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Oscillations in Notch Signaling Regulate Maintenance of Neural Progenitors

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SUMMARY

Expression of the Notch effector gene *Hes1* is required for maintenance of neural progenitors in the embryonic brain, but persistent and high levels of *Hes1* expression inhibit proliferation and differentiation of these cells. Here, by using a real-time imaging method, we found that *Hes1* expression dynamically oscillates in neural progenitors. Furthermore, sustained overexpression of *Hes1* downregulates expression of proneural genes, Notch ligands, and cell cycle regulators, suggesting that their proper expression depends on *Hes1* oscillation. Surprisingly, the proneural gene *Neurogenin2* (*Ngn2*) and the Notch ligand *Delta-like1* (*Dll1*) are also expressed in an oscillatory manner by neural progenitors, and inhibition of Notch signaling, a condition known to induce neuronal differentiation, leads to downregulation of *Hes1* and sustained upregulation of *Ngn2* and *Dll1*. These results suggest that *Hes1* oscillation regulates *Ngn2* and *Dll1* oscillations, which in turn lead to maintenance of neural progenitors by mutual activation of Notch signaling.

INTRODUCTION

Neural progenitors change their competency over time, giving rise to distinct types of cells during development (Alvarez-Buylla et al., 2001; Fishell and Kriegstein, 2003; Fujita, 2003; Götz and Huttner, 2005; Miller and Gauthier, 2007). Thus, maintenance of neural progenitors until later stages of development is essential for the generation of cells both in correct numbers and with a full spectrum of cell types. It has been shown that Notch signaling plays an important role in the maintenance of neural progenitors (Artavanis-Tsakonas et al., 1999; Gaiano and Fishell, 2002; Honjo, 1996; Selkoe and Kopan, 2003). Upon activation of Notch signaling by its ligands, such as Delta-like1 (Dll1), the intracellular domain of the transmembrane protein Notch (NICD) is released from the membrane region and transferred into the nucleus, where the NICD converts RBP-J from a repressor to an activator by forming a complex with it (Honjo, 1996; Selkoe and Kopan, 2003). The complex of NICD and RBP-J activates expression of the basic helix-loop-helix transcriptional repressors *Hes1* and *Hes5* (Ohtsuka et al., 1999), downregulates proneural gene expression, and inhibits neuronal differentiation (Bertrand et al., 2002; Ross et al., 2003; Kageyama et al., 2007). Inactivation of *Hes1* upregulates expression of proneural genes, accelerating neuronal differentiation (Ishibashi et al., 1995; Tomita et al., 1996; Hatakeyama et al., 2004), whereas misexpression of *Hes1* inhibits neuronal differentiation (Ishibashi et al., 1994; Ohtsuka et al., 2001), suggesting that *Hes1* is one of essential effectors of Notch signaling. It has been shown that postmitotic neurons express Notch ligands and activate Notch signaling of neighboring neural progenitors (Henrique et al., 1995; Myat et al., 1996; Dunwoodie et al., 1997). However, Notch ligands are already expressed in the developing nervous system before overt neuronal differentiation (Bettenhausen et al., 1995; Hatakeyama et al., 2004), raising the possibility that Notch ligands are also expressed by dividing neural progenitors.

Another issue is the expression mode of Hes1 in the developing nervous system. We previously found that Hes1 protein expression by neural progenitors that actively proliferate and differentiate is variable, with high levels in some cells, but lower levels or no expression in others (Baek et al., 2006). Furthermore, sustained *Hes1* expression inhibits proliferation of cultured neural progenitors by G1 phase retardation, suggesting that Hes1 expression should be downregulated at some points of the cell cycle (Baek et al., 2006). There are at least two possible explanations for such variable levels of Hes1 expression. One is that Hes1 expression is initially high in neural progenitors but is gradually downregulated and finally lost during neuronal differentiation. Another possibility is that Hes1 expression is oscillatory in neural progenitors. We previously found that Hes1 expression oscillates with a period of about 2 hr in cultured cells, such as fibroblasts (Hirata et al., 2002; Masamizu et al., 2006). This oscillatory expression is induced by serum stimulation or Notch activation and is regulated by negative feedback: Hes1 can repress its own expression by directly binding to its own promoter (Takebayashi et al., 1994), and repression of the promoter leads to rapid disappearance of both *Hes1* mRNA and Hes1 protein, because they are extremely unstable, which allows the next round of expression. In this way, Hes1 autonomously starts oscillatory expression (Hirata et al., 2002). However, it remains to be determined whether Hes1 expression oscillates in neural progenitors.

To address these questions, we examined the dynamics of *Hes1* expression in neural progenitors by taking advantage of a real-time imaging method that we previously developed (Masamizu et al., 2006). We found that *Hes1* expression dynamically oscillates in neural progenitors. Furthermore, we found that

