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Identification of *Nepro*, a gene required for the maintenance of neocortex neural progenitor cells downstream of Notch

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In the developing neocortex, neural progenitor cells (NPCs) produce projection neurons of the six cortical layers in a temporal order. Over the course of cortical neurogenesis, maintenance of NPCs is essential for the generation of distinct types of neurons at the required time. Notch signaling plays a pivotal role in the maintenance of NPCs by inhibiting neuronal differentiation. Although Hairy and Enhancer-of-split (Hes)-type proteins are central to Notch signaling, it remains unclear whether other essential effectors take part in the pathway. In this study, we identify *Nepro*, a gene expressed in the developing mouse neocortex at early stages that encodes a 63 kDa protein that has no known structural motif except a nuclear localization signal. Misexpression of *Nepro* inhibits neuronal differentiation only in the early neocortex. Furthermore, knockdown of *Nepro* by siRNA causes precocious differentiation of neurons. Expression of *Nepro* is activated by the constitutively active form of Notch but not by Hes genes. *Nepro* represses expression of proneural genes without affecting the expression of Hes genes. Finally, we show that the combination of *Nepro* and Hes maintains NPCs even when Notch signaling is blocked. These results indicate that *Nepro* is involved in the maintenance of NPCs in the early neocortex downstream of Notch.

KEY WORDS: Neural progenitor cell, Cerebral cortex, Notch, Mouse

INTRODUCTION

Cortical projection neurons are generated from neural progenitor cells (NPCs) residing in the ventricular zone (VZ) of the dorsal telencephalic vesicle (Molyneaux et al., 2007). The first cohort of neurons forms a transient layer called the preplate. Subsequently generated neurons migrate radially and accumulate within the preplate, thereby splitting it into the marginal zone and subplate, and forming the cortical plate (CP). As CP neurons continue to be generated, they migrate past the earlier generated ones and stop beneath the marginal zone. This sequence results in the six layers of the CP having an inside-out pattern of birth dates (Takahashi et al., 1999; Caviness et al., 2008). Cell transplantation studies indicate that NPCs produce distinct types of neurons by changing their differentiation potential (McConnell, 1988; Frantz and McConnell, 1996; Mizutani and Saito, 2005). Thus, molecular mechanisms underlying the maintenance of NPCs are essential for the generation of appropriate numbers of the various types of cortical neurons.

Notch signaling mediates cell-cell interactions during vertebrate and invertebrate development (Louvi and Artavanis-Tsakonas, 2006). Mutations in key components of the Notch signaling pathway have revealed that Notch signaling is required for the maintenance of NPCs (Hitoshi et al., 2002; Yoon and Gaiano, 2005). The intracellular domain of Notch cooperates with the DNA-binding protein Rbpjκ and its co-activator mastermind-like (Maml) to activate transcription of Hes genes, which encode basic helix-loop-helix (bHLH) transcription factors (Kageyama et al., 2007). Hes genes suppress the proneural genes, such as the neurogenins and *Mash1* (*Ascl1* – Mouse Genome Informatics), which are crucial for neuronal differentiation (Chen et al., 1997; Hatakeyama et al., 2004; Shimojo et al., 2008). It has been shown that *Hes1* and *Hes5* are required for proper cortical neurogenesis (Ohtsuka et al., 1999; Guillemot, 2007), yet it remains largely unknown whether other types of effectors are required for Notch signaling in the developing neocortex.

To gain insight into the molecular mechanisms underlying the maintenance of cortical NPCs, we identified genes expressed in NPCs at early stages by using digital differential display (DDD) and in situ hybridization. Through functional screening of the genes, we found that one of them, *Nepro*, exhibited an activity that inhibits neuronal differentiation. To investigate *Nepro* function in vivo, we analyzed its role in the developing neocortex by gain- and loss-of-function approaches using in vivo electroporation.

MATERIALS AND METHODS

Animals

ICR mice obtained from Clea (Tokyo, Japan) were used for all experiments. The plug date was designated as embryonic day 0.5 (E0.5). All experimental procedures with these mice were conducted in accordance with guidelines established by the Animal Care and Use Committee (Chiba University, Japan).

