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NUMB Localizes in the Basal Cortex of Mitotic Avian Neuroepithelial Cells and Modulates Neuronal Differentiation by Binding to NOTCH-1

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Summary

The importance of lateral inhibition mediated by NOTCH signaling is well demonstrated to control neurogenesis both in invertebrates and vertebrates. We have identified the chicken homolog of *Drosophila numb*, which suppresses NOTCH signaling. We show that chicken NUMB (c-NUMB) protein is localized to the basal cortex of mitotic neuroepithelial cells, suggesting that c-NUMB regulates neurogenesis by the modification of NOTCH signaling through asymmetrical cell division. Consistent with this suggestion, we show (1) that c-NUMB interferes with the nuclear translocation of activated c-NOTCH-1 through direct binding to the PEST sequence in the cytoplasmic domain of c-NOTCH-1 and (2) that c-NUMB interferes with c-NOTCH-1-mediated inhibition of neuronal differentiation.

Introduction

During development of the vertebrate central nervous system (CNS), neurons and glial cells differentiate from the neuroepithelium. To produce large numbers of distinct cell types, neuroepithelial cells behave as stem cells, giving rise to neuronal (or glial) precursors, while maintaining the neuroepithelial population. To accomplish this, neuroepithelial cells appear to undergo symmetrical and asymmetrical cell divisions (reviewed in McConnell, 1995). Time lapse analysis of ferret neocortex has revealed that symmetrical (vertical) division results in two identical neuroepithelial cell daughters, whereas asymmetrical (horizontal) division appears to produce an apical neuroepithelial cell and a basal prospective neuronal precursor (Chenn and McConnell, 1995). This observation may explain, in part, how neuroepithelial cells produce fate-restricted precursors while retaining the ability to duplicate themselves.

Asymmetrical localization of fate determinants and subsequent asymmetrical cell division have been shown to be important to produce distinct cell types in the development of the *Drosophila melanogaster* nervous system (reviewed in Doe and Spana, 1995; Vervoort et al., 1997; Fuerstenberg et al., 1998). For example, the homeodomain transcription factor PROSPERO localizes asymmetrically on the basal side of neuroblasts. After the neuroblast divides and after nuclear translocation of received PROSPERO, the basal daughter cell differentiates into a ganglion mother cell (GMC), whereas the PROSPERO-depleted apical daughter cell remains as a neuroblast (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995). *Prospero* function is important for the activation of GMC-specific genes, such as *even-skipped* (Doe et al., 1991), and the repression of neuroblast-specific genes (Vaessin et al., 1991).

Another example of an asymmetrically localized fate determinant in *Drosophila* is *numb*. Similar to PROSPERO, NUMB localizes on the basal side of neuroblast (Rhyu et al., 1994; Spana et al., 1995), although no significant defect has been noticed in the GMC lineage of *numb* mutants (Spana et al., 1995). NUMB also localizes asymmetrically in external sensory organ precursor cells and is essential for the production of IIa and IIb daughter cells that eventually produce hair and socket, and neuron and sheath cells, respectively (Uemura et al., 1989; Rhyu et al., 1994). Unlike PROSPERO, however, NUMB appears to regulate downstream gene expression indirectly. Thus, NUMB represses signaling through the NOTCH transmembrane receptor (Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996).

NOTCH signaling functions in both vertebrates and invertebrates to control the number of neurons that differentiate. It mediates so-called lateral inhibition among locally interacting cells (reviewed in Artavanis-Tsakonas et al., 1995). Among four *Notch* homologs found in mammals, *Notch-1* and *Notch-3* mRNAs have been detected widely in the ventricular zone of the developing CNS (Williams et al., 1995). NOTCH-1 protein has also been detected uniformly in the same area (Zhong et al., 1997). The tissue distribution of chicken *Notch-1* mRNA revealed a pattern similar to that of its mammalian homolog (Myat et al., 1996). Moreover, *c-Delta-1* (or *dlk-1* in mammals) and *c-Serrate-1* (or *Jagged-1* in mammals), which encode ligands for NOTCH proteins, are expressed in the same area, by postmitotic prospective neurons (Henrique et al., 1995; Lindsell et al., 1995; Myat et al., 1996). In transgenic mouse embryos carrying an activated form of the *Notch-3* transgene driven by the *nestin* promoter, the CNS revealed an expansion of the neuroepithelial layer, and fewer neurons, suggesting that NOTCH signaling represses neuronal differentiation (Lardelli et al., 1996). Other attempts to activate NOTCH signaling in *Xenopus* and zebrafish have demonstrated that neuronal differentiation is inhibited and that repression of NOTCH activation with a dominant negative form of X-DELTA-1 increased the number of neurons (Coffman et al., 1993; Chitnis et al., 1995; Dorsky et al., 1995; Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998). In chicken retina, moreover, similar results were obtained by the manipulation of NOTCH signaling (Austin et al., 1995; Henrique et al., 1997). Based on these observations, it has been proposed that activation of NOTCH signaling by NOTCH ligand–expressing neighbors prevents the neuroepithelial cells from undergoing neuronal differentiation.

Rat and mouse homologs of *numb* have also been identified (Verdi et al., 1996; Zhong et al., 1996, 1997).

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Mouse *Numb* (*m-Numb*) mRNA is expressed in a wide variety of tissues, and mouse *numblike* (*m-nbl*) is expressed in neurons (Zhong et al., 1996, 1997). Mouse *Numb* can rescue the external sensory organ phenotype of a *Drosophila numb* mutant (Zhong et al., 1996, 1997). In contrast to the basal localization of *Drosophila* NUMB, however, m-NUMB immunoreactivity has been reported to be localized on the apical side of dividing neuroepithelial cells (Zhong et al., 1996). These authors propose a model in which (1) repression of NOTCH signaling by m-NUMB in apical daughter cells maintains the undifferentiated progenitor state of neuroepithelial cells, (2) NOTCH signaling in basal daughter cells expresses a postmitotic, nondifferentiated migratory phenotype, and finally, (3) repression of NOTCH signaling by newly accumulated m-NUMB or NUMBLIKE (Nbl) induces the migratory cells to undergo terminal neuronal differentiation (Zhong et al., 1997). It is not clear in this model how both repression and activation of NOTCH signaling could prevent neuronal differentiation. Nor is this model consistent with previous reports that activation of NOTCH signaling results in decreased neuronal differentiation (Coffman et al., 1993; Austin et al., 1995; Chitnis et al., 1995; Dorsky et al., 1995; Lardelli et al., 1996; Dornseifer et al., 1997; Henrique et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998).

Here, we report that chicken NUMB (c-NUMB) localizes in the basal cortex of mitotic neuroepithelial cells similar to basal localization of *Drosophila* NUMB in neuroblasts and consistent with a role for *numb* in repressing NOTCH signaling in newly born prospective neurons. We also show that c-NUMB can modulate neurogenesis (1) by binding directly to the cytoplasmic domain of the activated form of c-NOTCH-1 and preventing its translocation to the nucleus and (2) by inhibiting the ability of the activated form of c-NOTCH-1 to repress neuronal differentiation.

