion of neuroepithelial progenitors in the VZ as the result of rapid, symmetric cell divisions. The nuclei of these neuroepithelial cells go through characteristic movements within the VZ, known as the interkinetic nuclear movements. M-phase nuclei are found at the ventricular surface, while S-phase nuclei are found away from the ventricle in the distal region of the VZ (Takahashi et al., 1993; Takahashi et al., 1995a). These early VZ cells (E11.5-E12.5) divide continuously – their cell cycle time is estimated to be about 8 hours (Takahashi et al., 1993; Takahashi et al., 1992) – leading to rapid lateral expansion of the neuroepithelium. From the VZ emerge the cells that give rise to the secondary proliferative population (SPP) in the subventricular zone (SVZ) (Takahashi et al., 1995b) and differentiating neurons of the telencephalon.

Many genes known to affect proliferation and differentiation of the developing mammalian nervous system are transcription factors. In particular, members of the basic helix-loop-helix (bHLH) family have received much attention because of the potency with which they are able to instruct cellular differentiation (Guillemot, 1999). Consistent with this role in cell fate determination and differentiation, many bHLH genes are expressed in a tissue-specific manner (class B bHLH proteins). A sub-class of HLH genes, which lack the basic DNA-binding domain, is known as Inhibitors of DNA binding or ID genes. The proteins encoded by these genes act as dominant-negative regulators of bHLH proteins by forming inactive heterodimeric complexes (Benezra et al., 1990; Norton, 2000; Yokota, 2001).

In mammals, there are four known ID gene family members. *Id1*, *Id2*, *Id3* and *Id4* are expressed in progenitors of the central nervous system (CNS) in overlapping but distinctive patterns

(Andres-Barquin et al., 2000; Jen et al., 1997). Consistent with their expression patterns, the activities of ID1 and ID3 seem functionally redundant in the nervous system. Deletion of either *Id1* or *Id3* individually does not lead to an observable phenotype, but when they are both inactivated, telencephalic neurogenesis and angiogenesis are greatly perturbed (Lyden et al., 1999). Mice lacking *Id2* are viable, although they have an abnormal immune system and a lactation defect (Mori et al., 2000; Yokota et al., 1999). *Id1*, *Id2* and *Id3* have been shown to interact with cell cycle regulatory molecules (Zebedee and Hara, 2001). For example, they negatively regulate the expression of cyclin D1, p16^{Ink4a} and p21$^{CIP1/WAF1/SDI1}$ (Alani et al., 2001; Lasorella et al., 1996; Prabhu et al., 1997). In addition, both genetic and biochemical evidence indicates that ID2 inhibits RB (Retinoblastoma) protein function to enhance the G1 to S transition in proliferating cells (Iavarone et al., 1994; Toma et al., 2000).

The role of the most recently identified member of the ID family, *Id4*, in nervous system development has not been explored. In vitro studies suggest that ectopic expression of *Id4* prevents oligodendrocytic differentiation (Kondo and Raff, 2000). However, its role in regulating neural stem cell proliferation and differentiation is not known. Here, we identify a role of *Id4* during cortical development in vivo and reveal a novel function for *Id4* in regulating cortical neurogenesis.

Materials and methods

Generation of *Id4* mutant animals
A *Nco*I-*Hin*dIII fragment, including the EGFP cDNA from the pEGFP-N1 plasmid vector (Clontech) ligated to a 1.1 kbp *Hin*dIII-*Nco*I *Id4*-5′ flanking genomic fragment and a 4 kb *Not*I-*Pst*I fragment (*Id4*-3′-flanking genomic fragment), was inserted into the neo-tk cassette (Qiu et al., 1995) and introduced into JM-1 ES cells by electroporation. Of the 24 clones examined, 4 clones were found to contain a 6.5 kb band corresponding to the mutant allele. PCR primers specific for the wild-type and targeted alleles that were used included:

Id4-wild-type sense, 5′-CTGCAGTGCGATATGAACG-3′;
Id4-wild-type antisense, 5′-GCGGCTGTCTCAGCAAAG-3′;
Id4-mutant sense, 5′-GTCGATTTCTGGAGCTCGGA-3′); and
Id4-mutant antisense, 5′-AACTTGTGGCCGTTTACGTC-3′.

The PCR cycling conditions were 95°C for 30 seconds, 60°C for 40 seconds and 72°C for 1 minute for 30 cycles. The amplified PCR products were analyzed on gels to separate the wild-type (166 bp) and targeted (220 bp) allele fragments.

Ventricular surface area measurements
Coronal and saggital sections of *Id4*$^{+/-}$ and *Id4*$^{-/-}$ tissues at matching levels of the CNS were stained with Cresyl Violet and compared by measuring the VZ surface area using the ImagePro program. Three separate measurements of the same sections were averaged and compared. In each age group, three separate littermate brain pairs, sectioned in coronal and saggital planes at similar angles and matched to comparable levels along the rostrocaudal and mediolateral axis were analyzed.

In situ hybridization
In situ hybridization on frozen sections was carried out using ^{35}S-labeled antisense-riboprobes (Yun et al., 2001). Each probe was examined on three to five different mutant-control littermate pairs from at least two independent crosses at each age shown. Photographs were taken using dark-field optics on an Olympus SZH10 microscope. The cDNAs used in this study included: *Wnt3a* (A. McMahon,

Harvard University, USA); *Emx1* (J. Rubenstein, UCSF, USA); *Dbx1* (T. Jessell, Colombia University, USA); *Ngn2* and *NeuroD* (F. Guillemot, NIMR, UK); *Tbr2* (A. Bulfone, TIGEM, Italy); and *Id2* and *Id4* (M. Israel, Dartmouth Medical School, USA).