Plasmids

For construction of *ΔNepro*, the *Bgl*II-*Nco*I fragment of mouse *Nepro* cDNA was deleted to remove the C-terminal half of the Nepro protein. *Nepro-HA* was constructed by inserting oligonucleotides encoding the hemagglutinin (HA) tag immediately upstream of the translation termination codon of *Nepro*. For misexpression of genes (see Table S1 in the supplementary material), their entire coding regions were inserted downstream of the second CAG promoter of pCAG-EYFP-CAG (Saito and Nakatsuji, 2001). pCAG-EYFP (enhanced yellow fluorescent protein) was used as a negative control in all studies (Saito and Nakatsuji, 2001).

In vivo electroporation

In vivo electroporation was performed as described (Saito and Nakatsuji, 2001; Saito, 2006). Chemically modified Stealth siRNAs (150 μM in PBS; Invitrogen, Carlsbad, CA, USA) were used. Among three *Nepro*-specific siRNAs, siRNA 5′-CAUCCCAUGCCUUACUUCAAAGAUU-3′, which

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corresponds to nucleotides 699-724 of *Nepro* (GenBank accession NM_145972), successfully repressed *Nepro* expression (see Fig. 3Z). As a control, Stealth RNA containing a scrambled sequence of the same GC content was used: 5′-CAUACGUCCUUCAUUACAAGCCAUU-3′. *Rbpjk*-specific siRNA (MSS208565) and the corresponding control siRNAs were purchased from Invitrogen. pCAG-EYFP was cotransfected with the siRNAs to visualize transfected cells. L-685,458 (Bachem, King of Prussia, PA, USA) was used at 50 μM. Each electroporation result was reproduced in multiple brains derived from at least three litters.

Immunohistochemistry (IHC) and in situ hybridization (ISH)

IHC and ISH were performed on coronal sections (12-14 μm) that were obtained with a cryostat as previously described (Kawauchi and Saito, 2008), with minor modifications. The following antibodies were used: rabbit anti-class III β-tubulin (RDI, Flanders, NJ, USA), mouse anti-nestin (Pharmingen, San Diego, CA, USA), rabbit anti-Tbr1 (Chemicon, Temecula, CA, USA), mouse anti-Ki67 (Pharmingen), rabbit anti-GFP (Invitrogen), rat anti-GFP (MBL, Nagoya, Japan), rat anti-HA (Roche Diagnostics, Mannheim, Germany) and Alexa Fluor-conjugated secondary antibodies (Invitrogen). For IHC with anti-HA, anti-Ki67 and anti-Tbr1 antibodies, sections were boiled for 5 minutes in 5 mM Tris-HCl and 1 mM EDTA (pH 8.0) and stained together with anti-GFP antibodies, which also recognize the EYFP protein. cRNA probes were prepared from plasmids listed in Table S1 in the supplementary material.

RESULTS AND DISCUSSION

Identification of *Nepro*, a gene expressed in the early neocortex

To identify genes that are specifically expressed in the mouse brain at early stages, we compared mouse expressed sequence tag (EST) libraries from early head tissues containing many NPCs with those from older head tissues with fewer NPCs, using DDD (see Table S2 in the supplementary material). In the early head libraries, 139 genes were over-represented. Among them, we focused on five genes, the functions of which are unknown and that encode proteins containing a nuclear localization signal (NLS), as many nuclear proteins are involved in cellular activity. Three of the five genes were expressed in the VZ of the early neocortex (see Table S3 in the supplementary material). To explore their function, we transfected the genes into NPCs using in vivo electroporation. Only one gene, which we termed *Nepro*, exhibited an activity that inhibits neuronal differentiation (Fig. 2B). *Nepro* encodes a 564 amino acid protein that contains a NLS but no other known structural motif, such as a HLH. Database analysis revealed that each vertebrate species appears to have a single *Nepro* homolog, and that the protein contains three conserved regions: QVEQC, a hydrophobic amino acid-rich region and DDIDDIF (Fig. 1A; see Fig. S1 in the supplementary material). No invertebrate homolog was found. *Nepro* expression was first detected faintly in the VZ of the mouse forebrain at E9.5, was clearly visible at E10.5 to E12.5, and subsequently declined and was almost absent by E15.5 (Fig. 1B-D; data not shown). We examined the subcellular localization of *Nepro* using *Nepro-HA*. *Nepro-HA* mimicked the function of *Nepro* (data not shown) and Nepro-HA was localized to the nucleus (Fig. 1E-G), suggesting that Nepro is a nuclear protein.