Results

Cloning of a Chicken Numb Homolog

We cloned a cDNA of the chicken homolog of the *Numb* gene family by low-stringency screening of a chicken embryonic brain cDNA library and by subsequent 5′ RACE (see Experimental Procedures). The deduced amino acid sequence obtained from the cDNA revealed the phospho-tyrosine-binding (PTB) domain, which was highly conserved in mammalian and *Drosophila* homologs (Figure 1A). Proline-rich sequences were also recognized in the downstream region of the PTB domain (Figure 1A). These sequences resemble the putative SH3-binding domains as previously suggested (Verdi et al., 1996; Zhong et al., 1996). The amino acid sequence comparison of the chicken *Numb* homolog with *m-Numb* and *m-nbl* showed 97% and 81% identities, respectively. We conclude, therefore, that the identified cDNA encodes the chicken homolog of *m-Numb*, and not *m-nbl*. Consistent with this conclusion, whole-mount in situ hybridization revealed wide spread expression of chicken *Numb* (*c-Numb*) mRNA (data not shown), similar to the expression of *m-Numb* mRNA (Zhong et al., 1996), but different from the neuron-specific expression of *m-nbl* (Zhong et al., 1997).

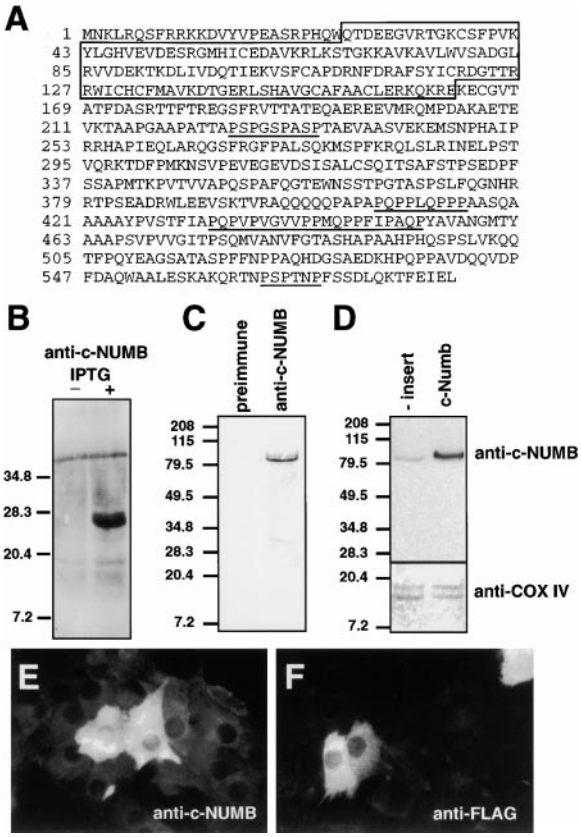

Figure 1. Amino Acid Sequence of c-NUMB and the Specificity of Anti-c-NUMB Antibody

(A) Amino acid sequence of c-NUMB was deduced from the cDNA sequence. A large box indicates phospho-tyrosine-binding (PTB) domain. Underlines reveal putative SH3-binding sequences.

(B) Immunoblot of the bacterially expressed His-tagged carboxy-terminal fragment of c-NUMB with anti-c-NUMB antibody. Induction of c-NUMB expression by IPTG produced an intense band (right lane). The same band was obtained with anti-His antibody, confirming the recognition of His-tagged c-NUMB with anti-c-NUMB antisera (data not shown).

(C) Immunoblot of neural tube extract from E3 chicken embryos. Anti-c-NUMB antibody revealed a band at approximately 80 kDa (right). No nonspecific signal was obtained with preimmune antisera (left).

(D) Immunoblot of NIH3T3 cell extracts. Anti-c-NUMB antibody revealed an intense band at approximately 80 kDa in an extract of *c-Numb* expression vector–transfected 3T3 cells, while a similar sized weak band was detected in a nontransfected cell extract. Reblotting the same membrane with anti-cytochrome oxidase subunit IV (COX IV) showed the same amount of the protein was loaded.

(E) FLAG-tagged *c-Numb*-transfected 3T3 cells showed strong c-NUMB immunoreactivity in the cytoplasm, while weak cytoplasmic staining of potentially endogenous expression of m-NUMB was also visible in the background.

(F) Cultures similar to E were stained with anti-FLAG, showing intense cytoplasmic localization of FLAG-tagged c-NUMB.

c-NUMB Protein Localization

Polyclonal rabbit antiserum was raised against a 20–amino acid peptide in the carboxy terminus of c-NUMB (see Experimental Procedures). This antiserum recognized a bacterially expressed, carboxy-terminal fragment of c-NUMB by Western blot analysis (Figure 1B). The antibody, affinity-purified with an antigen column,

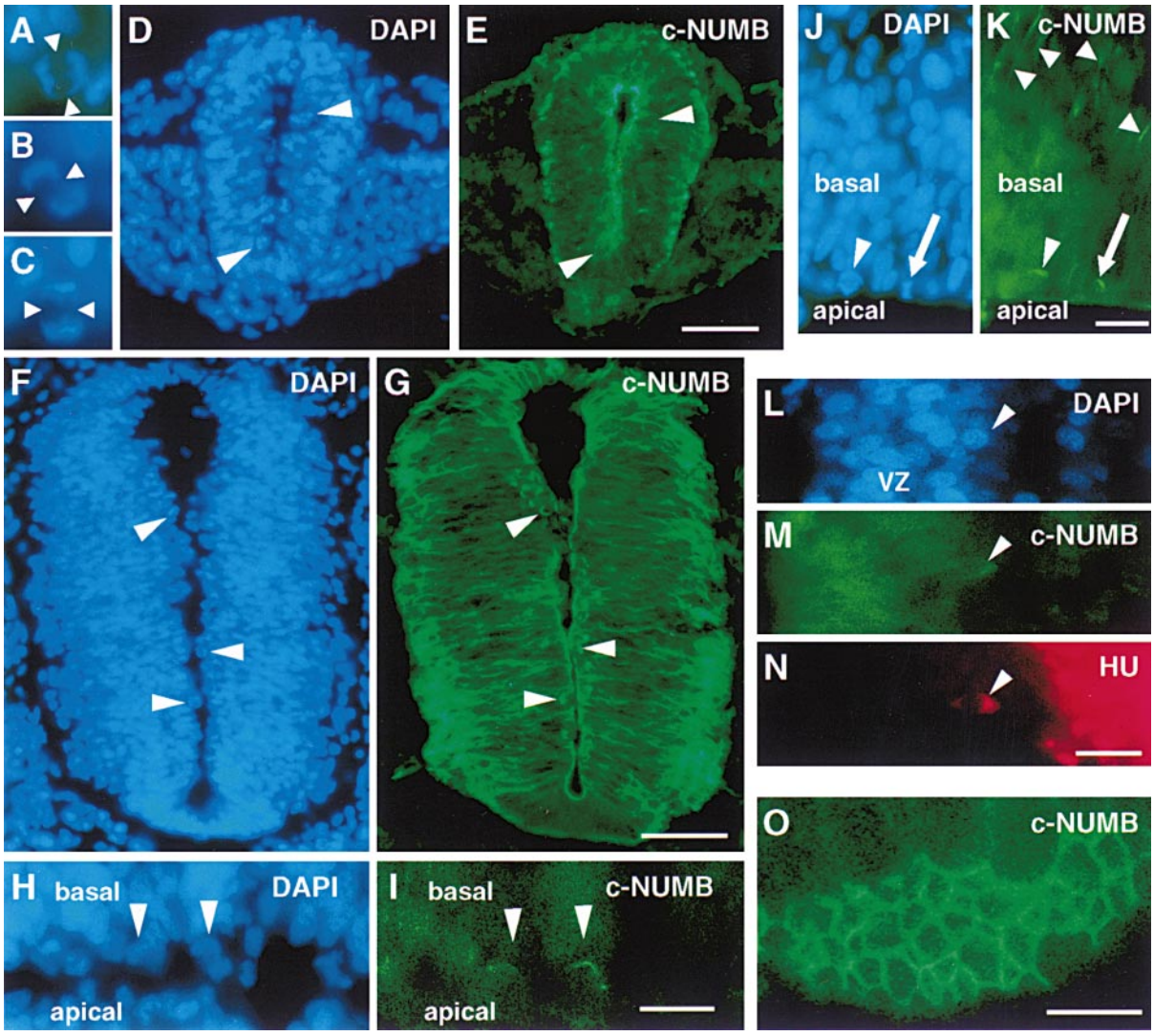

Figure 2. Tissue and Cellular Localization of Anti-c-NUMB Immunoreactivity

(A–C) Symmetric (A), oblique (B), and asymmetric (C) cell divisions in chicken neuroepithelium. E5.5 (stage 27) telencephalon was stained with DAPI.