Immunofluorescence
Labeling of S-phase cells by BrdU incorporation was performed by injecting BrdU (1 mg/10 g body weight) into pregnant females and waiting for 45-60 minutes before sacrificing. A standard immunofluorescence protocol was used. Primary antibodies used in this study included: phosphorylated Histone 3 (PH3) (rabbit IgG; Upstate Biotech), BrdU (rat; Harlen), MAP2 (mouse IgG; Sigma, catalog number M1406), PCNA (mouse IgG; Novocastra), TBR1 (gift of M. Sheng, Harvard Medical School, USA). Confocal images of PCNA staining were collected as 2 μm optical slices.

FACS scan analysis
E12.5 dorsal telencephalon from *Id4*$^{-/-}$ and control littermates was dissected in cold PBS. Cells were dissociated in 0.05% trypsin, rinsed, and resuspended in 100 μl of PBS. Cells were fixed in 70% ethanol, rinsed, resuspended in 1 ml of solution containing 100 μg/ml RNaseA/0.2% Triton X-100/50 μg/ml Propidium Iodide, and incubated at 37°C for 30 minutes. Stained cells were scanned in a Becton Dickinson (San Jose, CA) FACScan flow cytometer, and the cell cycle profile was quantitated using ModFit software.

Results

Smaller brain size in *Id4*$^{-/-}$ animals
To understand the in vivo function of *Id4*, we used homologous recombination to generate a mutant mouse in which the *Id4* locus was disrupted (Fig. 1A). Exons 1 and 2 of *Id4* were replaced by the coding regions of *GFP* (green fluorescent protein) and the neomycin-resistance gene. Following transfection into embryonic stem cells, recombination events were confirmed by PCR and Southern blot analysis (Fig. 1A). Two independent lines of recombinant animals carrying an altered *Id4* locus were identified. In both lines, animals lacking functional *Id4* survived postnatally in clean environments, but their brain (Fig. 1B) size was consistently smaller than that of their littermates (not shown).

Id4 is widely expressed in the developing mouse embryo nervous system (Andres-Barquin et al., 2000; Jen et al., 1997). Because we found that animals lacking *Id4* had smaller brains, we examined telencephalic development in the *Id4* mutant animal to better understand the physiological role of *Id4*. Although we did not observe a phenotype in the *Id4* heterozygote, the reduced brain size shown in Fig. 1B is apparent in homozygous animals from E11.5 onwards. When littermate brains were sectioned and compared at E12.5 (Fig. 1C,C′), the ventricular surface of the mutant telencephalon was found to be approximately 30% smaller than that of the wild type along the mediolateral axis (compare the distance between the arrows in Fig. 1C,C′,F). In particular, the dorsal and medial pallial regions, primordia for the neocortex and the hippocampus, respectively, appeared much smaller than the more ventral regions in both the cortical (lateral and ventral pallium) and subcortical (ganglionic eminences) tissues (Fig. 1C,C′,E,F). Saggital sections at E15.5 and E18.5 showed smaller lateral ventricles and reduced ventricular surface along the rostrocaudal axis as well (Fig. 1D,D′,G). Concurrently, the mutant cortical plates are consistently thicker than those of their littermates (Fig. 1D,D′,H). From this analysis, we
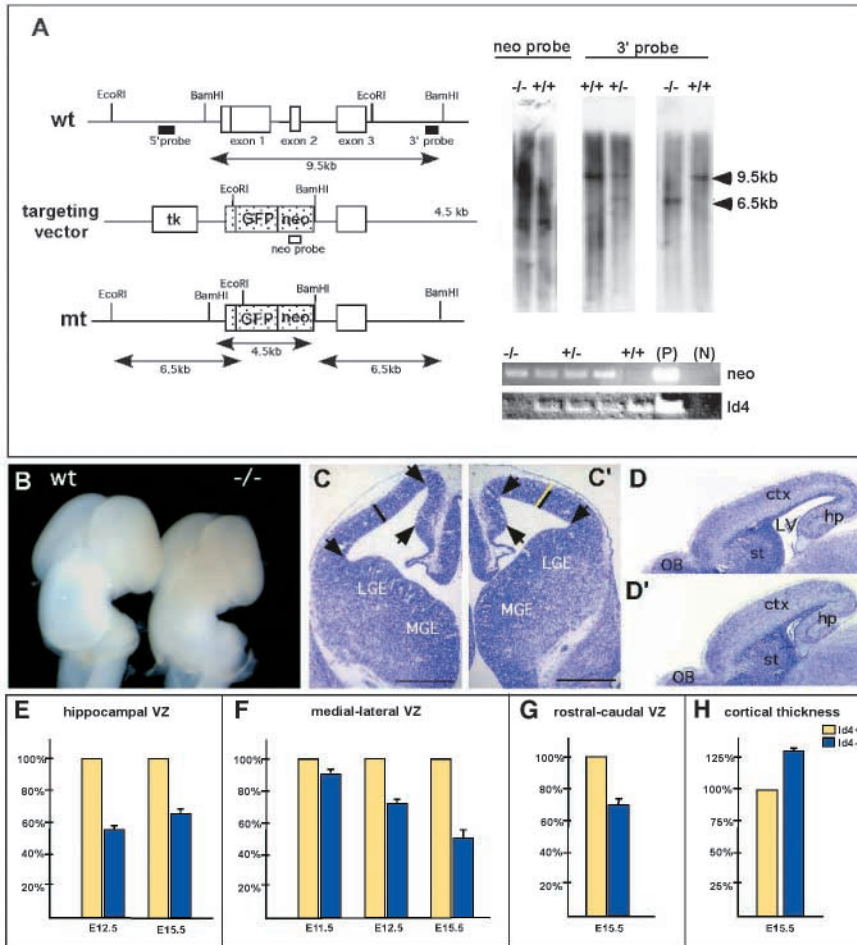
Fig. 1. (A) The recombinant targeting construct used to generate a mutant mouse with a disrupted *Id4* locus, and a Southern blot and PCR analysis demonstrating the successful targeting event. (B) E15.5 *Id4* mutant brain (right) and a littermate wild-type control brain (left). (C,C′) Cresyl violet-stained coronal sections through E12.5 wild-type and mutant telencephalon. Measurements of neocortex and hippocampal primordium shown in Fig. 2 were made between the arrows shown. Also, the thickening of the neocortex in the mutant (C′) is indicated by a comparison of the length of the black bar (wild-type thickness) to the yellow bar (mutant thickness). Basal ganglia (LGE and MGE) of the mutant appear normal in size. (D,D′) E18.5 saggital sections of control (D) and *Id4*⁻/⁻ (D′) telencephalon stained with cresyl violet. (E-H) Quantitation of the cortical VZ surface areas at E11.5, E12.5 and E15.5, in control and *Id4*⁻/⁻ littermates at matched levels on coronal sections (E,F) and saggital sections (G,H), normalized to littermate controls. Yellow bar, Id4⁺/⁻; blue bar, Id4⁻/⁻; wt, wild type; mt, mutant; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; hp, hippocampus; ctx, neocortex; OB, olfactory bulb; st, striatum; LV, lateral ventricle.