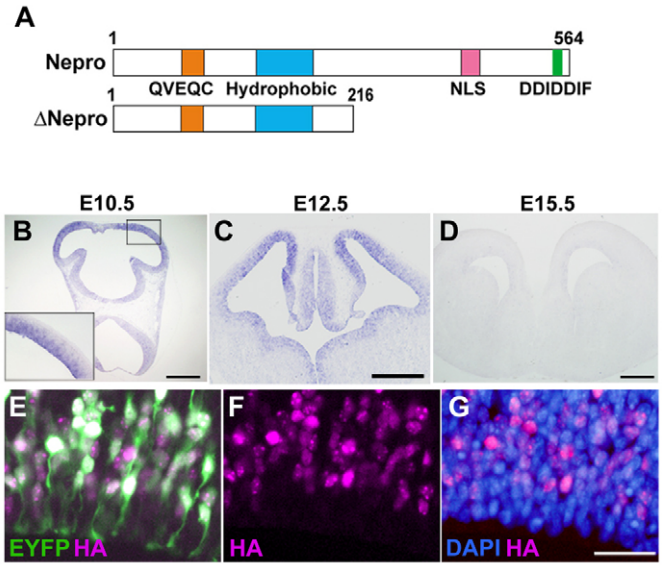

Fig. 1. Nepro protein and its expression. (**A**) Schematic illustration of the Nepro protein and a deletion mutant (*ΔNepro*). Colored boxes indicate conserved regions and the nuclear localization signal (NLS). (**B-D**) In situ hybridization (ISH) of mouse forebrain sections at E10.5, E12.5 and E15.5 to detect *Nepro* mRNA. The inset shows an enlarged view. (**E-G**) Immunostaining of Nepro-HA with an anti-HA antibody (magenta) 1 day after transfection of *Nepro-HA* at E13.5. EYFP fluorescence (E, green) and DAPI-stained nuclei (G, blue) are shown. Scale bars: 200 μm in B-D; 50 μm in G.

Nepro inhibits neuronal differentiation in the early neocortex

To examine Nepro function, we transfected *Nepro* into neocortex NPCs at E13.5, by which stage endogenous *Nepro* expression has declined. To visualize transfected NPCs and their daughter cells, *Nepro* was co-expressed with the *Eyfp* gene using a double promoter vector that carries both *Nepro* and *Eyfp*. As a control, NPCs transfected with *Eyfp* alone gave rise to neurons, and EYFP-positive neurons migrated out of the VZ into the CP (Fig. 2A). By contrast, transfection of *Nepro* greatly reduced the number of EYFP-positive neurons in the CP, and the majority of EYFP-positive cells remained in the VZ (Fig. 2B). This phenotype resembled that obtained by transfection of Hes genes (Fig. 2C). Indeed, the vast majority of *Nepro*-misexpressing cells continued to proliferate and were maintained as NPCs, as shown by the expression of the proliferation marker Ki67 and the NPC marker nestin (Fig. 2G,H). None of the *Nepro*-misexpressing cells in the VZ was positive for the neuronal marker βIII-tubulin (see Fig. S2 in the supplementary material).

To examine whether Nepro inhibits neuronal differentiation at a later stage, we transfected *Nepro* into NPCs at E15.5, at which stage *Nepro* expression was almost absent. In contrast to the transfection at E13.5, cells transfected with *Nepro* migrated out of the VZ into the CP, similar to when cells were transfected with *Eyfp* alone as a control (Fig. 2D,E). Conversely, the majority of cells that were transfected with Hes genes at E15.5 were maintained as NPCs in the VZ (Fig. 2F). These results indicate that Nepro exhibits an activity that inhibits neuronal differentiation only at early stages.

Nepro is required for proper differentiation of cortical NPCs

To determine whether *Nepro* is necessary for the maintenance of NPCs, we knocked down *Nepro* expression by transfecting *Nepro*-specific siRNA with *Eyfp* into neocortex NPCs at E11.5, at which stage *Nepro* is strongly expressed. *Nepro* mRNA levels were greatly reduced by *Nepro* siRNA, in contrast to control siRNA (Fig. 3Y,Z). When *Nepro* was knocked down, an increased fraction of EYFP-positive neurons was found in the CP, as shown by expression of