(D and E) Transverse section of E2 (stage 12) embryo stained with DAPI (D) and anti-c-NUMB (E). Neural tube revealed strong c-NUMB immunoreactivity, particularly in the apical and basal ends of the neuroepithelial cells (E). Mitotic cells (arrowheads) possessed diffuse anti-c-NUMB staining in their cytoplasm.

(F and G) A transverse section of E3.5 (stage 21) embryo stained with DAPI (F) and anti-c-NUMB (G). Virtually all the mitotic figures possessed c-NUMB staining on their basal side ([F and G], arrowheads). Filament-like staining in the apical and basal processes of neuroepithelial cells was also noted (G).

(H and I) A higher magnification of mitotic figures of stage 21 neural tube. Cortical localization was evident on the basal side of mitotic neuroepithelial cells (arrowheads).

(J and K) A transverse section of E4.5 (stage 25) chicken hindbrain. Both horizontally ([J], large arrowhead) and vertically ([J], arrow), dividing cells possessed anti-c-NUMB staining in their basal cortex (K). Filament-like staining in the basal processes of the nonmitotic cells was also observed (K).

(L–N) A transverse section of stage 21 neural tube. A Hu^+ migrating neuron precursor ([N], arrowhead) possessed c-NUMB immunoreactivity in the cytoplasm ([M], arrowhead). Left is central canal (apical), and right is neuronal cell layer (basal).

(O) A near sagittal section of stage 25 forebrain, looking at the apical surface of the ventricle. c-NUMB immunoreactivity in the apical endfeet appears as a meshwork.

Scale bars: (E) and (G), 50 μm; (I), (K), and (N), 20 μm; (O), 10 μm.

detected an approximately 80 kDa band on a Western blot of E3 chicken neural tube protein (Figure 1C). To confirm the specificity of anti-c-NUMB antibody, NIH3T3 cells transfected with a FLAG epitope-tagged *c-Numb* expression vector were stained with anti-c-NUMB antibody. Strong cytoplasmic staining was detected (Figure 1E). Similar strong cytoplasmic staining was obtained with anti-FLAG antibody (Figure 1F). With highly sensitive cy3-conjugated secondary antibody, however, weak immunoreactivity was also observed in nontransfected cells surrounding transfected cells, suggesting the presence of endogenous m-NUMB in NIH3T3 cells (Figure

1E). A Western blot of the *c-Numb*-transfected 3T3 cell extract revealed a strong 80 kDa band (Figure 1D), similar to the size of the band detected in the neural tube extract (Figure 1C). A similarly sized, but weak, band was obtained from nontransfected NIH3T3 cell extract (Figure 1D), confirming the presence of endogenous m-NUMB.

Sections of various stages (stages 12–30) and axial levels of chicken embryos were stained with the anti-c-NUMB antibody. Strong anti-c-NUMB immunoreactivity (IR) was detected throughout the neuroepithelium (Figures 2E and 2G). In nonmitotic neuroepithelial cells, c-NUMB-IR was observed mainly in basal and apical processes and was diminished or absent in the soma (Figure 2K; see also Figures 2E and 2G). Oblique (grazing) sections of the apical surface of the neuroepithelium revealed junctional staining that appeared as a meshwork on the apical surface (Figure 2O). This meshwork-like appearance suggests that the apical c-NUMB-IR belongs to the endfeet of nonmitotic neuroepithelial cells, rather than being localized at the apical side of mitotic cells.

In mitotic neuroepithelial cells, c-NUMB-IR was detected within the basal cortex (Figures 2I and 2K; see also Figure 2G), contrary to previously reported apical localization of m-NUMB-IR (Zhong et al., 1996; see Discussion). This asymmetrical localization of c-NUMB-IR could be detected at all ages examined in virtually all mitotic cells throughout the CNS including the telencephalic hemispheres, diencephalon, mesencephalon, metencephalon, neural retina, and spinal cord. In E3.5 (stage 21) neural tube, E4.5 (stage 25) telencephalon, and E4.5 diencephalon, approximately 80%–85% of mitotic figures possessed c-NUMB-IR basally. However, in the most posterior part of E2 (stages 11–13) neural tube and in the presumptive choroid plexus of E4.5 (stage 25) telencephalon, only diffuse c-NUMB-IR was observed throughout the cell body of mitotic cells (Figure 2E and data not shown). The absence of expression of a panneuronal marker (Hu-IR; Marusich et al., 1994; Wakamatsu and Weston, 1997) revealed that no neuronal differentiation took place in these locations (data not shown). We conclude, therefore, that the timing of the asymmetrical localization of c-NUMB-IR is correlated with neurogenesis.

To confirm the basal localization of c-NUMB-IR in mitotic neuroepithelial cells, in vivo transfection experiments were performed (see Experimental Procedures). FLAG-tagged *c-Numb* expression vector was injected into the right telencephalic hemisphere of E4 (stages 22 and 23) chicken embryos. Brains were fixed, sectioned, and stained with anti-FLAG and anti-c-NUMB antibodies 8–10 hr after injection (Figure 3). Although FLAG$^+$ cells undergoing mitosis were extremely rare, none of these contained apically localized FLAG-IR. Some cells (3/11; Figures 3A and 3B) clearly revealed basal localization, similar to c-NUMB-IR, whereas in the remainder of the transfected cells (8/11), expression of the transgene was high and FLAG-IR was detected throughout the cytoplasm (data not shown). In addition, overexpressed FLAG-tagged c-NUMB also localized throughout the cytoplasm of nonmitotic neuroepithelial cells with high concentration in both the basal processes and the apical endfeet of these cells, supporting the suggestion above

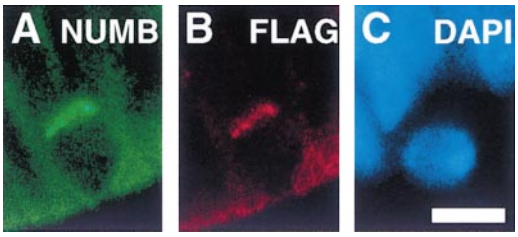

Figure 3. Exogenous c-NUMB Localizes in the Basal Cortex of a Mitotic Neuroepithelial Cell

A transverse section of E4.5 (stage 24) chicken embryonic forebrain, 8 hr after in vivo transfection with FLAG-tagged *c-Numb* expression vector. Both anti-c-NUMB (A) and anti-FLAG (B) immunoreactivities localized basally in the cortex of an early prophase mitotic figure revealed by DAPI staining ([C], arrows). Scale bar: 10 μm.

that endogenous c-NUMB-IR in the vicinity of mitotic cells belonged to the apical endfeet of nonmitotic cells (data not shown). Taken together, these data allow us to conclude that c-NUMB localizes in the basal cortex of mitotic neuroepithelial cells.