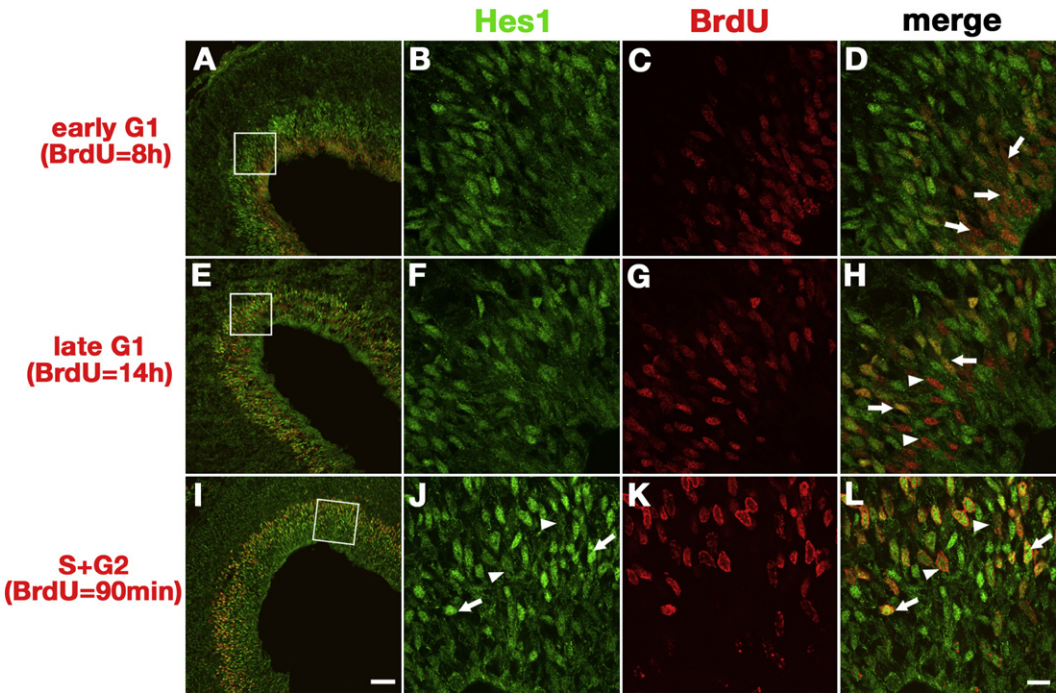


Figure 1. Hes1 Expression in Neural Progenitors

BrdU was administered to E14.5 mouse embryos, and the telencephalon was examined 90 min, 8 hr, and 14 hr later, which corresponds to labeling of cells in S-G2 phase (I–L), early G1 phase (A–D), and late G1 phase (E–H) , respectively. Hes1 protein expression and BrdU incorporation were immunohistochemically analyzed. Hes1 expression occurred at variable levels from S to G2. Cells expressing Hes1 protein at high and low levels are indicated by arrows and arrowheads, respectively (J and L). In early G1 phase, when cell bodies were located near the ventricular surface, Hes1 protein expression was mostly absent (B–D), (some of them are indicated by arrows). After early G1 phase, various levels of Hes1 expression occurred again in about 50% of the BrdU-labeled cells, whereas no expression occurred in other $BrdU^+$ cells. Some $Hes1^+$ and $Hes1^-$ cells are indicated by arrows and arrowheads, respectively (H). Scale bars, 50 μm (A), (E), and (I) and 10 μm (B–D), (F–H), and (J–L).

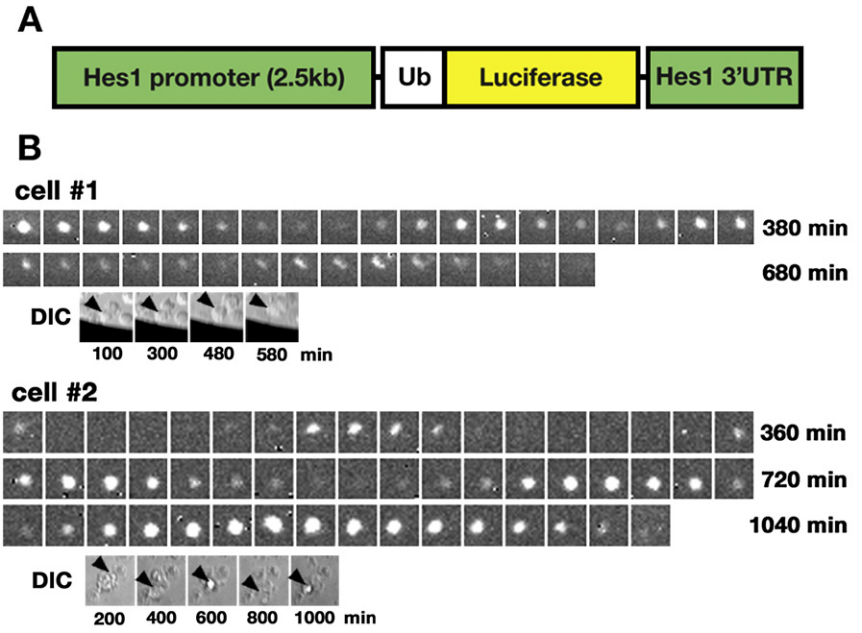
the proneural gene *Neurogenin2* (*Ngn2*) and the Notch ligand *Dll1* are expressed in an oscillatory manner by neural progenitors, and that these oscillations are regulated by Hes1 oscillation. In contrast, downregulation of Hes1 expression, which is known to induce neuronal differentiation, leads to sustained upregulation of *Ngn2* and *Dll1* expression. These results suggest that oscillations in Notch signaling play an important role in maintenance of neural progenitors.

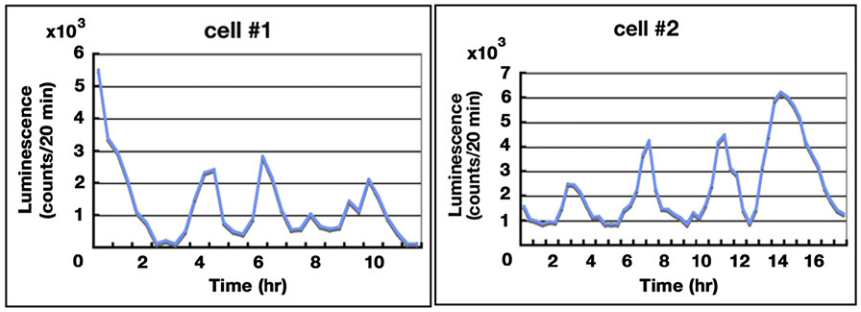
RESULTS

Hes1 Is Expressed at Various Levels by Neural Progenitors

We first examined Hes1 expression in neural progenitors in the developing telencephalon. Hes1 protein was expressed in the ventricular zone of the developing nervous system (Figures 1A, 1E, and 1I). Virtually all Hes1-expressing cells were found to be positive for Ki67, a marker for mitotic cells (see Figures S1A–S1D available online), indicating that Hes1 is expressed only by dividing cells. To reveal the relationship between Hes1 expression and the cell cycle, we administered BrdU to mouse embryos at embryonic day (E) 14.5 and examined brain sections 30 min, 90 min, 8 hr, and 14 hr later, which corresponds to the labeling, respectively, of cells in S phase, S-G2 phase, early G1 phase, and late G1 phase (Takahashi et al., 1995). Cells in M phase were labeled by antiphosphorylated histone H3 (pH3) antibody. In addition, the cell cycle phases were assessed according to the location of cell bodies of neural progenitors. During S phase, cell bodies are present at the outer region of the ventricular zone but descend toward the ventricular surface during G2 phase (Fujita, 2003; Takahashi et al., 1993). Cell division occurs at the ventricular surface, and cell bodies ascend during G1 phase (Fujita, 2003; Takahashi et al., 1993).

Hes1 protein was expressed in the nuclei of neural progenitors in S and G2 phases (Figures 1J–1L, $Hes1^+BrdU^+$). Some cells expressed Hes1 protein at high levels (Figures 1J–1L, arrows) whereas others expressed it at low levels (Figures 1J–1L, arrowheads). During M phase ($pH3^+$), Hes1 protein was not expressed by some cells but was present in the cytoplasm of others (Figures S1E–S1H). However, in early G1 phase, when cell bodies were located near the ventricular surface, Hes1 protein expression was mostly absent (Figures 1B–1D; many $BrdU^+$ cells near the ventricular surface were negative for Hes1). After early G1 phase, various levels of Hes1 expression occurred again in about 50% of the BrdU-labeled cells, whereas no expression occurred in other $BrdU^+$ cells (some $Hes1^+$ and $Hes1^-$ cells are indicated by arrows and arrowheads, respectively, in Figures 1F–1H). Because neural progenitors undergo asymmetric cell division at this stage, which produces a new neural progenitor and a neuron or a neuronal precursor, it is likely that half of the





D

stage	total cell number	cell number (%)		period time (top to top)	
		≧2 peaks	≧3 peaks	≧2 peaks	≧3 peaks
E9.5	176	29 (16.5)	7 (4.0)	176.7±4.7 min	154.0±36.8 min
E11.5	172	66 (38.4)	34 (19.8)	146.0±8.5 min	146.0±8.5 min
E13.5	211	73 (34.6)	46 (21.8)	149.0±20.4 min	143.5±20.4 min
E14.5	141	38 (27.0)	21 (14.9)	133.4±21.0 min	144.4±18.7 min

Figure 2. Real-Time Imaging of *Hes1* Expression in Dissociated Neural Progenitor Cultures

(A) Structure of the Hes1 reporter. Ubiquitinated luciferase was expressed under the control of the 2.5 kb Hes1 promoter (Masamizu et al., 2006).