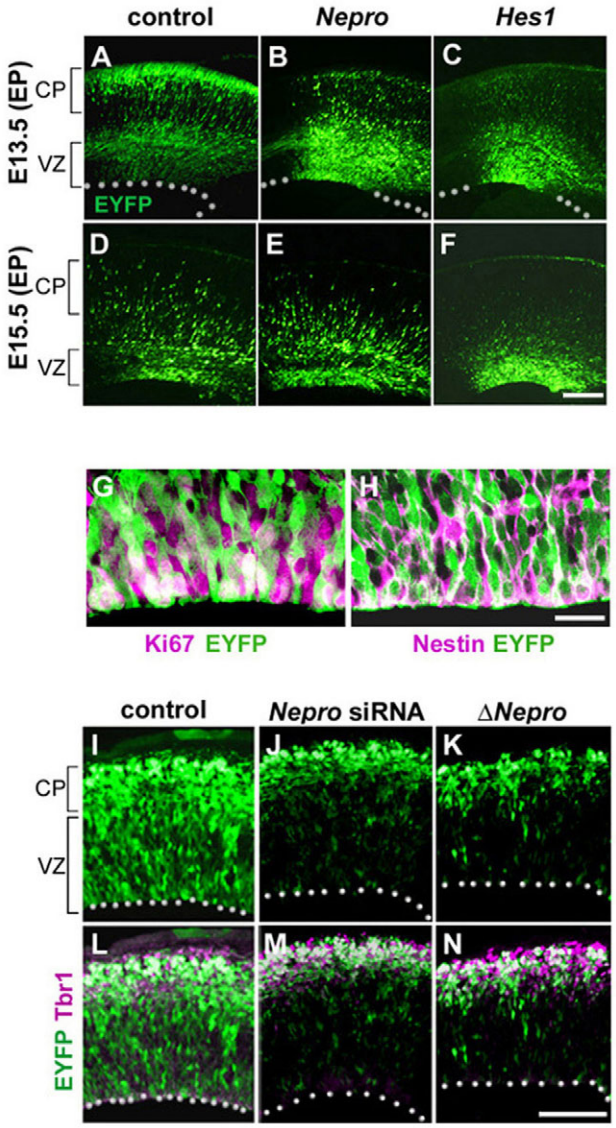

Fig. 2. *Nepro* is involved in the maintenance of NPCs. (**A-F**) The neocortex 3 days after transfection of *Eyfp* alone as a control (A,D) and *Eyfp* with either *Nepro* (B,E) or *Hes1* (C,F) at E13.5 (A-C) and E15.5 (D-F). (**G**,**H**) Immunostaining with anti-Ki67 (G) and anti-nestin (H) antibodies in the VZ of the *Nepro*-transfected brain. (**I-N**) The neocortex 2 days after transfection of *Eyfp* alone as a control (I,L) and *Eyfp* with either *Nepro*-specific siRNA (J,M) or *ΔNepro* (K,N) at E11.5. Sections were immunostained with an anti-Tbr1 antibody. Apoptosis was not significantly increased by transfection of *Nepro*, *Nepro* siRNA or *ΔNepro* (data not shown). Scale bars: 100 μm in F,N; 25 μm in H.