Our observations suggest that, upon asymmetrical (horizontal) cell division, the basal daughter cells retain c-NUMB and differentiate into neurons, whereas c-NUMB-depleted apical daughter cells remain as neuroepithelial cells. To examine this inference further, we analyzed the occurrence of asymmetrical cell division in the chicken CNS. Day 5.5 (stage 27) telencephalon was stained with DAPI, and the orientation of metaphase to telophase mitotic figures was examined in neuroepithelial cells. Approximately 40% of mitotic figures indicated either oblique (22%; Figure 2B) or asymmetrical cell division (18%; Figure 2C), similar to the ferret neocortex (Chenn and McConnell, 1995). This observation suggested that basally localized c-NUMB in mitotic neuroepithelium would be segregated into basal daughter cells. Consistent with this idea, we found that the migrating neuronal precursor cells, which expressed the panneuronal marker (Hu-IR), also possessed c-NUMB-IR (Figures 2M and 2N). Although it is still possible that the c-NUMB in these migrating neuronal precursors was newly synthesized after the mitosis, this observation suggests that NOTCH signaling in neuronal precursors is repressed by c-NUMB.

Activation of NOTCH Signaling and Misexpression of c-Numb Modifies Neurogenesis in Neuroepithelium

To elucidate the role of *c-Numb* in neuronal differentiation, in vivo transfections of E4 (stages 22 and 23) embryos were performed with various FLAG-tagged expression vectors: (1) green fluorescent protein (*GFP*) as a negative control, (2) an activated form of *c-NOTCH-1* (*CNIC*$^{\Delta C89}$), containing most of the cytoplasmic domain of chicken NOTCH-1 but lacking an 89–amino acid sequence within the carboxy terminus that includes the PEST sequence (see Experimental Procedures), and (3) FLAG-tagged *c-Numb* (see above). Since the DNA solutions were injected into the brain ventricle, the transfection was achieved mostly in neuroepithelial cells. First, since the activated form of c-NOTCH-1 localizes in the nucleus, neuroepithelium was cotransfected with *E. coli* β-galactosidase in order to visualize the location and

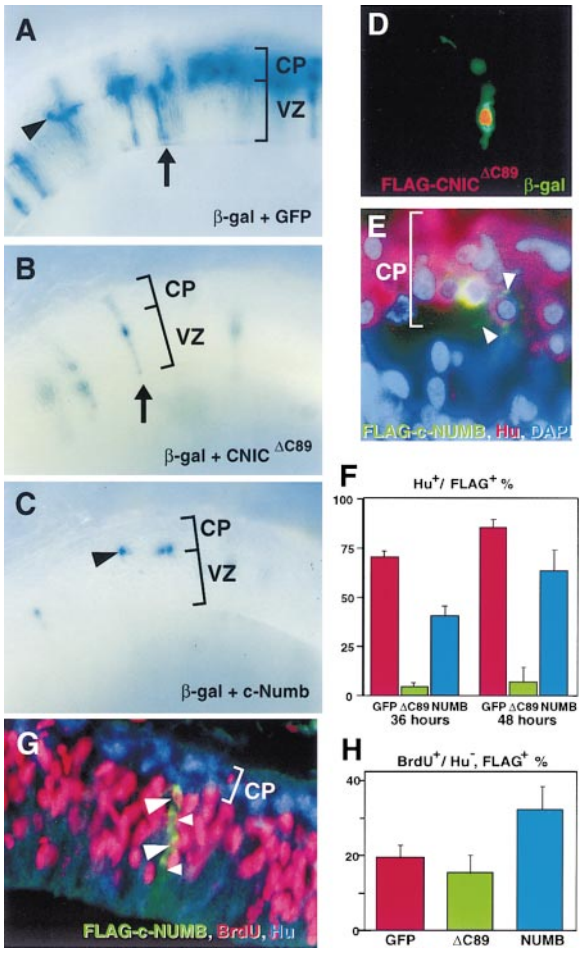

Figure 4. Misexpression of the Activated Form of *c-Notch-1* and *c-Numb* Modulates Neurogenesis in Chicken Nervous Tissues

(A–F) Effect of *CNIC*$^{\Delta C89}$ and *c-Numb* misexpression in neuronal differentiation of chicken forebrain.

(A–C) Localization and morphology of transfected cells revealed by X-gal staining of β-galactosidase activity (48 hr after transfection). Cells transfected with β-galactosidase and *GFP* showed both neuroepithelial (arrow) and neuronal (arrowhead) morphologies (A). *CNIC*$^{\Delta C89}$-transfected cells primarily possessed bipolar neuroepithelial cell shape (B). Most of *c-Numb*-transfected cells showed neuronal morphology and were localized in the cortical plate (C).

(D) Coexpression of β-galactosidase and CNIC$^{\Delta C89}$.

(E) An example of a FLAG-tagged *c-Numb*-transfected neuron (36 hr after transfection). Anti-FLAG, anti-Hu, and DAPI nuclear staining is shown in green (FITC), red (TRITC), and blue, respectively. The transfected cell shows yellow color due to the overlap of green and red fluorescences. Arrowheads indicate neurites of the transfected cell.

(F) Cell counts of Hu$^+$ cells in FLAG$^+$-transfected populations 36 and 48 hr after transfection. In vivo transfected brains were sectioned and stained with anti-Hu and anti-FLAG. To obtain each bar, anti-Hu immunoreactivity of 100–300 FLAG$^+$ cells of three brains was examined.

(G) An example of a nest of undifferentiated FLAG-tagged *c-Numb*-transfected cells (36 hr after transfection). Anti-FLAG, anti-Hu, and anti-BrdU stainings are shown in green (FITC), blue (AMCA-S), and red (TRITC), respectively. Large and small arrowheads indicate BrdU$^+$- and BrdU$^-$-transfected cells, respectively.

(H) Cell counts of BrdU$^+$ cells in FLAG$^+$, Hu$^-$ populations 36 hr after transfection.

Abbreviations: CP, cortical plate; VZ, ventricular zone.

the morphology of the transfected cells (Figures 4A–4D). The transfection was performed with a transgene: β-galactosidase DNA ratio of 3:1 to assure that virtually all the β-galactosidase$^+$ cells also coexpressed the transgene. Coexpression of transgenes was confirmed by anti-β-galactosidase and anti-FLAG antibody staining (Figure 4D). *GFP*-transfected cells revealed both neuronal and neuroepithelial morphology (Figure 4A). In contrast, virtually all *CNIC*$^{\Delta C89}$-transfected cells revealed neuroepithelial morphology with long basal and apical processes (Figures 4B and 4D). Most of the *c-Numb*-transfected cells revealed round morphology and were located in the cortical plate, suggesting their neuronal identity (Figure 4C), although some neuroepithelial cells were also observed in the ventricular zone.