(B) Neural progenitors were prepared from the telencephalon of Hes1 reporter mice. Bioluminescence images of individual neural progenitors were taken using 20 min exposures and binning of pixels 4 × 4 to increase signal-to-noise ratios (Movie S1). Bright images (DIC) are also shown.

(C) Quantification of bioluminescence of individual neural progenitors shown in (B).

(D) Average period of *Hes1* oscillations in neural progenitors. The average period of *Hes1* oscillation was 2–3 hr during E9.5 to E14.5. Thus, it was not significantly changed during this period, although there was some tendency that the period was longer at earlier stages.

Standard deviation is shown with each value.

BrdU-labeled cells (i.e., those that expressed Hes1) remained as neural progenitors, whereas the other half (i.e., those that did not express Hes1) differentiated into neurons or neuronal precursors. These results suggest that Hes1 expression is downregulated during early G1 phase in all cells, and remains suppressed thereafter in neurons and neuronal precursors, but occurs again at various levels in neural progenitors.

Hes1 Expression Oscillates in Neural Progenitors in Dissociation Cultures

Hes1 was expressed at various levels by neural progenitors, but it was not clear how Hes1 expression changes in these cells. To address this question, we examined the dynamics of Hes1 expression by taking advantage of a real-time imaging method, one that used a ubiquitinated firefly luciferase reporter under the control of the *Hes1* promoter (pHes1-Ub1-Luc) (Figure 2A) (Masamizu et al., 2006). Because the half-lives of *Hes1* mRNA and Hes1 protein are about 20 min (Hirata et al., 2002), an unstable reporter with a half-life of 20 min or less is necessary to monitor the dynamic changes of Hes1 expression. The ubiquitinated firefly luciferase reporter has a half-life of about 10 min and is unstable enough to monitor the precise dynamics of oscillatory expression of *Hes1* in the somite segmentation clock and in cultured fibroblasts (Masamizu et al., 2006). Accordingly, we prepared dissociation cultures of telencephalic neural progenitors from the transgenic mice carrying pHes1-Ub1-Luc reporter at E9.5, E11.5, E13.5, and E14.5 and monitored their bioluminescence using a highly sensitive CCD camera.

We found that 60%–85% of neural progenitors soon lost *Hes1* expression in dissociation cultures, probably because Notch signaling was disrupted by dissociation (Figure S2B, cells 1–4). The rest of the neural progenitors (15%–40%) expressed *Hes1* for at least several hours, and *Hes1* expression oscillated in all of them (Figures 2B and 2C, Movie S1, and Figure S2B, cells 5–8), suggesting that the variability in Hes1 expression observed in neural progenitors is due to oscillation. The periods of *Hes1* oscillation were not stable but varied from cycle to cycle and from cell to cell (Figures 2B and 2C and Figure S2A). In some cases, *Hes1* expression was repressed for several hours but then suddenly increased and started oscillating (Figure S2B, cells 9–11). These

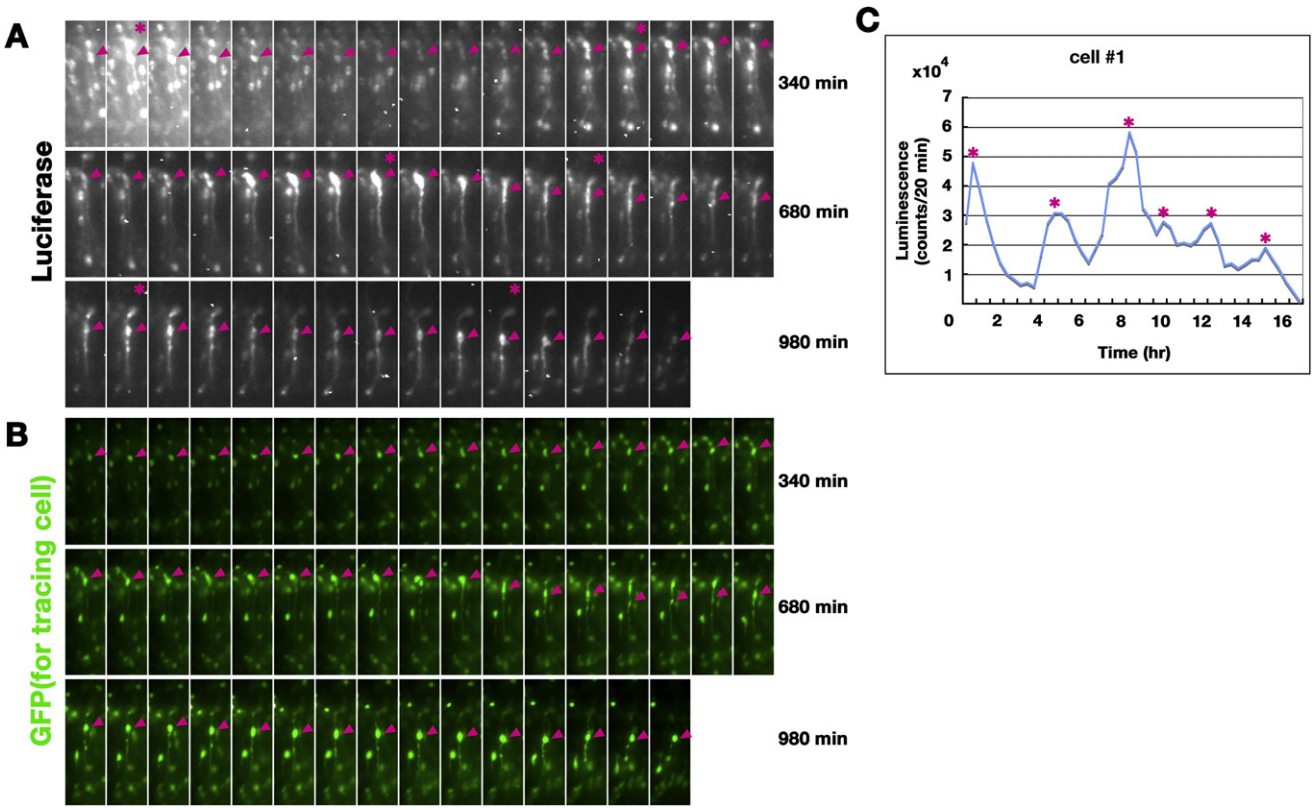


Figure 3. Real-Time Imaging of *Hes1* Expression in a Slice Culture of the Dorsal Telencephalon

pHes1-Ub1-Luc reporter was introduced into telencephalic neural progenitors of mouse embryos in utero by electroporation at E13.5, and a slice culture was prepared one day later. pEF-EGFP vector was also introduced to visualize cell morphology.