conclude that *Id4* function is required for the development of a normal size brain, and especially for the lateral expansion of the cortical neuroepithelium.

Reduced dorsomedial progenitor zones in the *Id4*⁻/⁻ mutant

To determine whether the smaller cortex in *Id4*⁻/⁻ animals reflected the loss of a specific region or an overall reduction in all cortical regions, we examined the expression of molecular markers that distinguish different progenitor zones. Genes expressed at the dorsal and ventral extremes of the pallial (cortical) progenitor zone appeared normal. For example, *Wnt3a* expression in the cortical hem (Lee et al., 2000) was unchanged in mutant animals (Fig. 2A,A′). In addition, no consistent differences were found in the expression patterns of genes marking the ventral and lateral pallium progenitors (*Dbx1*, *Emx1*, *Tbr2* and *Wnt7b*; Fig. 2B-D′, and not shown), or the mantle (not shown) (Puelles et al., 2000; Yun et al., 2001) in *Id4*⁻/⁻ animals. To quantitate the difference in the surface area of the cortical proliferative zone, we measured the cortical VZ width on matched coronal sections from littermate control and *Id4*⁻/⁻ animals. We found progressive shortening of the VZ surface area (Fig. 2G) and consistent reduction in the hippocampal primordium (approximately 50%, Fig. 2H) in *Id4*⁻/⁻ animals. These findings, in the context of the histological analysis shown in Fig. 1, indicate that the hypoplasia of the

Id4⁻/⁻ telencephalon most severely affects dorsomedial structures.

As *Id4* is highly expressed in all cortical progenitor zones (Fig. 2F), we were surprised to find these regional differences. We hypothesized that ID4 function is unique in the dorsomedial cortex and that other ID genes compensate for *Id4* loss in other regions of the telencephalon. Consistent with this possibility, we observed elevated levels of *Id1*, *Id2* and *Id3* mRNA in the *Id4*⁻/⁻ telencephalon by RT-PCR (data not shown). Because *Id2* is expressed in a lateral-to-medial gradient in the VZ of the cortex (levels are undetectable between the arrowheads in Fig. 2E), we used in situ hybridization to determine whether an altered pattern of *Id2* expression could be identified. We found that *Id2* expression was increased beyond its normal dorsal boundary in the cortex extending all the way to the midline in Id4′ animals (Fig. 2E′). This observation raises the possibility that *Id4* normally represses *Id2* expression in the dorsomedial region, or that the hypoplastic region of the *Id4*⁻/⁻ mouse cortex corresponds to areas in which *Id2* is normally not expressed.