To assess neurogenesis further, expression of the Hu-IR was examined in FLAG$^+$-transfected cells (Figures 4E and 4F). It should be noted that, since the transfection efficiency was relatively low, the transfectants often formed a nest of cells, and we could examine the results at a semiclonal level. This analysis revealed that 70.5% and 85.4% of *GFP*-transfected cell population were Hu$^+$ 36 and 48 hr after transfection, respectively. In contrast, only a few of *CNIC*$^{\Delta C89}$-transfected cells coexpressed Hu-IR (4.1% and 6.7% at 36 and 48 hr after transfection, respectively). Therefore, activation of NOTCH signaling repressed Hu expression in a cell-autonomous manner and likely inhibited neuronal differentiation. In the case of *c-Numb* transfection, neuronal differentiation was clearly observed. *c-Numb*-transfected Hu$^+$ cells often possessed long neurites, confirming their identity as maturing neurons (Figure 4E). Paradoxically, however, the proportion of Hu$^+$ cells in *c-Numb*-transfected cell populations was smaller than that in *GFP*-transfected cell populations (40.1% and 63.2% at 36 and 48 hr after transfection, respectively; Figure 4F). Although *c-Numb* was expected to repress NOTCH signaling, detailed observation revealed that neuroepithelial cells were heterogeneous in response to the overexpression of *c-Numb*. Hence, although most of the *c-Numb*-transfected cells were sparsely distributed in the cortical layer and coexpressed Hu-IR, a minor population of transfected cells formed clusters of undifferentiated neuroepithelial cells (Figure 4G), suggesting the stimulation of proliferation in some of the transfected clones. If we exclude these neuroepithelial clusters, the percentage of Hu$^+$ cells in *c-Numb*-transfected population is nearly as high as the percentage in *GFP*-transfected population (64.5% and 74.4% at 36 and 48 hr after transfection, respectively). To test the effect of *c-Numb* overexpression on cell proliferation, brains were pulse labeled with BrdU for 40 min after 36 hr of transfection. Transfected, BrdU-labeled brains were sectioned and triple-stained with anti-FLAG, Hu, and BrdU antibodies. In these preparations, the proportion of BrdU$^+$ cells among FLAG$^+$, Hu$^-$ cells (transfected, undifferentiated cells) was higher in the *c-Numb*-transfected population than in the *CNIC*$^{\Delta C89}$- or *GFP*-transfected cells, suggesting that NUMB increased the proliferation rate (Figures 4G and 4H) in a subset of neuroepithelial cells. Because of the low transfection efficiency, a nest of undifferentiated cells was likely to be a cluster of daughter cells derived from a single transfected cells. Taken together, we conclude that, although *c-Numb* overexpression in many

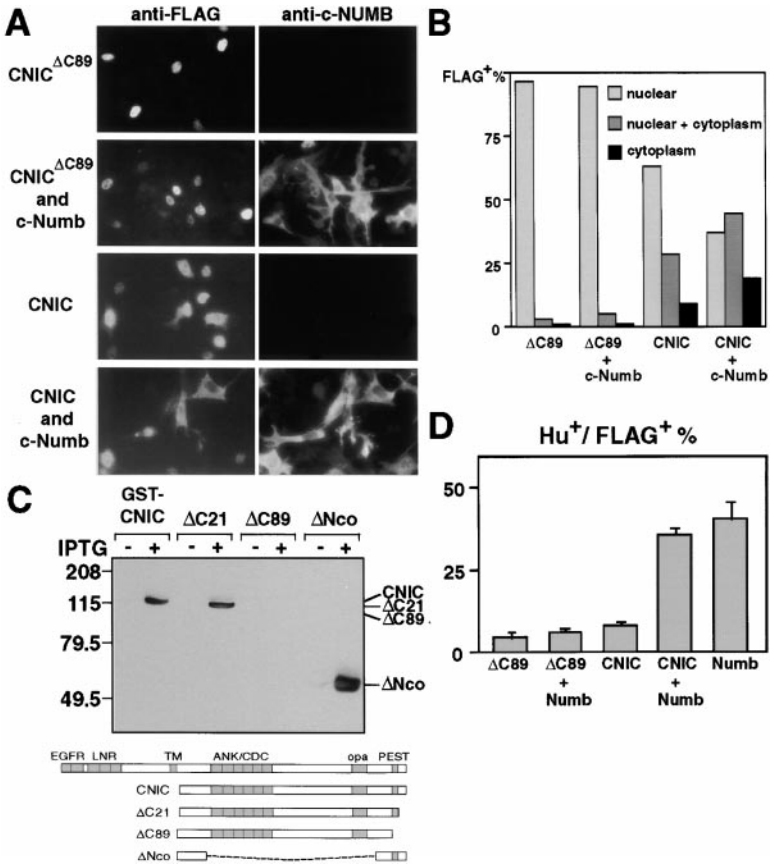

Figure 5. The Carboxy-Terminal Portion of c-NOTCH-1 Is Required for c-NUMB Function

(A) Staining of NIH3T3 cells transfected with FLAG-tagged *c-Notch-1* expression vectors with or without *c-Numb* expression vector. CNIC$^{\Delta C89}$ localized in the nuclei. CNIC localization was mostly in the nucleus but was also diffusely present in the cytoplasm, whereas, in the presence of c-NUMB, CNIC tended to localize primarily in the cytoplasm.

(B) Cell counts of (A). Data for each bar were based on examination of 800–1000 FLAG$^+$ cells.

(C) c-NUMB binds to the PEST domain of c-NOTCH-1. c-NUMB expressed in NIH3T3 cells binds to bacterially expressed CNIC, CNIC$^{\Delta C21}$ CNIC$^{\Delta Nco}$, but not to CNIC$^{\Delta C89}$. Expression of GST fusion proteins was induced by IPTG. c-NUMB was detected with anti-c-NUMB antibody. Expression of GST fusion proteins was confirmed by CBB gel staining (data not shown). Molecular weight markers are indicated on the left (kDa). Abbreviations: EGFR, EGF repeats; TM, transmembrane domain; ANK/CDC, ankyrin/cdc repeats.

(D) Cell counts of Hu$^+$ cells in FLAG$^+$-transfected populations 36 hr after transfection. In vivo transfected brains were sectioned and stained with anti-Hu and anti-FLAG. In the case of cotransfection, the ratio of *CNIC* (or *CNIC*$^{\Delta C89}$) and *c-Numb* plasmid DNA was adjusted to 1:3 to assure the coexpression of *c-Numb* in *CNIC* (or *CNIC*$^{\Delta C89}$) –transfected cells, and transfectants were detected by FLAG-tag on the CNIC proteins. To obtain each bar, Hu-IR of 100–300 FLAG$^+$ cells of three brains was examined.

cases permitted neuroepithelial cells to differentiate into neurons, *c-Numb* also stimulated a minor population of neuroepithelial cells to proliferate, so that overall, the proportion of Hu$^+$ neuronal cells in *c-Numb*-transfected population ended up being lower than that in the *GFP*-transfected population.

c-NUMB Binding to C-NOTCH-1 Interferes with Nuclear Translocation and the Subsequent Inhibition of Neurogenesis

As previously described, the cytoplasmic domain of NOTCH protein localizes in the nucleus (Fortini et al., 1993; Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993; Schroeter et al., 1998; Struhl and Adachi, 1998). Previous studies have also suggested that the inhibition of NOTCH-signaling by *numb* is mediated by the direct binding of NUMB to the cytoplasmic domain of NOTCH protein (Guo et al., 1996; Zhong et al., 1996). To test the effect of c-NUMB on nuclear localization of the cytoplasmic domain of c-NOTCH-1, therefore, FLAG-tagged *CNIC*$^{\Delta C89}$ and *c-Numb* expression vectors were cotransfected into NIH3T3 cells (Figures 5A and 5B). Cells cotransfected with *CNIC*$^{\Delta C89}$ and *c-Numb* expression vectors revealed c-NUMB-IR in the cytoplasm (Figure 5A), similar to cells transfected with *c-Numb* expression vector alone (Figure 1E). Likewise, cells transfected with *CNIC*$^{\Delta C89}$ possessed FLAG-IR mostly in the nucleus, regardless of whether cells were transfected with *CNIC*$^{\Delta C89}$ expression vector alone or cotransfected with *c-Numb*. Since the *CNIC*$^{\Delta C89}$ construct lacked the C-terminal portion of the cytoplasmic domain of c-NOTCH-1 that includes the PEST sequence, we also prepared a FLAG-tagged expression vector carrying the whole cytoplasmic domain of c-NOTCH-1 (CNIC). Although the *CNIC* expression vector alone gave rise to diffuse staining in the cytoplasm in addition to a strong staining in the nucleus, cotransfection with *CNIC* and *c-Numb* expression vectors resulted in a dramatic increase in cytoplasmic localization of FLAG-IR and a decrease in nuclear localization. It should be noted that some of the *c-Numb*-transfected cells possessed CNIC in their nuclei (Figure 5A), suggesting that some other, as yet unknown, factor(s) is involved in the c-NUMB-mediated cytoplasmic localization of CNIC.