(A) Bioluminescence images were taken using 20 min exposures and binning of pixels 4 × 4 to increase signal-to-noise ratios (Movie S2). *Hes1* expression dynamically oscillated in a neural progenitor.

(B) To trace cells, EGFP expression was monitored. Labeled cells displayed an elevator movement of their nuclei and cell bodies in a slice culture.

(C) Quantification of bioluminescence of a neural progenitor shown in ([A], arrowheads). Peaks are indicated by asterisks.

cells probably proceeded from early G1 to late G1 phase in culture, because Hes1 is not expressed during early G1 phase (Figures 1A–1D). If such long silence was excluded, the average period of Hes1 oscillation from E9.5 to E14.5 was 2–3 hr (Figure 2D). Thus, the period of Hes1 oscillation was not significantly changed during development, although there was some tendency for the period to be longer at earlier stages.

Hes1 Expression Oscillates in Neural Progenitors in Slice Cultures

We next examined the dynamics of Hes1 expression in slice cultures, which do not disrupt Notch signaling and thus represent in vivo situations better than dissociation cultures. The pHes1-Ub1-Luc reporter was introduced into telencephalic neural progenitors of mouse embryos in utero by electroporation at E13.5, and slice cultures were prepared one day later to examine Hes1 expression. pEF-EGFP vector, which directs GFP expression from the elongation factor 1α promoter, was also introduced. Neural progenitors, which were visualized by GFP expression, displayed an elevator movement of their nuclei and cell bodies in slice cultures (Figure 3B). The intensity of GFP labeling was not significantly changed according to the cell movement (Figure 3B). In contrast, the luciferase activity representing *Hes1* expression was oscillatory in neural progenitors, when they were located in the outer region of the ventricular zone or descending toward the ventricular surface (corresponding to late G1, S, and G2 phases) (Figure 3A, arrowheads; Figure S3; and Movie S2). The average period of *Hes1* oscillation in slice cultures was about 3 hr (Figure 3C). However, neural progenitors ascending near the ventricular surface toward the outer region, which were in early G1 phase, did not express *Hes1* (data not shown). All these data agreed well with the above results.

Persistent and High Levels of *Hes1* Repress Expression of Proneural Genes, Notch Ligands, and Cell Cycle Regulators

Real-time monitoring experiments revealed that Hes1 expression oscillates in neural progenitors. We next investigated the significance of Hes1 oscillation. It was previously shown that persistent and high levels of Hes1 expression not only inhibit neuronal differentiation but also retard cell cycle progression of cultured cells (Castella et al., 2000; Ström et al., 2000; Hartman

A

Fold Change	Gene Name	Genbank
0.1265		AI323358
0.2095	Ascl1	BB425719
0.2395	Neurod4	BB250949
0.259	Cnnm3	BB236001
0.2615	Gadd45g; CR6	AK007410
0.3025	Neurog2; ngn2	NM_009718
0.3035	Gprc5b; Raig2	BC020004
0.308	Insm1; IA-1	NM_016889
0.312	Hes5	AV337579
0.317	Ccnd1; Cyl-1	NM_007631
0.322		BB561515
0.3265	Dll1; Delta1	NM_007865
0.3565	Myt1; Nzf2	NM_008665
0.371	Bmf	BB212341
0.375		BB468410
0.3785	Jag1	AV359819
0.3855	Sstr2; sst2	NM_009217
0.389	Eomes	BB128925
0.391	Ccnd1; Cyl-1	NM_007631
0.3975	Socs3	BB241535
0.4125		BM244697
0.419	Bcor	AV318805
0.4245	D16Ertd472e	AV381575
0.428	Hes6	AI326893
0.4285	Sertad4	BQ174721
0.429	Bcl7c	BM234097
0.4325	Rev1l; MGC66950	NM_019570
0.4405	Btg2; Pc3; TIS21	NM_007570
0.4445	Tbr2; Eomes	AB031037
0.4465	Mfng	NM_008595
0.4525		BB331017
0.4535	BC031353	BG276629
0.4555	A630082K20Rik	BM238680
0.457	Tcfap2c; Ap-2.2	BC003778
0.4575	Elk3	AW544251
0.46	Fgfr3; HBGFR	NM_008010
0.4625	Ccne2	AF091432
0.4665	5033413D16Rik	AK017164
0.4745	Rhbdl4	BM118307
0.483	E130115E03Rik	AV299725

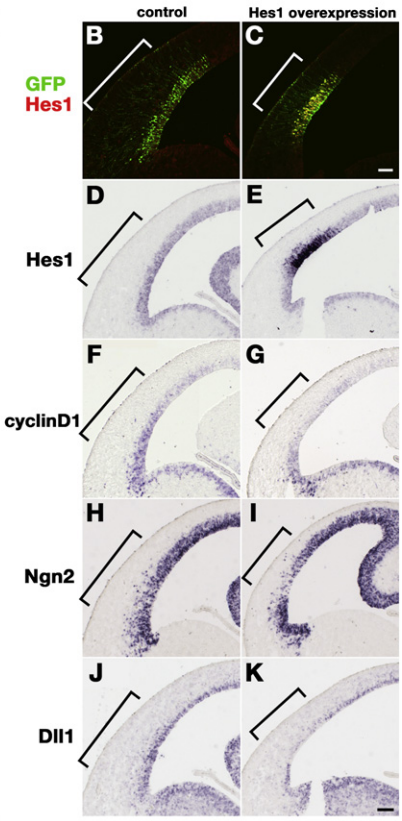


Figure 4. Effects of Sustained Overexpression of *Hes1*

(A) List of genes whose expression was repressed by sustained overexpression of *Hes1*. Genes that display >2.0-fold higher repression by *Hes1* are indicated. pEF or pEF-Hes1 together with pEF-EGFP was electroporated into telencephalic neural progenitors at E13.5. Eighteen hours later, the telencephalon was dissociated, and EGFP-positive cells were collected by a cell sorter. Biotinylated cRNAs were made from each sample and hybridized to high-density microarrays. Data from two independent experiments are shown.