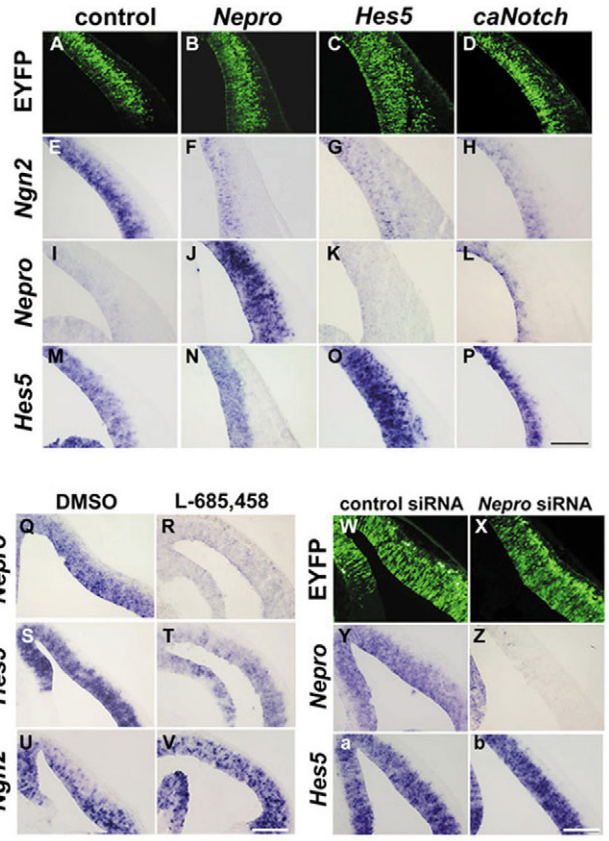

Fig. 3. Genetic interaction between *Nepro* and Notch. (**A-P**) The neocortex 12 hours after transfection of *Eyfp* (A,E,I,M) as a control and *Eyfp* with *Nepro* (B,F,J,N), *Hes5* (C,G,K,O) or caNotch (D,H,L,P) at E13.0. The expression of *Ngn2*, *Nepro* and *Hes5* in the neocortex was analyzed by ISH. *Hes1* also repressed the expression of *Ngn2* and *Mash1*, as previously described (data not shown) (Shimojo et al., 2007). (**Q-V**) The neocortex 5 hours after injection of DMSO (Q,S,U) as a control and L-685,458 (R,T,V) at E11.5. In contrast to *Nepro* and *Hes5*, *Ngn2* mRNA levels were not decreased (U,V). (**W-Z**,**a**,**b**) The neocortex 16 hours after transfection of *Eyfp* together with control or *Nepro* siRNA at E11.5. *Nepro* mRNA expression was clearly reduced by *Nepro* siRNA, whereas expression of *Hes5* was unchanged. Scale bars: 200 μm.

βIII-tubulin and the CP neuronal marker Tbr1 (Hevner et al., 2001), compared with transfection of *Eyfp* with or without control siRNA (Fig. 2J,M; data not shown). The number of EYFP-positive NPCs in the VZ was greatly reduced by *Nepro* siRNA, and none of the EYFP-positive cells in the CP was immunolabeled for nestin (see Fig. S3 in the supplementary material). These findings suggest that NPCs are not properly maintained in the absence of Nepro. We next examined whether the C-terminal part of Nepro is important for its activity by transfecting a deletion mutant (*ΔNepro*; Fig. 1A) into NPCs. In contrast to full-length Nepro, the mutant did not inhibit neuronal differentiation (Fig. 2K,N), suggesting that the C-terminal part is required for Nepro function. Furthermore, misexpression of *ΔNepro* mimicked the effect of *Nepro* siRNA (Fig. 2J,M), suggesting that ΔNepro can act as a dominant-negative form of Nepro.

Nepro represses the expression of proneural genes

We next examined whether *Nepro* is able to repress proneural genes. Misexpression of *Nepro* decreased mRNA levels of *Ngn2* and *Mash1*, as did Hes and the constitutively active form of Notch (caNotch), whereas no change in these mRNA levels was seen with transfection of *Eyfp* alone (Fig. 3E-H; see Fig. S4 in the supplementary material). These results suggest that *Nepro* inhibits neuronal differentiation, presumably by repressing proneural genes.

Nepro expression is regulated by a Notch receptor

We next examined whether Notch signaling involves *Nepro* expression. Misexpression of caNotch induced expression of *Nepro* as well as of *Hes5*, whereas misexpression of *Eyfp* alone did not