The intracellular localization of c-NOTCH-1 and c-NUMB suggested that c-NUMB interacts with the C-terminal portion of c-NOTCH-1 (possibly the PEST domain) and inhibits nuclear translocation of c-NOTCH-1. This idea was supported by Far Western blotting analyses. Thus, in addition to CNIC and CNIC$^{\Delta C89}$, two other deletion mutant forms of c-NOTCH-1 were constructed (see Experimental Procedures). CNIC$^{\Delta C21}$ lacked only a 21–amino acid sequence of the C-terminal end and still possesses the PEST sequence. CNIC$^{\Delta Nco}$ lacks most of the cytoplasmic domain of c-NOTCH-1, including the ankyrin/cdc 10 repeats and opa sequence, but includes the C-terminal portion containing the PEST sequence. Lysates of bacteria carrying GST fusion of these constructs were resolved on SDS-PAGE and transferred

onto a nitrocellulose membrane. This membrane was then incubated with a lysate from NIH3T3 cells transfected with *c-Numb* expression vector. Binding of c-NUMB to bacterially expressed GST–CNIC, CNIC$^{\Delta C21}$, and CNIC$^{\Delta Nco}$ was detected with anti-c-NUMB antibody. In contrast, no binding was observed to CNIC$^{\Delta C89}$ (Figure 5C). Although a previous study reported the binding of m-NUMB to both the N- and C-terminal portion of the cytoplasmic domain of NOTCH-1 (Zhong et al., 1996), we conclude that the major c-NUMB-binding site of c-NOTCH-1 is located in the C-terminal portion and that the PEST sequence is essential and probably sufficient for this binding. These results, taken together, suggest that c-NUMB may function as a cytoplasmic anchor for the activated form of c-NOTCH-1 and thereby prevents translocation to the nucleus.

We also tested whether c-NUMB could interfere with the ability of activated c-NOTCH-1 to inhibit neurogenesis in the neuroepithelium of chicken brain (Figure 5D). In vivo transfection of FLAG-tagged *CNIC* and *CNIC*$^{\Delta C89}$ revealed such inhibition of neurogenesis. Only 7.9% and 4.1%, respectively, of FLAG$^+$ cells were Hu$^+$. Cotransfection of *c-Numb* failed to antagonize the effect of *CNIC*$^{\Delta C89}$ (6.1%), whereas cotransfection of *c-Numb* with *CNIC* resulted in neurogenesis (35.7%) at a level comparable to when cells were transfected with *c-Numb* alone (40.1%). These results suggested that, upon asymmetrical cell division, the binding of c-NUMB to the PEST domain of c-NOTCH-1 permits neuronal differentiation by inhibiting the nuclear translocation of c-NOTCH-1 in basal daughter cells.

Since this model depends on the distribution of NOTCH proteins in mitotic neuroepithelial cells, it is important to establish where NOTCH proteins are localized in avian neuroepithelial cells. To do so, we used a synthetic polypeptide corresponding to the C-terminal portion of c-NOTCH-1 to produce a polyclonal antibody that recognized recombinant CNIC–GST fusion protein on a Western blot (data not shown). This antibody also recognized NIH3T3 cells transfected with *CNIC* expression vector, but not cells transfected with *CNIC*$^{\Delta C89}$ vector (Figure 6A). Immunological staining of chicken CNS revealed punctate c-NOTCH-1-IR throughout the ventricular zone (Figure 6B), which resembled the cell surface staining of NIH3T3 cells transfected with full-length rat *Notch-1* expression vector (data not shown). c-NOTCH-1-IR was observed uniformly in the mitotic neuroepithelial cells (Figures 6C and 6D).

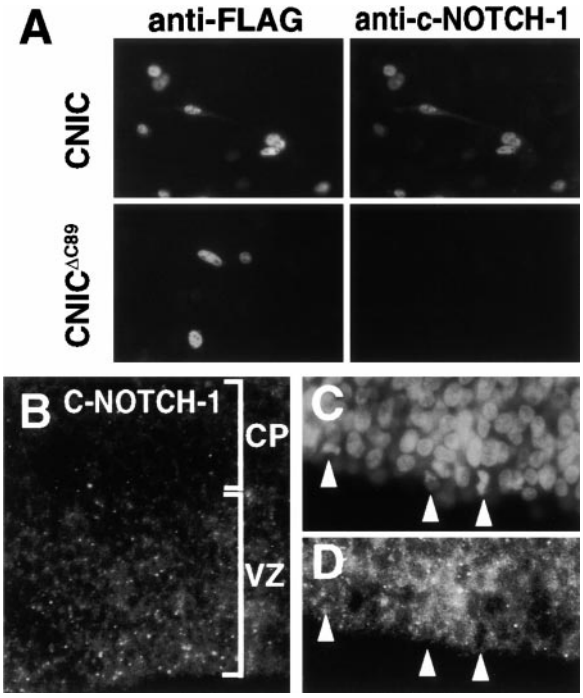

Figure 6. *c-NOTCH-1* Is Localized Homogeneously in Mitotic and Nonmitotic Neuroepithelial Cells

(A) Immunostaining of NIH3T3 cells transfected with FLAG-tagged *CNIC* and *CNIC*$^{\Delta C89}$ vectors. Only the nuclei of *CNIC*-transfected cells were double stained with anti-FLAG and anti-c-NOTCH-1 antibodies.

(B) Immunostaining of E5.5 (stage 28) telencephalon. c-NOTCH-1-IR was mainly observed in the ventricular zone (VZ) and was weak in the cortical plate (CP).

(C) DAPI staining of (D). Arrowheads indicate the metaphase mitotic figures.

(D) Higher magnification of ventricular zone. c-NOTCH-1-IR was homogeneously observed in the mitotic neuroepithelial cells (arrowheads).

Discussion

Asymmetrical cell division has been reported to occur in the neuroepithelium of vertebrate (ferret) neocortex. After such asymmetrical division, basal daughter cells appear to differentiate into neurons, and apical daughter cells remain undifferentiated (Chenn and McConnell, 1995). We have now shown that the proportion of asymmetrical cell division in chicken brain is similar to that in ferret brain. In recent years, the importance of asymmetrical localization of cell fate determinants has been widely recognized in invertebrate development. For example, PROSPERO and NUMB are asymmetrically localized during neuroblast division in the developing *Drosophila* CNS. Since extensive similarities in the regulatory mechanism of early development have been revealed between insects and vertebrates, and since vertebrate homologs of both *prospero* and *numb* have now been identified (Oliver et al., 1993; Verdi et al., 1996; Zhong et al., 1996, 1997), it is important to test whether *prospero* and *numb* are involved in asymmetrical cell division and subsequent fate determination of vertebrate CNS precursors.