(B–K) pEF (B, D, F, H, and J) or pEF-Hes1 (C, E, G, I, and K) together with pEF-EGFP was electroporated into the developing telencephalon at E13.5, and brain sections were examined by immunohistochemistry (B and C) or in situ hybridization (D–K). Sustained overexpression of *Hes1* repressed the endogenous expression of *cyclin D1* (G), *Ngn2* (I), and *Dll1* (K). Electroporated regions are shown by brackets. Scale bars, 100 μm (C) and (K).

et al., 2004; Baek et al., 2006), raising the possibility that persistent Hes1 expression represses expression of genes required for efficient proliferation and differentiation of neural progenitors. To determine the target genes for Hes1, we introduced pEF-Hes1 and pEF-EGFP vectors into the developing telencephalon by electroporation at E13.5. As a control, pEF was used instead of pEF-Hes1. The telencephalon was dissociated 18 hr after electroporation, at which time the control cells did not initiate overt neuronal differentiation; thus, the earliest changes in gene expression could be detected. Transfected cells (GFP^+) were collected by a cell sorter, and biotinylated cRNAs of each sample were hybridized to high-density microarrays. We found that 40 genes displayed more than two-fold repression by persistent and high levels of Hes1 expression in telencephalic neural progenitors (Figure 4A). These genes included the proneural genes *Mash1* (*Ascl1*), *Math3* (*Neurod4*), and *Ngn2*, the Notch ligands *Dll1* and *Jag1*, and the cell cycle regulators *cyclin D1* (*Ccnd1*) and *cyclin E2* (*Ccne2*).

To confirm that *Hes1* represses the endogenous expression of these genes, we introduced pEF-Hes1 and pEF-EGFP (Hes1 + EGFP) into the developing dorsal telencephalon by electroporation at E13.5. As a control, pEF was introduced instead of pEF-Hes1 (EGFP only). In situ hybridization and immunohistochemical analysis indicated that sustained overexpression of *Hes1* repressed the endogenous expression of *cyclin D1*, *Ngn2*, and *Dll1* (Figures 4F–4K and Figures S4A–S4F). Furthermore, *Hes1* inhibited BrdU uptake (Figures S4G–S4L) and led to G1 phase retardation (Figure S5). These in vivo results agreed well with the previous in vitro data (Baek et al., 2006). Hes1 + EGFP and the EGFP-only telencephalic cells were also collected by sorting, and gene expression levels were examined by quantitative PCR. *Cyclin D1*, *Ngn2*, and *Dll1* expression was significantly downregulated in *Hes1*-overexpressing (Hes1 + EGFP) cells, compared with control (EGFP only) cells (Figure S6). These results suggest that persistent and high levels of *Hes1* expression inhibit neuronal differentiation and cell cycle progression by repressing expression of proneural genes and cell cycle regulators. These results also suggest that proper expression of these genes depends on Hes1 oscillation.

In situ hybridization and quantitative PCR analysis indicated that *Hes1* also repressed other genes, such as the Notch signaling molecules *Hes5* and *Manic fringe* (*Mfng*) (Figures S6 and S7A–S7D) (Ohtsuka et al., 1999; Chen et al., 2001) and the cell cycle regulators *Gadd45g*, *Myt1*, and *Tis21* (Figures S6 and S7E–S7J), which induce G2-M arrest (Mueller et al., 1995; Rouault et al., 1996; Vairapandi et al., 2002), suggesting that *Hes1* regulates cell proliferation and differentiation via multiple factors.

Ngn2 and *Dll1* Are Expressed at Various Levels by Dividing Neural Progenitors

Ngn2 and *Dll1* not only were repressed by overexpression of *Hes1* (Figures 4I and 4K) but also are known to be upregulated in the absence of *Hes1* (Hatakeyama et al., 2004; Baek et al., 2006), raising the possibility that expression of *Ngn2* and *Dll1* dynamically changes under the control of *Hes1* oscillation. We therefore examined whether *Ngn2* and *Dll1* are expressed at various levels by neural progenitors. BrdU was administered to embryos at E10.5, E12.5, and E14.5 to monitor dividing neural progenitors at S to G2 phase.

Immunohistochemical analysis showed that Ngn2 protein was expressed at various levels in the ventricular zone at E10.5,

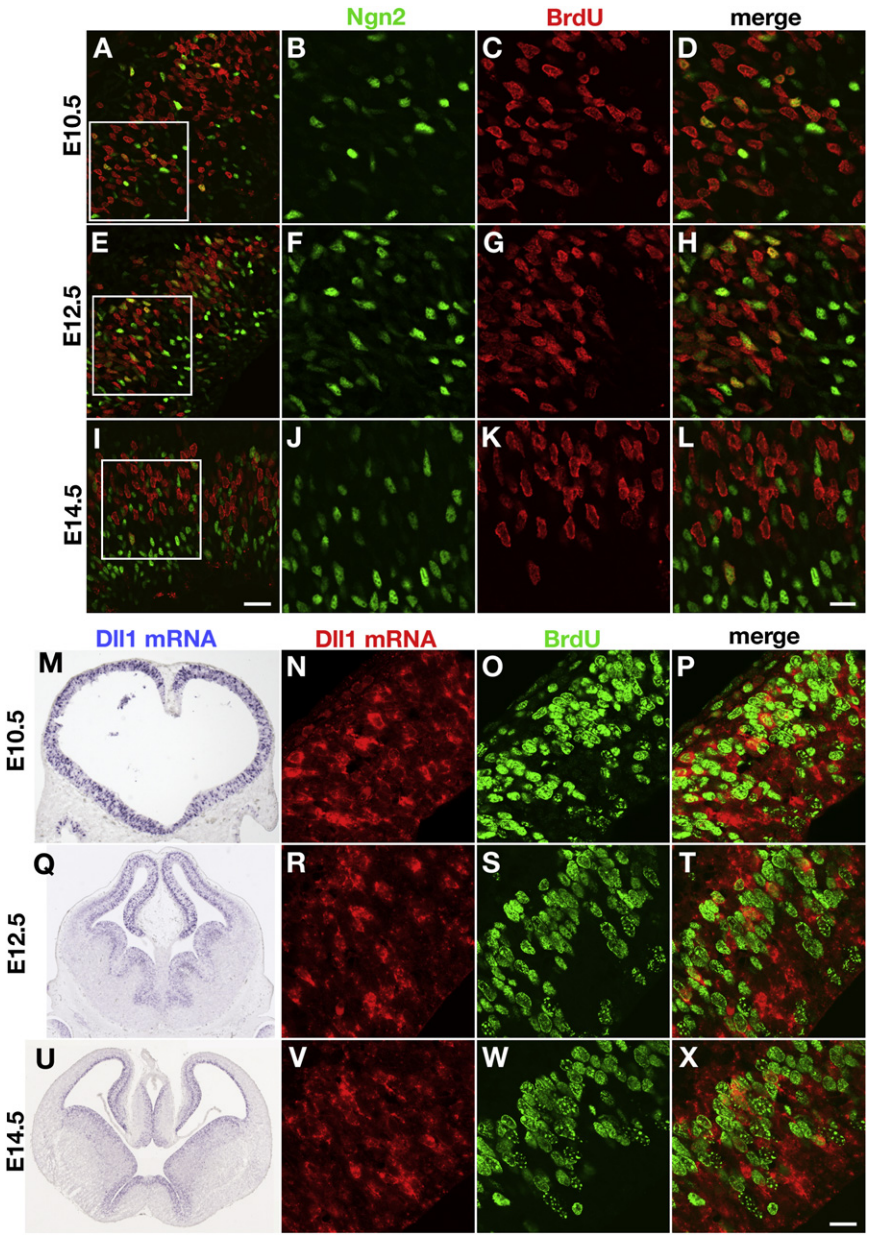


Figure 5. Ngn2 and *Dll1* Expression in Dividing Neural Progenitors

BrdU was administered to mouse embryos, and the telencephalon was examined 1–2 hr later to monitor the cells at S to G2 phase.