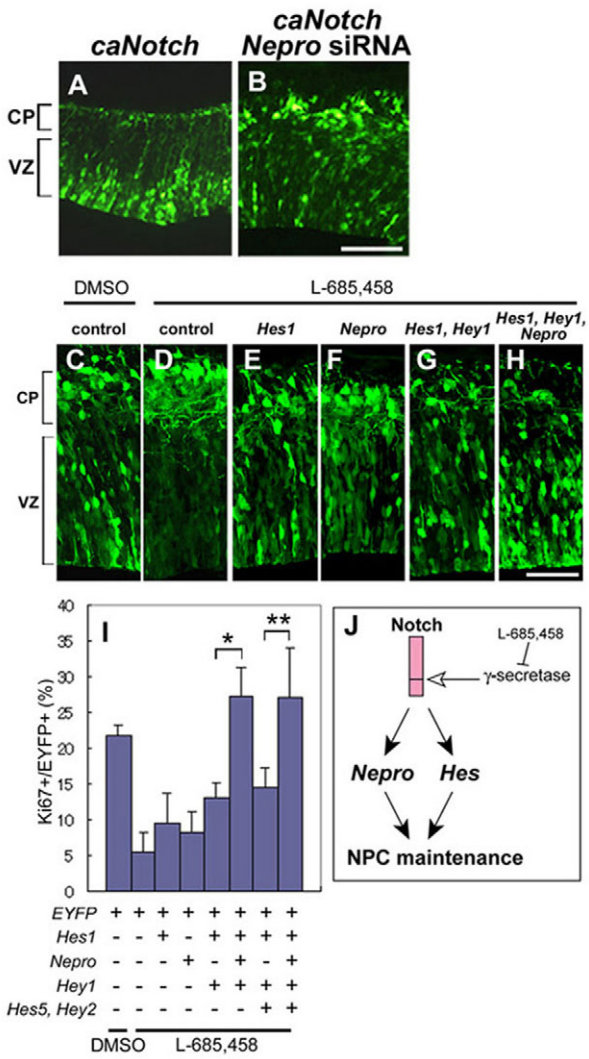

Fig. 4. *Nepro* is required to maintain NPCs. (**A**,**B**) The neocortex 2 days after transfection of *Eyfp* and caNotch with (A) or without (B) *Nepro* siRNA at E11.5. (**C-H**) The neocortex 2 days after transfection of *Eyfp* as a control and *Eyfp* with *Hes1*, *Hey1* and/or *Nepro* in the presence of L-685,458 at E11.5 The distribution of EYFP-positive cells was not affected by injection of DMSO as a control (C). (**I**) Quantification of the percentage of Ki67-positive/EYFP-positive cells in sections transfected with the genes shown along the *x*-axis. Error bars indicate the s.d., *, $P<0.001$, **, $P<0.005$. *P*-values were calculated by Student's *t*-test. (**J**) The Nepro and Notch pathways in the early neocortex. After cleavage by γ-secretase, activated Notch induces the expression of *Nepro* and Hes in the early neocortex. Scale bars: 100 μm in B; 50 μm in H.

influence its expression (Fig. 3I-P). Conversely, a γ-secretase inhibitor, L-685,458, which blocks cleavage and activation of Notch (Martys-Zage et al., 2000), drastically reduced mRNA levels of *Nepro* and *Hes5*, compared with the control solvent dimethylsulfoxide (DMSO), which did not affect mRNA levels (Fig. 3Q-V). Furthermore, *Nepro* expression was decreased by *Rbpjk*-specific siRNA (Fukushima et al., 2008) and the dominant-negative form of *Maml1* (*DN-Maml1*) (Weng et al., 2003; Maillard et al., 2004) (see Fig. S5 in the supplementary material). These results indicate that *Nepro* is activated downstream of canonical Notch signaling.

Misexpression of *Hes1* and *Hes5* did not affect *Nepro* expression, and misexpression of *Nepro* did not change *Hes1* or *Hes5* expression (Fig. 3K,N; see Fig. S6A in the supplementary material). Moreover, *Nepro* knockdown did not affect the expression of *Hes1* or *Hes5* (Fig. 3b; see Fig. S6B in the supplementary material). These findings suggest that *Nepro* is activated in parallel with Hes, but neither downstream nor upstream of Hes.

Nepro is an essential Notch effector

To determine whether Nepro is an essential effector for Notch signaling, *Nepro* siRNA or *ΔNepro* was transfected together with caNotch. Whereas caNotch maintained the vast majority of cells as NPCs in the VZ, cells transfected with caNotch together with *Nepro* siRNA or *ΔNepro* differentiated into CP neurons positive for Tbr1 (Fig. 4A,B; see Fig. S7 in the supplementary material), indicating that *Nepro* is necessary downstream of Notch for maintenance of NPCs.

We then examined whether misexpression of *Nepro* and Hes is sufficient to maintain NPCs when Notch activity is blocked (Fig. 4C-H). L-685,458 markedly reduced NPCs in the VZ (Fig. 4D), consistent with the precocious differentiation caused by the blocking of Notch signaling. The number of proliferating NPCs was measured by immunostaining of Ki67 (Fig. 4I; see Fig. S8 in the supplementary material). The reduction of NPCs by L-685,458 was not suppressed by misexpression of *Hes1* or *Nepro* (Fig. 4E,F). Co-transfection of *Hes1* and hes-related 1 (*Hey1*) was also not sufficient to suppress the reduction (Fig. 4G), indicating that the lack of Hes1 and Hey1 heterodimers, which are known to be more stable than homodimers (Iso et al., 2001), is not the reason for the absence of activity. By contrast, misexpression of *Hes1* and *Hey1* with *Nepro* suppressed the reduction in NPCs (Fig. 4H). Similarly, whereas overexpression of Hes and Hey genes (*Hes1*, *Hes5*, *Hey1* and *Hey2*), which are expressed in the embryonic neocortex, was also not sufficient to suppress the reduction in NPCs, the addition of *Nepro* was sufficient to suppress the reduction (Fig. 4I; data not shown). Furthermore, the combinatorial effect of *Hes1* and *Nepro* was also observed when Notch signaling was blocked by *DN-Maml1* (see Fig. S9 in the supplementary material). These results indicate that when Notch activity is blocked, *Nepro* and Hes are necessary for the maintenance of NPCs.