Initial studies on the distribution of NUMB and NOTCH in asymmetrically dividing neuroepithelial cells in vertebrates, however, have challenged our understanding of NOTCH function in neurogenesis. Thus, it has been reported that NUMB-IR is localized on the apical side of mitotic neuroepithelial cells in mice (Zhong et al., 1996). Further, NOTCH-IR has been reported to be localized basally in mitotic neuroepithelial cells in the developing ferret neocortex (Chenn and McConnell, 1995). Since the basal daughter cells of asymmetrically dividing neuroepithelial cells appear to undergo neuronal differentiation in these vertebrate systems, it has been proposed that apical daughter cells that receive NUMB remain undifferentiated (Zhong et al., 1996, 1997). Activation of NOTCH signaling in the basal daughter cells has also

been proposed to be responsible for causing the postmitotic, but nondifferentiated, state of migratory daughter cells (Chenn and McConnell, 1995; Zhong et al., 1996, 1997). This unprecedented role for NOTCH in promoting a nondividing, but nondifferentiated, intermediate neuronal phenotype and the implied role of NUMB in preventing neuronal differentiation by repressing NOTCH function in apical cells seem paradoxical and difficult to reconcile with previous results. In contrast, the basal localization of NUMB that we have observed suggests a more parsimonious model in which NUMB inhibits NOTCH signaling and thereby permits neuronal differentiation in the basal daughter cells. This model is consistent both with the perceived function of NOTCH to inhibit neuronal differentiation in vertebrate neurogenesis (Coffman et al., 1993; Austin et al., 1995; Chitnis et al., 1995; Dorsky et al., 1995; Lardelli et al., 1996; Dornseifer et al., 1997; Henrique et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998) and with the role of NUMB to suppress that inhibition in the development of the *Drosophila* nervous system (Rhyu et al., 1994; Spana et al., 1995; Frise et al., 1996; Guo et al., 1996). At present, both of these models remain to be tested further. Perhaps, future experiments using m-NUMB targeted loss-of-function mutations will be useful to elucidate the issue.

In avian CNS development, however, we have observed homogeneous distribution of c-NOTCH-1 immunoreactivity in the mitotic neuroepithelial cells. Uniform localization of NOTCH-1 has also been observed in mouse neocortex (Zhong et al., 1997). This supports the idea that NOTCH-1 will be equally distributed into both basal and apical daughter cells upon asymmetrical cell division. Consequently, the activation or repression of NOTCH-1 function will depend on the localization of other factors such as NUMB. At present, we do not know the reason for the discrepancy between our results and the pattern of NOTCH immunoreactivity in ferret brain (Chenn and McConnell, 1995). One possible explanation, however, is that the NOTCH-1 antibody, which was developed with human NOTCH-1 sequence and not fully characterized in ferret, might recognize a NUMB-inactivated form of NOTCH-1 in the ferret brain.

We have also shown that c-NUMB localizes asymmetrically in the basal cortex of mitotic neuroepithelial cells, and we observed c-NUMB inheritance in migrating neuronal precursors. These observations suggest that, upon asymmetric cell division, c-NOTCH-1-mediated signaling is activated in apical daughter cells, and that c-NUMB inhibits the activation of NOTCH signaling in basal daughter cells, so that these cells are allowed to differentiate into neurons. This model is consistent with the function of vertebrate *Notch-1* and *Drosophila Numb* (see above), but not with the reported apical localization of m-NUMB (Zhong et al., 1996). We cannot, at present, explain the apparent discrepancy between our data demonstrating basal c-NUMB localization and the data reporting apical localization of m-NUMB (Zhong et al., 1996, 1997). However, several possibilities might be suggested that support our findings. First, although the differences in localization of NUMB-IR might reflect species differences, this possibility is weakened by the fact that our results correspond to the localization of *Drosophila* NUMB in the basal cortex of neuroblasts (Rhyu et al., 1994; Spana et al., 1995). Second, it is possible that the antibodies used in one or both of the studies cross-react with the closely related Nbl protein, which, in turn, could alter the interpretation of localized immunoreactivity. More complete characterization of the antibodies used in these studies might be useful. However, based on the results of our Western analysis and correspondence of FLAG- and NUMB-IR in transfected cells (Figure 1) and the localization of exogenous c-NUMB protein (Figure 3) it is unlikely that our immunostaining represents Nbl. Moreover, since Nbl is reported to be expressed in differentiating neurons present in the cortical plate (Zhong et al., 1997), it also seems unlikely that the localized immunoreactivity that we report in early neural tubes (see Figures 2D–2G) can be attributed to Nbl. In addition, our inferences about the localization and function of c-NUMB are strengthened by our observation that c-NUMB localizes basally in mitotic cells only when and where neurogenesis is occurring.

We have also shown that overexpression of *c-Numb* permits neuroepithelial cells to differentiate into neurons in chicken brain, whereas overexpression of activated *c-Notch-1* dramatically inhibits neurogenesis. Moreover, coexpression of exogenous *c-Numb* antagonizes the effect of the activated *c-Notch-1*. These observations are consistent with the idea that *c-Numb* suppresses NOTCH signaling and permits neuronal differentiation.

However, c-NUMB overexpression also causes increased proliferation in a minor population of neuroepithelial cells. Although we do not know the difference between this minority and the majority that undergoes neuronal differentiation, a few possibilities can be considered. First, *c-Numb*-transfected cells might interact. Inhibition of NOTCH signaling by c-NUMB may cause the upregulation of NOTCH ligands, so that gross activation of NOTCH signaling will be increased among the transfectants (e.g., Parks et al., 1997). Second, the role of NOTCH signaling might be bimodal. It has been reported that, in *Drosophila* eye development, NOTCH signaling is required both for specification of R8 neurons and for subsequent lateral inhibition of neighboring cells not to take R8 fate (Baker and Yu, 1997; Ligoxygakis et al., 1998). Therefore, repression of NOTCH signaling by c-NUMB may inhibit neurogenesis in a minor population of cells that has not completed the initial specification. Finally, fate-restricted precursors might be present in the neuroepithelium. As described previously, rat cerebral cortex has fate-restricted precursors as well as multipotent cells (Williams and Price, 1995). Thus, the chicken neuroepithelium used in this study may be composed of heterogeneous cell populations that differ in their response to the overexpression of *c-Numb*. If this is so, however, the identity of cells whose proliferation is stimulated by overexpression of *c-Numb* remains to be determined.

Previous studies have shown that *Drosophila* NUMB and mouse NUMB directly bind to the cytoplasmic domain of NOTCH and NOTCH-1, respectively (Guo et al., 1996; Zhong et al., 1996). Recent studies have revealed that cleavage of the NOTCH cytoplasmic domain is required upon activation, which subsequently leads to translocation of this domain to the nucleus (Schroeter et al., 1998; Struhl and Adachi, 1998). Consistent with this interpretation, we have demonstrated that c-NUMB

binds to the PEST domain within the C terminus of c-NOTCH-1 and reduces the nuclear translocation of c-NOTCH-1. However, since the combination of high expression level of transgenes and heterologous assay systems may misrepresent the real function of *c-Numb*, further biochemical studies remain to be performed. Interestingly, as described above, the presence of c-NUMB appears not to be sufficient to inhibit nuclear translocation of activated c-NOTCH-1. Consistent with our inference, it has been shown that rat NUMB binds to SH3 domains of SRC family kinases, possibly through the proline-rich sequences (Verdi et al., 1996). Moreover, it has recently been shown that PON (*partner of numb* gene product) binds and colocalizes with NUMB in *Drosophila* (Lu et al., 1998). Therefore, c-NUMB may be an adaptor protein and requires other proteins such as cytoskeletal components and other adaptor proteins to anchor the c-NOTCH-1 in the cytoplasm. Further studies on the interaction of NUMB and these molecules will provide opportunities to understand the regulatory mechanism of NOTCH signaling and asymmetrical cell division in vertebrate systems.