To determine whether the molecular abnormalities associated with the loss of *Id4* also contributed to cortical hypoplasia by enhancing progenitor cell death, we examined apoptosis in the developing telencephalon of *Id4*⁻/⁻ animals. We did not detect differences in the number of apoptotic cells in the *Id4*⁻/⁻ and control telencephalon at E11.5, E12.5 or E15.5
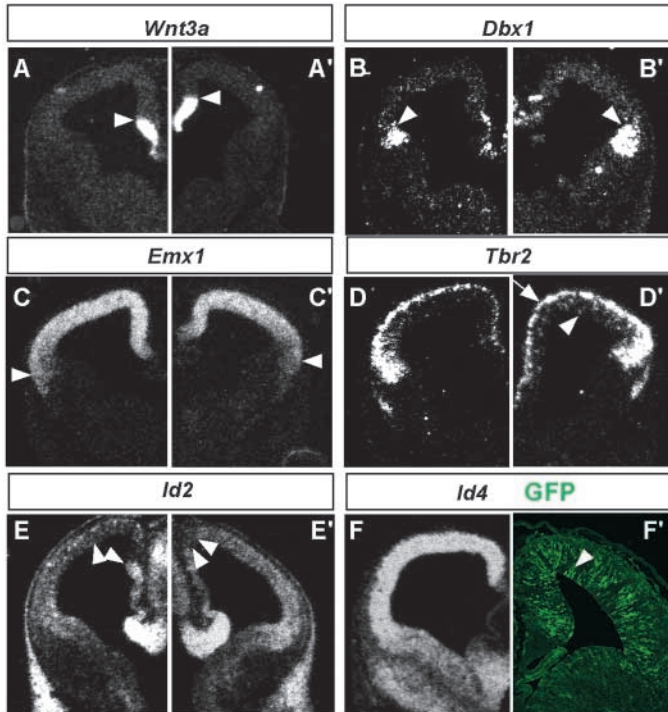
Fig. 2. E11.5 coronal sections through wild-type (A-F) and *Id4⁻/⁻*
(A′-F′) telencephalon. *Wnt3a*, *Dbx1*, *Emx1* and *Tbr2* expression in
LP and VP progenitor zones appears normal (A-D′). (A-B′) The
limits of the Wnt3a and Dbx1 expression domains are indicated by
the arrowheads. (C,C′) Arrowheads indicate mantle expression of
Emx1 in the lateral pallium. (D′) Arrowhead indicates ectopic *Tbr2*-
expressing cells in the VZ; arrow points to the increased number of
Tbr2-expressing postmitotic cells in the dorsomedial mantle of the
Id4⁻/⁻ animal. (E) Endogenous *Id2* expression shows a ventral-to-
dorsal gradient of expression and is undetectable between the
arrowheads. (E′) The boundaries of the reduced *Id2*-negative region
in the *Id4⁻/⁻* brain is indicated by arrowheads. (F) *Id4* expression is
uniformly high in all cortical areas. (F′) GFP expression from the *Id4*
locus is detectable in the *Id4⁻/⁻* brain (arrowhead). LGE, lateral
ganglionic eminence; VP, ventral pallium; LP, lateral pallium; DP,
dorsal pallium; MP, medial pallium; ctx, neocortex; LV, lateral
ventricle.
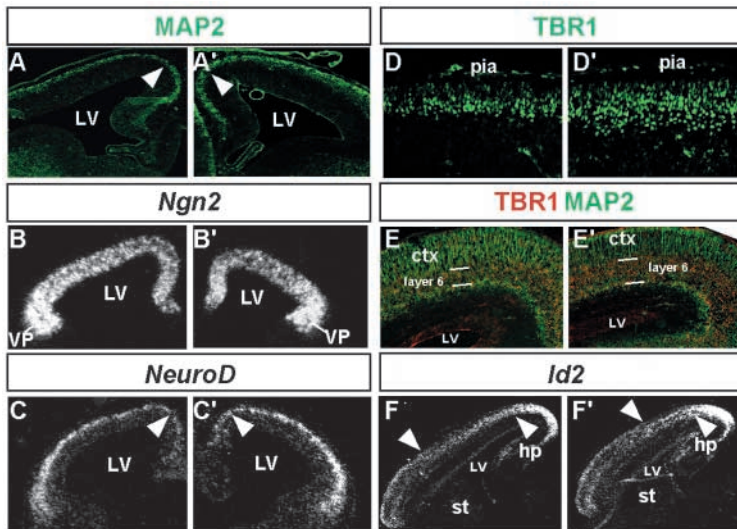
(data not shown). Furthermore, the insertion of DNA encoding
GFP into the *Id4* locus allowed the identification of cells in
which *Id4* transcription would occur. GFP-expressing cells
were clearly present in *Id4⁻/⁻* mutants at E12.5 (Fig. 2F′),
indicating that the loss of *Id4* function does not result in
extensive cell death.

Precocious differentiation of early cortical progenitors

To determine whether the dorsomedial cortical progenitor
zones are reduced as a result of precocious differentiation
or compromised proliferation of neural stem cells, we first
examined early neurogenesis in the mutant telencephalon. At
E11.5, cortical neurogenesis is just beginning in the dorsal and
medial pallium, whereas in the more ventral regions of the
cortex, the ventral and lateral pallium, neurons are produced
from E10.5. Expression of pan-neuronal markers at E11.5
(MAP2 and βIII-tubulin, Fig. 3A,A′, and not shown) was
reproducibly increased throughout the postmitotic zone of the
Id4⁻/⁻ cortex, suggesting precocious neurogenesis. Therefore,
we examined the expression of other genes that either regulate
neurogenesis in cortical progenitors or are markers of cortical
neurons. At E11.5, neurogenin 2 (*Ngn2*; *Neurog2* – Mouse
Genome Informatics), a proneural bHLH gene (Fode et al.,
2000; Gradwohl et al., 1996), was expressed in a similar
pattern in both wild type and *Id4⁻/⁻* animals. However, the
expression of markers identifying early differentiating neurons,
NeuroD (Fig. 3C,C′) and *Tbr2* (arrow in Fig. 2D,D′), was
consistently increased in the mantle zone of the dorsomedial
cortex in *Id4⁻/⁻* animals. Furthermore, scattered *Tbr2*-
expressing cells were found ectopically in the VZ (dorsal to its
normal expression domain in the VP and LP (Bulfone et al.,
1999; Yun et al., 2001)) of the dorsal and medial pallium
(arrowhead in Fig. 2D,D′). Each of these findings is indicative
of precocious differentiation of early cortical progenitors.