In this study, we identified *Nepro*, which is a novel Notch effector for the maintenance of NPCs in the early stages of neocortex development (Fig. 4J). *Nepro* is an atypical Notch effector for the following reasons: first, *Nepro* does not contain an HLH motif, unlike Hes and Hey proteins; and second, although members of the Notch pathway are conserved from invertebrates to vertebrates and often make up protein families, there is a single homolog of *Nepro* in each vertebrate and no invertebrate homolog. This might suggest that the development of the neocortex involves a different mechanism from that used in invertebrates.

Finally, unlike Hes genes, which are expressed throughout the developing nervous system, *Nepro* expression is mainly restricted to the neocortex at early stages. Consistent with this, *Nepro* was not activated by misexpression of caNotch at later stages (data not shown). At E15.5, NPCs were maintained by misexpression of *Hes1*, but not of *Nepro* (Fig. 2E,F). These findings suggest that *Nepro* is involved in the maintenance of NPCs only at early stages, and thus the machinery to maintain NPCs is not the same throughout development.

Nepro mutants in which the NLS is disrupted by amino acid substitutions did not exhibit Nepro activity (see Fig. S1 in the supplementary material; data not shown), suggesting that the NLS is important for *Nepro* function. The requirement of both *Nepro* and Hes for the maintenance of NPCs suggests that their direct or

indirect interaction may be crucial. *ΔNepro*, which lacks the C-terminal portion, mimicked the *Nepro* knockdown phenotype (Fig. 2K), suggesting that *Nepro* interacts with key factors. It remains to be determined what proteins interact with *Nepro* and how NPCs are maintained by those interactions in the early cortex.