(A–L) Many BrdU$^+$ cells expressed Ngn2 at variable levels at E10.5 and E12.5 (A–H), suggesting that many dividing neural progenitors express Ngn2 at this stage. At E14.5, some of BrdU$^+$ cells expressed Ngn2, although the number was reduced (I–L). At this stage, many Ngn2-expressing cells did not incorporate BrdU, suggesting that many of them differentiated into postmitotic neurons. Boxed regions in (A), (E), and (I) are enlarged in (B–D), (F–H), and (J–L), respectively.

(M–X) *Dll1* was expressed in the ventricular zone at E10.5, E12.5, and E14.5 (M, Q, and U). Many BrdU$^+$ cells expressed *Dll1* at variable levels at E10.5 and E12.5 (N–P and R–T), suggesting that many dividing neural progenitors express *Dll1* at this stage. At E14.5, the number of *Dll1*-expressing neural progenitors was reduced (V–X). Scale bars, 20 μm in (A), (E), and (I); 20 μm in (N–P), (R–T), and (V–X); and 10 μm in (B–D), (F–H), and (J–L).

E12.5, and E14.5 (Figures 5A, 5B, 5E, 5F, 5I, and 5J). At E10.5 and E12.5, many of Ngn2-expressing cells (39.0% ± 15.1% at E10.5 and 38.9% ± 6.4% at E12.5) were labeled with BrdU (Figures 5A–5H). Furthermore, many Ngn2-expressing cells were positive for Ki67 at this stage (Figures S8A–S8H). At E14.5, some of BrdU$^+$ or Ki67$^+$ cells expressed Ngn2, although the number was reduced (Figures 5I–5L and Figures S8I–S8L). At this stage, many Ngn2-expressing cells did not incorporate BrdU, suggesting that many of them differentiated into postmitotic neurons (Figures 5I–5L). Similarly, in situ hybridization analysis showed that *Dll1* mRNA was expressed at various levels in the ventricular zone at E10.5, E12.5, and E14.5 (Figures 5M, 5N, 5Q, 5R, 5U, and 5V). At E10.5 and E12.5, many *Dll1*-expressing cells (46.0% ± 2.1% at E10.5 and 42.0% ± 4.5% at E12.5) were labeled with BrdU (Figures 5N–5P and 5R–5T), but this ratio was reduced at E14.5 (Figures 5V–5X). Thus, Ngn2 and *Dll1* expression occurred at various levels in many dividing neural progenitors at E10.5 and E12.5, but the number of Ngn2- and *Dll1*-expressing neural progenitors was reduced at E14.5.

Inverse Correlation between Hes1 and Ngn2/*Dll1* Expression Levels

Our results indicated that Ngn2 and *Dll1* are indeed expressed at various levels by neural progenitors. We next sought to determine the relationship between Hes1 protein and Ngn2/*Dll1* expression levels. Many cells coexpressed Hes1 and Ngn2 or *Dll1* at E12.5 (Figures 6A–6D and 6I–6L). Interestingly, in most cells, when the levels of Hes1 protein were high, levels of Ngn2 expression were low (Figures 6A–6D, arrows), and vice versa (Figures 6A–6D, arrowheads). Similarly, when the levels of Hes1 protein were high, *Dll1* expression was mostly undetectable, and when the levels of Hes1 protein were low, *Dll1* expression was observed in neural progenitors (Figures 6I–6L). Thus, there was an inverse correlation between Hes1 and Ngn2/*Dll1* expression levels (Figure S9). At E14.5, there was also an inverse correlation between Hes1 and Ngn2/*Dll1* expression levels, although the number of cells coexpressing Hes1 and Ngn2 or *Dll1* was reduced (Figures 6E–6H and 6M–6P and Figure S9).

The above results suggest that variable levels of *Ngn2* and *Dll1* expression are controlled, at least in part, by *Hes1*. To examine this possibility, we next misexpressed low, intermediate, and

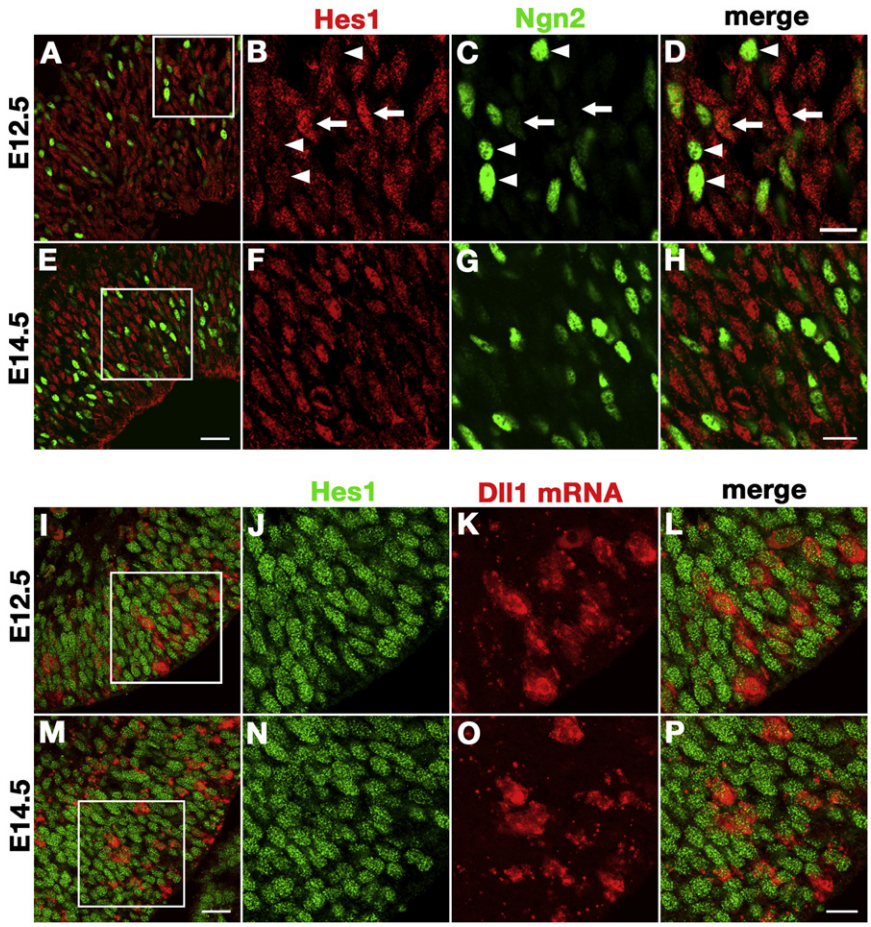


Figure 6. Inverse Correlation Between Hes1 and Ngn2/*Dll1* Expression

The telencephalon at E12.5 and E14.5 was examined.