Experimental Procedures

Experimental Animals

Fertilized chicken (F_1 of White Leghorn × New Hampshire) and Japanese quail (*Coturnix coturnix japonica*) eggs were obtained from Oregon State University, Poultry Program, Animal Sciences Department, Corvallis, Oregon. Embryos were staged according to Hamburger and Hamilton (1951).

cDNA Cloning

A short fragment of mouse *Numb* (Zhong et al., 1996) was amplified by PCR from mouse 11-day embryonic cDNA (Clontech). The amplified cDNA fragment was used as a probe to screen a chicken embryonic brain cDNA library (Funahashi et al., 1993). One identified clone, cNb13 (2.5 kb), contained coding region corresponding to the downstream region of the PTB domain and also contained a 3′ untranslated sequence followed by a polyA tail. Based on sequence similarity, we have concluded that this clone encoded the chicken homolog of mouse *Numb* (*c-Numb*; see Results). To obtain the full coding sequence, total RNA was prepared from E4 chicken embryos with Trizol (GIBCO), polyA$^+$ RNA was selected with Oligotex beads (Qiagen), and cDNA synthesis and 5′RACE was performed with the Marathon RACE kit (Clontech) with the *c-Numb* specific primers. To obtain full-length *c-Numb* cDNA, the cNb13 and 5′RACE products were connected.

A 1.7 kb fragment of quail *Notch-1* was PCR amplified from oligo-dT-primed cDNA of E6 quail embryo dorsal root ganglia, generated with Superscript II (GIBCO). This quail *Notch-1* cDNA fragment was used to screen the cDNA library described above, and a 3.7 kb fragment of the chicken *Notch-1* (CN1) was obtained. This fragment covered the coding region for the entire intracellular portion of the protein, as well as the Lin-12/Notch repeats and two EGF repeats.

Expression Constructs

Eukaryotic Expression Vectors

A 2.2 kb fragment of *c-Notch-1*, consisting of the intracellular portion of the protein, was PCR amplified from CN1. This fragment lacked an 89–amino acid sequence at the carboxy terminus, including the PEST sequence, and was designated CNIC$^{\Delta C89}$ and subcloned into pyDF31 for FLAG epitope-tagging. The entire cytoplasmic domain of *c-Notch-1* (CNIC) was obtained from CN1 by MscI–NotI digestion, then subcloned into pyDF30 for FLAG epitope-tagging. These FLAG-tagged cDNAs were subcloned into the pmiwSV expression vector (Wakamatsu et al., 1997). The expression vector containing rat full-length Notch-1 (pBOS Notch-1) was kindly provided by Dr. G. Weinmaster.

To remove the noncoding sequences of *c-Numb*, the coding region was PCR amplified and subcloned into either pyDF30 for FLAG epitope-tagging or directly into pmiwSV. FLAG-tagged *c-Numb* cDNA was also subcloned into pmiwSV.

Bacterial Expression Vectors

c-Numb cDNA corresponding to the carboxy-terminal portion (aa [amino acids] 436–582) was PCR amplified and subcloned into pTrcHisA (Invitrogen) to produce a His-tagged c-NUMB fragment. *CNIC*$^{\Delta C21}$, which lacks a C-terminal 21–amino acid sequence, was PCR amplified. *CNIC*$^{\Delta Nco}$, which lacks ankyrin/cdc ten repeats and opa sequence, was created by the NcoI digestion and subsequent self ligation of *CNIC*. GST fusions of *CNIC* and deletion constructs (see above) were made in pGEX2T plasmid (Pharmacia). Expression of these tagged or fusion proteins were induced according to manufacturer's protocol.

Antibody Production

A 21–amino acid synthetic polypeptide corresponding to the carboxy-terminal end of c-NUMB, with an additional cysteine for KLH conjugation (CNPSPTNPFSSDLQKTFEIEL), and a 20–amino acid polypeptide corresponding to the carboxy-terminal end of c-NOTCH-1 (CSSPPTSMQGHIPEAFK) were obtained from Alpha Diagnostic International and used to immunize rabbits. Both anti-c-NUMB and anti-c-NOTCH-1 polyclonal antibodies were purified from antiserum with antigen-coupled affinity columns (Sulfolink kit; Pierce) according to the manufacturer's instructions.

In Situ Hybridization and Immunological Staining

Immunological staining was performed as described previously (Wakamatsu et al., 1997). Anti-FLAG (Zymed), c-NUMB, and c-NOTCH-1-IR were detected either by FITC-conjugated (Jackson) or cy3-conjugated (Cappel) anti-rabbit IgG antibodies. Anti-Hu 16A11 (mouse IgG2b; Marusich et al., 1994), anti-FLAG M2 (mouse IgG1; Sigma), and anti-BrdU (mouse IgG1; Boehringer) antibodies were detected by anti-mouse IgG-cy3 (Chemicon) or TRITC, FITC, or biotin-conjugated anti-mouse IgG2b, IgG1 antibodies (Southern Biotechnologies) in combination with Neutra-Avidin AMCA-S (Molecular Probe).

Whole-mount in situ hybridization was performed as described previously (Wakamatsu and Weston, 1997).

Transfection into Cultured Cells

NIH3T3 mouse fibroblast cell line was transfected with Lipofect-Amine PLUS (GIBCO), according to the manufacturer's instruction.

In Vivo Transfection into Chicken Brain

Superfect transfection solution was prepared according to the manufacturer's procedure (Qiagen). Extraembryonic membranes were partially torn to expose the head of E4 (stages 22 and 23) chicken embryos. Fluid in the brain ventricle was partially removed by gentle suction with a mouth pipette, then transfection solution was injected into the ventricle. Initially, most of the transfected cells were found in the ventricular zone 8–12 hr after the transfection. X-gal staining was performed as described previously (Wakamatsu et al., 1997).

BrdU Pulse Labeling of Chicken Brain

BrdU solution (10 μg/ml in PBS) was injected into the ventricle of chicken telencephalon, and injected embryos were fixed in 4% PFA/PBS after 40 min of the incubation. Anti-BrdU staining was performed as described previously (Marusich et al., 1994).

Immunoblots and Far Westerns

Bacterial lysates or cell extracts were separated on SDS-PAGE gel, transferred onto nitrocellulose membrane, and processed further. For standard Western analyses, anti-c-NUMB polyclonal, anti-His tag polyclonal (SantaCruz), and anti-cytochrome oxidase subunit IV monoclonal (Capaldi et al., 1995) antibodies were used as primary antibodies. Horseradish peroxidase (HRP)–conjugated anti-rabbit IgG (Cappel) and alkaline phosphatase–conjugated anti-mouse IgG (Southern Biotechnologies) antibodies were used as secondaries. An ECL chemoluminescence system (Amersham) was used to detect HRP, and NBT/BCIP was used to detect alkaline phosphatase activity.

For Far Western analysis, lysates of *E. coli* carrying pGEX2T-*CNIC*, *CNIC*$^{\Delta C21}$, *CNIC*$^{\Delta C89}$, and *CNIC*$^{\Delta Nco}$ were separated on SDS-PAGE, transferred onto a nitrocellulose membrane, incubated with 5% skim milk/PBS, then incubated with extracts of pmiw*c-Numb*-transfected NIH3T3 cells for 3 hr at room temperature. After washes with PBS, the membrane was fixed with 0.5% paraformaldehyde/PBS for 20 min, then was incubated with 2% glycine/PBS for 10 min. Subsequently, c-NUMB was detected with standard Western procedures as described above.

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