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Supplementary material
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References
Caviness, V. S., Bhide, P. G. and Nowakowski, R. S. (2008). Histogenetic processes leading to the laminated neocortex: migration is only a part of the story. *Dev. Neurosci.* **30**, 82-95.
Chen, H., Thiagalingam, A., Chopra, H., Borges, M. W., Feder, J. N., Nelkin, B. D., Baylin, S. B. and Ball, D. W. (1997). Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. *Proc. Natl. Acad. Sci. USA* **94**, 5355-5360.
Frantz, G. D. and McConnell, S. K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. *Neuron* **17**, 55-61.
Fukushima, H., Nakao, A., Okamoto, F., Shin, M., Kajiya, H., Sakano, S., Bigas, A., Jimi, E. and Okabe, K. (2008). The association of Notch2 and NF-kappaB accelerates RANKL-induced osteoclastogenesis. *Mol. Cell. Biol.* **28**, 6402-6412.
Guillemot, F. (2007). Cell fate specification in the mammalian telencephalon. *Prog. Neurobiol.* **83**, 37-52.
Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F. and Kageyama, R. (2004). Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. *Development* **131**, 5539-5550.
Hevner, R. F., Shi, L., Justice, N., Hsueh, Y., Sheng, M., Smiga, S., Bulfone, A., Goffinet, A. M., Campagnoni, A. T. and Rubenstein, J. L. (2001). Tbr1 regulates differentiation of the preplate and layer 6. *Neuron* **29**, 353-366.
Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A. J., Nye, J. S., Conlon, R. A., Mak, T. W., Bernstein, A. and van der Kooy, D. (2002). Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes Dev.* **16**, 846-858.
Iso, T., Sartorelli, V., Poizat, C., Iezzi, S., Wu, H. Y., Chung, G., Kedes, L. and Hamamori, Y. (2001). HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling. *Mol. Cell. Biol.* **21**, 6080-6089.
Kageyama, R., Ohtsuka, T. and Kobayashi, T. (2007). The Hes gene family: repressors and oscillators that orchestrate embryogenesis. *Development* **134**, 1243-1251.
Kawauchi, D. and Saito, T. (2008). Transcriptional cascade from Math1 to Mbh1 and Mbh2 is required for cerebellar granule cell differentiation. *Dev. Biol.* **322**, 345-354.
Louvi, A. and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat. Rev. Neurosci.* **7**, 93-102.
Maillard, I., Weng, A. P., Carpenter, A. C., Rodriguez, C. G., Sai, H., Xu, L., Allman, D., Aster, J. C. and Pear, W. S. (2004). Mastermind critically regulates Notch-mediated lymphoid cell fate decisions. *Blood* **104**, 1696-1702.
Martys-Zage, J. L., Kim, S. H., Berechid, B., Bingham, S. J., Chu, S., Sklar, J., Nye, J. and Sisodia, S. S. (2000). Requirement for presenilin 1 in facilitating lagged 2-mediated endoproteolysis and signaling of notch 1. *J. Mol. Neurosci.* **15**, 189-204.
McConnell, S. K. (1988). Fates of visual cortical neurons in the ferret after isochronic and heterochronic transplantation. *J. Neurosci.* **8**, 945-974.
Mizutani, K. and Saito, T. (2005). Progenitors resume generating neurons after temporary inhibition of neurogenesis by Notch activation in the mammalian cerebral cortex. *Development* **132**, 1295-1304.
Molyneaux, B. J., Arlotta, P., Menezes, J. R. and Macklis, J. D. (2007). Neuronal subtype specification in the cerebral cortex. *Nat. Rev. Neurosci.* **8**, 427-437.
Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F. and Kageyama, R. (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *EMBO J.* **18**, 2196-2207.
Saito, T. (2006). In vivo electroporation in the embryonic mouse central nervous system. *Nat. Protoc.* **1**, 1552-1558.
Saito, T. and Nakatsuji, N. (2001). Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. *Dev. Biol.* **240**, 237-246.
Shimojo, H., Ohtsuka, T. and Kageyama, R. (2008). Oscillations in notch signaling regulate maintenance of neural progenitors. *Neuron* **58**, 52-64.
Takahashi, T., Goto, T., Miyama, S., Nowakowski, R. S. and Caviness, V. S., Jr. (1999). Sequence of neuron origin and neocortical laminar fate: relation to cell cycle of origin in the developing murine cerebral wall. *J. Neurosci.* **19**, 10357-10371.
Weng, A. P., Nam, Y., Wolfe, M. S., Pear, W. S., Griffin, J. D., Blacklow, S. C. and Aster, J. C. (2003). Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol. Cell. Biol.* **23**, 655-664.
Yoon, K. and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat. Neurosci.* **8**, 709-715.

Table S1. Genes used for ISH and misexpression

Gene	Accession number	Plasmid
Nepro	NM_145972	FANTOM clone ID# G730009P16*
Hes1	NM_008235	FANTOM clone ID# 5133400H24*
Hes5	NM_010419	FANTOM clone ID# A830039A02*
Hey1	NM_010423	FANTOM clone ID# 2600002K17*
Hey2	NM_013904	FANTOM clone ID# F930015I01*
Ngn2	NM_009718	FANTOM clone ID# E130304I06*
Mash1	NM_008553	A gift from Dr D. J. Anderson†
DN-Maml1-GFP		A gift from Dr W. Pear‡

*__Carninci, P., Kasukawa, T., Katayama, S., Gough, J., Frith, M. C., Maeda, N., Oyama, R., Ravasi, T., Lenhard, B., Wells, C. et al.__ (2005). The transcriptional landscape of the mammalian genome. *Science* **309**, 1559-1563.

†__Johnson, J. E., Birren, S. J. and Anderson, D. J.__ (1990). Two rat homologues of Drosophila achaete-scute specifically expressed in neuronal precursors. *Nature* **346**, 858-861.

‡Weng et al. (2003)

Table S2. Overview of screening

ESTs derived from E9.5-E10.5 brain	14933
ESTs derived from E13.5-P15 brain	301493
Enriched EST clusters derived from E9.5-E10.5 brain*	139
NLS-coding genes in the clusters	43
Unpublished genes among those containing NLS	5
Unpublished NLS-coding genes expressed by early cortical NPCs	3

*The genes expressed in the NPCs were examined by DDD (http://www.ncbi.nlm.nih.gov/UniGene/info_ddd.shtml). Amino acid sequence motifs were searched by PSORTII (http://psort.ims.u-tokyo.ac.jp/form2.html).

Table S3. Unpublished NLS-coding genes that are expressed in the early cortical NPCs

Mm.310573	BC027231 (*Nepro*)
Mm.132381	Prr11
Mm.312204	4930534B04Rik