(A–H) Immunohistochemistry for Hes1 and Ngn2. At E12.5, when the levels of Hes1 protein were high, levels of Ngn2 expression were low ([A–D], arrows), and vice versa ([A–D], arrowheads). Thus, there was an inverse correlation between Hes1 and Ngn2 expression levels. At E14.5, there was also an inverse correlation between Hes1 and Ngn2 expression levels, but fewer cells coexpressed Hes1 and Ngn2, suggesting that Ngn2 expression is gradually restricted to Hes1$^-$ cells (E–H). Boxed regions in (A) and (E) are enlarged in (B–D) and (F–H), respectively.

(I–P) Immunohistochemistry for Hes1 and in situ hybridization for *Dll1*. At E12.5, when the levels of Hes1 protein were high, *Dll1* expression was mostly undetectable, and when the levels of Hes1 protein were low, *Dll1* expression was observed in neural progenitors (I–L). Thus, there was an inverse correlation between Hes1 and *Dll1* expression levels. At E14.5, there was also an inverse correlation between Hes1 and *Dll1* expression levels, but fewer cells coexpressed Hes1 and *Dll1*, suggesting that *Dll1* expression is gradually restricted to Hes1$^-$ cells (M–P). Boxed regions in (I) and (M) are enlarged in (J–L) and (N–P), respectively. Scale bars, 20 μm in (A), (E), (I), and (M); 10 μm in (B–D), (F–H), (J–L), and (N–P).

high levels of *Hes1* by electroporating different amounts of pEF-Hes1 into the developing dorsal telencephalon at E13.5. As *Hes1* expression levels increased (Figures S10A, S10B, S10H, S10I, S10O, and S10P), *Ngn2* and *Dll1* expression levels decreased (Figures S10C, S10D, S10J, S10K, S10Q, and S10R). We also obtained the same results when we electroporated the Tet-Off promoter-driven *Hes1* expression vector and applied different amounts of doxycycline (data not shown). These results support the notion that *Ngn2* and *Dll1* expression levels dynamically change depending on *Hes1* expression levels.

Interestingly, when *Hes1* was persistently misexpressed at an intermediate or a high level, many nontransfected cells (GFP-negative) prematurely differentiated into neurons (TuJ1$^+$) in the ventricular zone (Figures S10L–S10N and S10S–S10Y). This was probably because *Dll1* expression was persistently repressed in transfected cells (GFP$^+$) and thus Notch signaling was kept inactive in their neighboring nontransfected cells (GFP$^-$). These data suggest that *Hes1* oscillation is required for mutual activation of Notch signaling and maintenance of neural progenitors in the ventricular zone.

Oscillatory Expression of *Ngn2* and *Dll1* Is Regulated by Hes1 Oscillation

To reveal the dynamics of *Ngn2* and *Dll1* expression, we imaged their expression at the single cell level by using a ubiquitinated firefly luciferase reporter under the control of the *Ngn2* or *Dll1* promoter (pNgn2-Ub1-Luc and pDll1-Ub1-Luc) (Figures 7A and 7H). The half-lives of *Ngn2* and *Dll1* mRNAs are shorter than 30 min in neural progenitors (data not shown); thus, this reporter is unstable enough to monitor the precise dynamics of *Ngn2* and *Dll1* expression. The pNgn2-Ub1-Luc or pDll1-Ub1-Luc reporter was introduced into telencephalic neural progenitors of mouse embryos in utero by electroporation at E13.5, and dissociation cultures were prepared one day later. pEF-EGFP vector was also introduced together, and the bioluminescence of GFP$^+$ cells was monitored. Both *Ngn2* and *Dll1* expression were found to oscillate in about a half of GFP$^+$ cells (Figures 7B, 7D, 7I, and 7K and Movies S3 and S4) but were relatively persistent in the other half (Figures 7C, 7E, 7J, and 7L and Movies S3 and S4). Slice cultures were also prepared from pNgn2-Ub1-Luc- or pDll1-Ub1-Luc-electroporated telencephalon, and both oscillating and persistently expressing cells were found in either case (data not shown). In dissociation cultures, the oscillating cells were negative for TuJ1 expression, and thus it is likely that they were neural progenitors (Figures 7F and 7M). In contrast, the persistently expressing cells were positive for TuJ1, and thus they were differentiating neurons (Figures 7G and 7N). These results indicated that both *Ngn2* and *Dll1* expression are oscillating in neural progenitors but persistent in differentiating neurons.