Early-born (E11.5-E13.5) neurons of the cortex form deep
layer neurons (layers 5 and 6) (Takahashi et al., 1999). At
E15.5 and E18.5, TBR1 expression in postmitotic cortical
neurons (Hevner et al., 2001) identified an increased number
of deep layer neurons in *Id4⁻/⁻* animals (Fig. 3D-E′). In
addition, TBR1-expressing neurons were found in more
dispersed positions in Id4⁻/⁻ animals at E15.5 (Fig. 3D′).
Consistently, *Id2* expression at E18.5 (Rubenstein et al.,
1999) revealed disorganized layering/differentiation
within the cortical layers (Fig. 3F,F′). These observations

Fig. 3. E11.5 (A,A′), E12.5 (B-C′), E15.5 (D,D′) and E18.5
(E-F′) coronal (A-D′) and saggital (E-F′) sections of wild-type
(A-F) and *Id4⁻/⁻* (A′-F′) telencephalon are compared.
Immunofluorescence was detected using the antibodies listed
above each panel (A,A′,D-E′) and in situ hybridization was
performed using the S-35-labeled riboprobes listed above each
panel (B-C′,F,F′). A pan-neuronal marker, MAP2 (A,A′),
shows reproducibly increased expression in the mutant
dorsomedial cortex (arrowhead). Markers of differentiating
neurons, *NeuroD* (C,C′) and *Tbr2* (see Fig. 2D,D′), also show
increased expression in the dorsomedial mantle (arrowheads
in C,C′). TBR1 expression marks differentiating cortical
neurons that will form deep cortical layers (D-E′).
Arrowheads in F,F′ indicate disorganized cortical layers and
areas. hp, hippocampus; ctx, neocortex; LV, lateral ventricle;
VP, ventral pallium; st, striatum.

provide additional evidence that early cortical progenitors are precociously differentiating in the absence of the functional *Id4* gene.

Prolonged G1-S transition in the Id4$^{-/-}$ cortex

To determine whether defects in progenitor proliferation also contributed to the *Id4*$^{-/-}$ mutant phenotype, we examined markers of cell cycle phases and found that G1-S transition of the Id4$^{-/-}$ neuroepithelial cells is compromised at E12.5. We compared progenitor cells in wild-type and *Id4*$^{-/-}$ cortex by analyzing the expression of markers that identify all cells actively proliferating (PCNA, proliferating cell nuclear antigen), or cells in M-phase (PH3: Phosphorylated Histone 3), or cells in S-phase, by a short pulse of BrdU (Bromodeoxyuridine)-labeling. At E12.5, the density of proliferating cells in the VZ is indistinguishable in wild-type and *Id4*$^{-/-}$ animals (PCNA$^+$ cells in Fig. 4A,A′,D′), but the VZ is approximately 15% thicker in the *Id4*$^{-/-}$ mutant (see Fig. 1C,C′, Fig. 4A,A′). To determine whether this increased thickness could be attributed to cells at a particular phase of the cell cycle, we first examined cells in M phase. We found that the actual number of cells in M-phase was indistinguishable in the wild-type and *Id4*$^{-/-}$ animals at this age (Fig. 4C,C′, and not shown). BrdU labeling at E12.5 identified cells that were in S phase (Fig. 4B,B′). Although the thickness of the S-phase zone varied within the cortical VZ (thinner dorsomedially) in the mutant animal, the most striking difference between the control and *Id4*$^{-/-}$ animals we found was in the thickness of the zone corresponding to the G-phases of

the cell cycle. The G-phase zone is sandwiched between the BrdU-positive S-phase cells (Fig. 4B,B′) and the PH3-positive M-phase cells (Fig. 4C,C′). The thickening of this zone (distance between the arrowheads in Fig. 4B,B′) clearly accounts for the overall increased thickness of the VZ in mutant animals. The presence of cycling cells in this region, as identified by PCNA expression (Fig. 4A,A′,D′), suggested that progenitor cells in the *Id4*$^{-/-}$ animal might make a prolonged transition between G1-S and/or G2-M phases of the cell cycle. We, therefore, evaluated the cell cycle distribution of cortical cells isolated from E12.5 dorsal telencephalon by FACS analysis. In multiple experiments (*n*=7) examining cells from E12.5 or E13.5 littermate embryos, we invariably observed a reduction in the percentage of cells in S-phase and a corresponding increase in the percentage of cells in G0/G1, as indicated in representative experiments from E12.5 shown in Table 1. In wild-type animals at E12.5, virtually all VZ cells are dividing and no G0 cells are found among them (Takahashi et al., 1995a). Our finding that the thicker mutant VZ is composed of PCNA$^+$ proliferating cells (Fig. 4A′,D′) indicates that the increased percentage of cells in G0/G1 detected by FACS analysis (Table 1) represents a prolonged G1 in the mutant cells at this age. Although the increased number of prematurely differentiating cells observed at this age may also contribute to the higher percentage of G0/G1 cells in the ID4 mutants, the fact that most cells in the G-phase zone are PCNA positive (Fig. 4A′,D′) indicates that a contribution of prematurely differentiating cells to the thickening of the VZ is unlikely. Together, these two independent lines of investigation indicate that *Id4* is required for normal G1-S transition in early neural stem cells. Thus, the reduced brain size in the *Id4*$^{-/-}$ cortex results from both premature differentiation and compromised cell cycle transition of early neural stem cells.
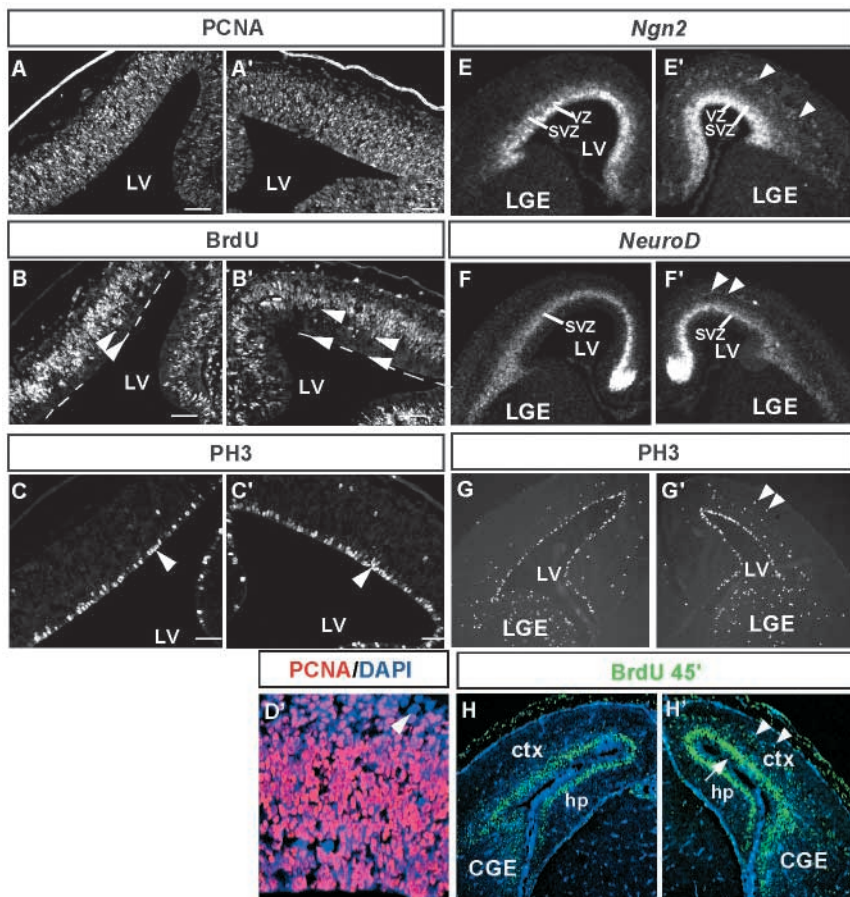
Fig. 4. E12.5 (A-C′,D′) and E15.5 (E-H′) coronal sections through control (A-H) and *Id4*$^{-/-}$ (A′-H′) mutant cortex. Markers of cell cycle used are: proliferating cells, PCNA (A,A′,D′); S-phase, BrdU pulse (B,B′,H,H′); and M-phase, PH3 (C,C′,G,G′). (B,B′) The G-phase zone is indicated by the distance between the arrowheads; yellow dashes indicate the ventricular surface. (D′) High magnification image of PCNA staining (red), double labeled with DAPI (blue) to visualize all nuclei. At E15.5, ectopically positioned *Ngn2*- and *NeuroD*-expressing cells were detected outside of the VZ and SVZ (arrowheads in E′,F′). Immunofluorescence analysis was performed using antibodies against PH3 and BrdU (following a 45-minute labelling pulse) in littermate control and *Id4* null animals (arrowheads in G′,H′ indicate ectopically proliferating cells). BrdU is shown in green and DAPI in blue (H,H′). Arrow in H′ indicates increased BrdU labeling in the *Id4*$^{-/-}$ SVZ at E15.5. LV, lateral ventricle; hp, hippocampus; ctx, neocortex; LGE, lateral ganglionic eminence; CGE, caudal ganglionic eminence; VZ, ventricular zone; SVZ, subventricular zone.

Table 1. Cell cycle analysis of E12.5 _Id4_⁺/⁻ and _Id4_⁻/⁻ dorsal telencephalon

Experiment	Genotype	Sample size, n	Percentage in G0/G1*	Percentage in S*	Percentage in G2/M*
1	+/–	2	60.70±1.85	27.16±0.03	12.14±1.83
	–/–	2	63.03±2.87	25.31±4.65	11.67±1.77
2	+/–	1	52.41	41.31	6.28
	–/–	3	55.94±1.29	35.96±1.14	8.10±0.16
3	+/–	6	58.04±1.51	38.80±2.42	3.17±1.74
	–/–	1	60.84	37.65	1.51
4	+/–	3	61.03±1.01	31.82±2.63	7.15±2.92
	–/–	3	65.36±0.50	27.45±0.28	7.19±0.71

Shown here are typical experiments from PI labeling and FACS scanning of _Id4_⁺/⁻ and _Id4_⁻/⁻ dorsal telencephalon cells at E12.5. _n_ identifies the numbers of _Id4_⁻/⁻ or control littermates in each experiment. Quantitation using ModFit software revealed the increased percentage of G1-phase cells and the decreased percentage of S-phase cells in _Id4_⁻/⁻ samples. Consistent shifts in cell cycle profile were observed in every litter examined, a total of seven experiments representing seven independent litters (four litters from E12.5 and three litters from E13.5, not shown). The total number of embryos we examined was nine Id4⁻/⁻ and 12 Id4⁺/⁻ at E12.5, and seven Id4⁻/⁻ and eight Id4⁺/⁻ at E13.5. The data from Experiment 4 was evaluated by _t_-test, a representative experiment with a large enough sample size for a _t_-test, resulting in a _P_ value of 0.0026. If there were no association, the probability of always observing an increased percentage of cells in G0/G1 in Id4⁻/⁻ animals in seven total experiments would be 0.00781.

*Values are ±s.e.m. where applicable.

Ectopically positioned proliferating cells

To determine whether the proliferation defect in _Id4_⁻/⁻ animals at E12.5 was also detectable at later stages of development, we examined markers of cortical progenitors and cellular proliferation at E15.5. Surprisingly, we found a greater number of proliferating cells outside of the VZ in the _Id4_⁻/⁻ animals. In addition to progenitors in the SVZ, many cells in the mantle (postmitotic) region ectopically expressed markers of VZ and SVZ, such as _Ngn2_, _Ngn1_, _NeuroD_, _Emx1_ and cadherin 8 (Fig. 4E-F′, and not shown). We interpret this expression pattern to reflect the presence of proliferating cells in the postmitotic region. Therefore, we sought evidence for ectopic proliferation at E15.5 by evaluating cortical tissue following a 45-minute BrdU pulse to identify S-phase cells and PH3⁺ immunostaining to identify M phase cells. As indicated in Fig. 4G-H′, we observed many BrdU-positive, as well as PH3-positive, cells in the mantle zone. This observation indicates that, in contrast to the compromised proliferation observed at E12.5, cells in the postmitotic region are aberrantly proliferating in the _Id4_ mutant at E15.5.

Discussion

Unlike other mouse lines in which inactivation of a single ID gene family member does not show an observable CNS phenotype (Lyden et al., 1999; Yokota, 2001), the _Id4_⁻/⁻ mouse has an obviously smaller brain. For example, _Id1_ and _Id3_ single mutants do not show an obvious CNS phenotype, although a phenotype in the telencephalon does emerge when _Id1_ and _Id3_ are simultaneously inactivated (Lyden et al., 1999). Here, we provide multiple lines of evidence indicating that the _Id4_ mutant phenotype arises from the requirement, in vivo, of ID4 function in cortical stem cells for their proliferation and differentiation. Importantly, we show different effects of _Id4_ loss of function on cortical cell proliferation at different times during development.

Requirement for _Id4_ during early stem cell proliferation

Neural epithelium of the developing cortex expands laterally through the rapid division of early stem cells. Cortical neurogenesis commences at around E11.5 and the onset of an observable Id4⁻/⁻ phenotype coincides with this major milestone in cortical development. At E12.5, the mutant cortical VZ lateral expansion is greatly compromised, and a progressively reduced cortical VZ area is found from this time forward. For example, at E15.5, the cortical VZ surface area is less than 50% of the control littermate brains (~60% mediolaterally×~70% rostrocaudally). At E12.5, the _Id4_ mutant VZ is thicker and this thickening can be attributed to a prolonged G1 phase of the cell cycle (Fig. 4 and Table 1). This phenotype is consistent with in vitro studies indicating that ID genes enhance proliferation by facilitating the G1-S transition. For example, ID2 has been shown to bind to the RB (Retinoblastoma) protein thereby enhancing the G1-S transition (Iavarone et al., 1994), and other ID genes have been shown to regulate the expression of the G1 cyclin-dependent kinase inhibitors p16 and p21 at the transcriptional level (Alani et al., 2001; Lasorella et al., 1996; Prabhu et al., 1997). ID genes are in turn regulated during the cell cycle where their peak level of expression is observed early in G1, after serum stimulation (Christy et al., 1991; Hara et al., 1994). However, our analysis of these G1-S regulators in the _Id4_⁻/⁻ telencephalon did not reveal significant changes in their expression levels (data not shown), suggesting that _Id4_ does not regulate their expression during cortical development. We are currently examining the possibility that ID4 interacts with these molecules at the protein:protein level to affect their function.

Our findings provide evidence supporting an integrated proliferative regulatory mechanism that coordinates the timing, the number and the neuronal specificity of cortical neurons, as proposed by Caviness and his colleagues in their theoretical model (Caviness et al., 2003). Their model predicts that increasing the proportion of daughter cells exiting the cell cycle early will lead to reduced lateral expansion of the neuroepithelium, as a result of the reduced proportion of daughter cells available to expand the proliferating population. It also predicts that the early cortical plate will be transiently thicker (because of an increased output of postmitotic cells), but significantly thinner at the end of the neurogenic period because of the loss of stem cells early on. In addition, an

increase in the proportion of daughter cells exiting the VZ is associated with a lengthening of the cell cycle time, particularly G1, during normal development. With the exception of a thicker, rather than thinner, cortical plate in the *Id4* mutant at E18.5, the *Id4* mutant phenotype is consistent with this model. Hence, our findings generally support the existence of an integrated regulatory mechanism, and identify *Id4* as a key regulator that controls the time domain, the output domain, and neuronal specification during cortical development.

Role of ID family members in telencephalic development

We were intrigued by the region-specific requirement for ID4 function, and the relatively mild mutant phenotype at P0 despite the early loss of stem cells due to both premature differentiation and compromised cell cycle transition. As *Id1*, *Id2* and *Id3* are also expressed in the developing telencephalon, these ID genes may functionally compensate for *Id4* in non-affected areas. For example, mutational studies of *Id1* and *Id3* have revealed that they are functionally redundant during telencephalic development. To examine this possibility, we monitored expression levels of other ID genes in *Id4* mutant telencephalon. Indeed *Id1*, *Id2* and *Id3* expression levels were increased in the *Id4* mutant, when compared with a control littermate (Fig. 2E′ and not shown). In particular, *Id2* expression was altered both spatially (Fig. 2E,E′) and quantitatively (not shown). Hence, we examined possible functional redundancies between *Id2* and *Id4* by analyzing *Id2/4* double null embryos. We were unable to detect evidence of altered telencephalic development in *Id2*^{−/−} animals (data not shown). Interestingly, the brain size of the *Id2/4* double null animal was only slightly smaller than that of the *Id4* single mutant (data not shown), and no obvious defects were found in other areas of the telencephalon. This observation indicates that *Id2* is not necessary to compensate for *Id4* in other areas, and supports the idea that ID4 function is unique and necessary for the development of the telencephalic midline tissue. *Id2/4* double mutant analysis also suggests the existence of other compensatory mechanisms, either at the molecular level by *Id1* or *Id3*, or at the cellular level (see below).

Role of *Id4* in differentiating neurons

In contrast to the reduced proliferation observed in the early neuroepithelium, the number of proliferating cells outside of the VZ in the *Id4*^{−/−} brain after E15.5 is dramatically increased (Fig. 4). These ectopically positioned cells proliferate, as indicated by both S-phase and M-phase markers, and differentiate, as indicated by NeuroD expression, in ectopic positions. These results indicate a potential compensation mechanism at the cellular level for the reduced proliferation of early VZ progenitors, and may account for the relatively mild *Id4* mutant phenotype observed at birth.

Ectopically positioned proliferating cells found in the *Id4*^{−/−} cortex are reminiscent of changes observed in animals lacking the *Rb* gene. In *Rb*^{−/−} animals, ectopically positioned proliferating cells are found in the cortex, and this phenotype has been interpreted as indicating that differentiating mantle cells have aberrantly re-entered the cell cycle in the absence of functional RB (Clarke et al., 1992; Lee et al., 1992; Lipinski et al., 2001; MacPherson et al., 2003). We therefore examined

Rb levels in *Id4*^{−/−} animals, but we did not observe a significant difference in its expression, either at the RNA or protein level (not shown). ID2 has been shown to bind RB protein and to inhibit its function post-translationally (Iavarone et al., 1994). Hence, a possible explanation for the observed phenotype is that the increased level of *Id2* expression in the *Id4* mutant (Fig. 2E′) leads to inactivation of RB. However, direct examination of this hypothesis indicates that this phenotype is not the result of an epigenetic event (increased *Id2* levels). The *Id2/4* double mutant cortex also contained many ectopically proliferating cells in the mantle region (not shown). These observations suggest that if the *Id4* phenotype were mediated through the RB pathway, ID4 most likely acts to enhance RB-mediated inhibition of proliferation, in contrast to ID2, which negatively regulates RB. Among the many possibilities, direct interactions can be envisioned in which ID4 competes with ID2 for RB binding in differentiating neurons. Or, ID4 may directly interact with other molecules in the cell cycle machinery, and depending on the interacting partner and cellular context, either enhance or inhibit cell cycle transition.

Id4 interaction with bHLH factors

ID genes have been shown to inhibit differentiation in many different cell types in vitro and in vivo (Norton, 2000). The main mechanism of this potent inhibitory effect is attributed to its ability to form dominant-negative dimers with bHLH proteins. In the developing cortical progenitor zone, at least three proneural bHLH genes, *Ngn1*, *Ngn2* and *Mash1*, are important for progenitor proliferation, cell fate specification, and differentiation (Fode et al., 2000; Nieto et al., 2001; Parras et al., 2002; Ross et al., 2003). As ID proteins are thought to act as negative regulators of bHLH proteins by sequestering available E-proteins, we have postulated that proneural bHLH proteins are hyper-active in the *Id4* mutant cortex. Consistent with this model, the *Id4* mutant phenotype correlates well with both loss-of-function and gain-of-function studies of these proneural genes (Fode et al., 2000; Parras et al., 2002; Sun et al., 2001). For example, ectopic expression of *Ngn1* clearly promotes neuronal differentiation (Sun et al., 2001), and the simultaneous loss of *Ngn2* and *Mash1* function results in reduced neurogenesis (and *NeuroD* expression), particularly in the dorsomedial cortex (Fode et al., 2000). In *Id4*^{−/−} animals, where proneural bHLH proteins are presumably free from ID4 inhibition, neural stem cells prematurely differentiate and this is most obvious in the dorsomedial region (Fig. 2D,D′, Fig. 3A,A′,C,C′). It is unclear, at present, why the dorsomedial region of the telencephalon is particularly sensitive to both positive and negative HLH protein activity, but these in vivo studies reveal an important requirement for bHLH transcription factor function in this area, and emphasize the role of *Id4* in regulating its development.

CNS development requires complex molecular interactions that control progenitor proliferation and differentiation, and *Id4* can now be recognized to be important for these processes. Our finding that *Id4* inhibits neural progenitor differentiation is consistent with the assignment of this function to other ID family members. In addition, we provide in vivo evidence for a novel function of *Id4* in preventing aberrant cell cycle entry in differentiating neurons. These observations underscore the importance of cell context for ID gene function, and in particular for *Id4*, which either enhances or blocks cell cycle transition in

the same developing tissue at different times. This study reveals a crucial role for *Id4* in cortical development, and identifies the molecular pathways over which its effects are mediated, while suggesting important new areas for future study.

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