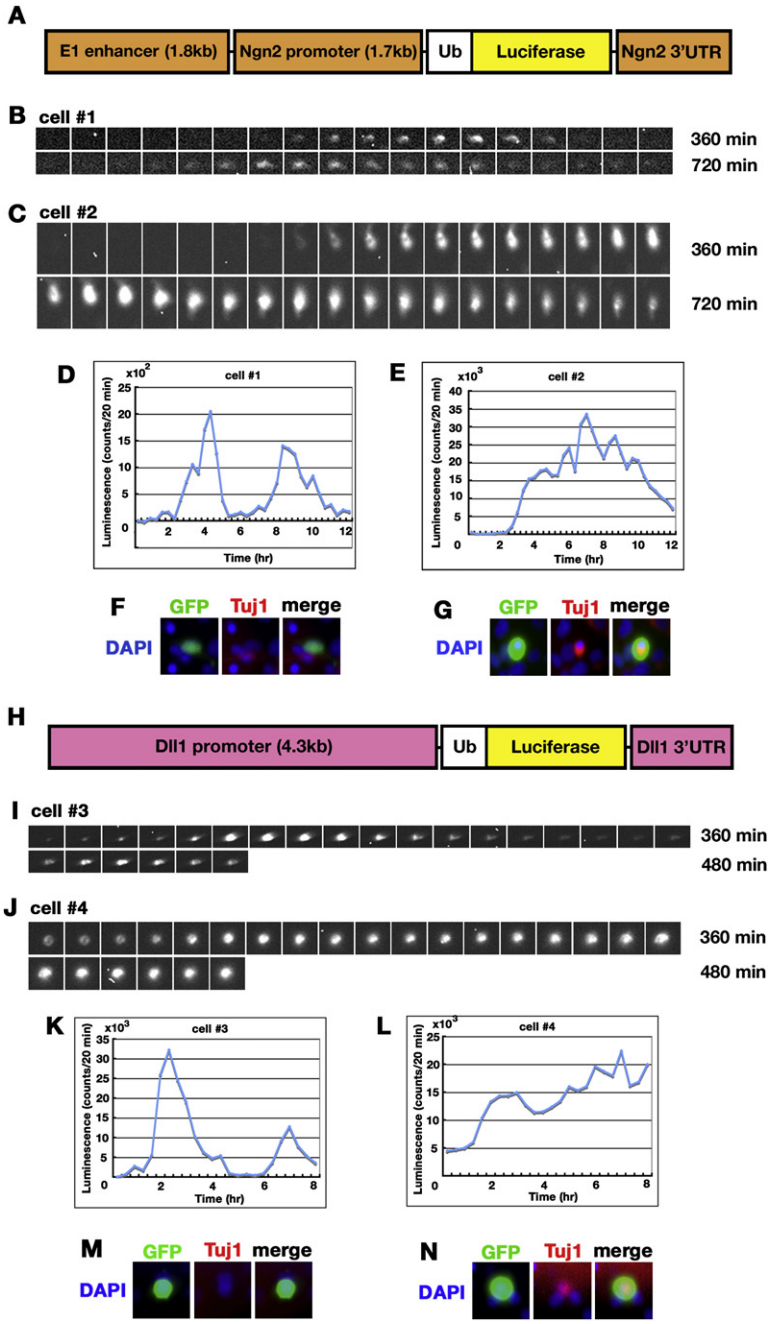


Figure 7. Oscillatory Expression of *Ngn2* and *Dll1* in Neural Progenitors

The pNgn2-Ub1-Luc (A–G) or pDll1-Ub1-Luc reporter (H–N) was introduced into telencephalic neural progenitors of mouse embryos in utero by electroporation at E13.5, and dissociation cultures were prepared one day later. pEF-EGFP vector was also introduced together, and the bioluminescence of GFP^+ cells was monitored.

(A) Schematic structure of pNgn2-Ub1-Luc.

(B and C) Bioluminescence images of individual neural progenitors were taken using 20 min exposures and binning of pixels 2 × 2 (Movie S3).

(D and E) Quantification of bioluminescence of individual neural progenitors shown in (B) and (C).

(F and G) After bioluminescence images were taken, the cells were immunostained. A cell expressing *Ngn2* in an oscillatory manner was negative for TuJ1 (F), whereas one expressing *Ngn2* persistently was positive for TuJ1 (G).

(H) Schematic structure of pDll1-Ub1-Luc.

(I and J) Bioluminescence images of individual neural progenitors were taken using 20 min exposures and binning of pixels 2 × 2 (Movie S4).

(K and L) Quantification of bioluminescence of individual neural progenitors shown in (I) and (J).

(M and N) After bioluminescence images were taken, the cells were immunostained. A cell expressing *Dll1* in an oscillatory manner was negative for TuJ1 (M), whereas one expressing *Dll1* persistently was positive for TuJ1 (N).

We observed *Hes1* oscillation more frequently in neural progenitor cultures, which were plated at higher density (data not shown). We thus speculated that cell-cell interaction via Notch signaling is required for *Hes1* oscillation and that *Dll1* oscillation mutually activates Notch signaling in neighboring neural progenitors. To determine whether Notch signaling is required for *Hes1* oscillation in these cells, we examined the effect of a γ-secretase inhibitor, which blocks Notch signaling. In the presence of the γ-secretase inhibitor DAPT, *Hes1* expression was persistently repressed (Figure S11A), indicating that *Hes1* oscillation depends on Notch signaling. Under this condition, both *Ngn2* and *Dll1* expression was persistently upregulated (Figures S11B and S11C). Real-time imaging analysis showed that, in the presence of DAPT, the number of cells expressing *Ngn2* and *Dll1* in an oscillatory manner decreased, whereas that of cells exhibiting persistent expression of *Ngn2* and *Dll1* increased. Thus, blockade of Notch signaling, a condition known to induce neuronal differentiation, leads to repression of *Hes1* expression and sustained upregulation of *Ngn2* and *Dll1* expression.

It was recently shown that, in addition to Notch signaling, Jak2-Stat3 signaling is required for *Hes1* oscillation in fibroblasts (Yoshiura et al., 2007). To determine whether Jak2-Stat3 signaling is involved in *Hes1* oscillation in neural progenitors, AG490, an inhibitor of this signaling, was added to the cultures. Under this condition, *Hes1* oscillation disappeared (Figure S12), suggesting that Jak2-Stat3 signaling also regulates *Hes1* oscillation in neural progenitors.

DISCUSSION

Oscillatory Expression of *Hes1*, *Ngn2*, and *Dll1* in Neural Progenitors

We examined the dynamics of Hes1 expression using a real-time imaging method and showed that *Hes1* expression dynamically oscillates in neural progenitors in the embryonic brain (Figure 8A). To our surprise, we found that expression of the

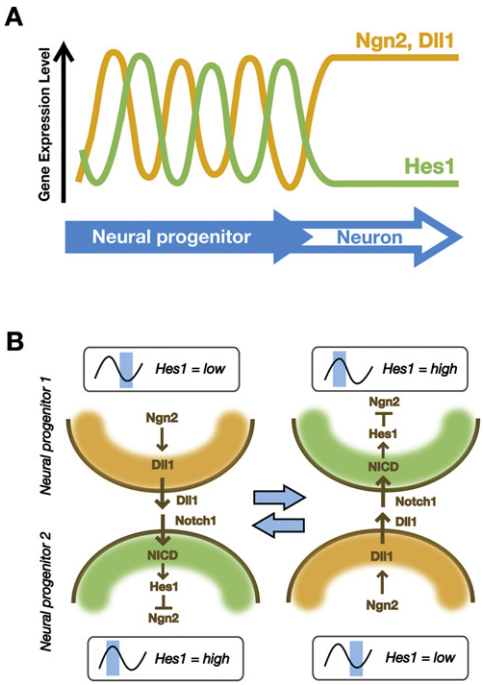


Figure 8. Model for Oscillations in Notch Signaling

(A) Expression of *Hes1*, *Ngn2*, and *Dll1* oscillates in dividing neural progenitors. In immature postmitotic neurons, *Hes1* is downregulated, whereas *Ngn2* and *Dll1* are upregulated in a sustained manner. It is likely that oscillatory expression of Ngn2 is not sufficient but sustained upregulation is required for neuronal differentiation.

(B) *Ngn2* and *Dll1* oscillations are regulated by *Hes1* oscillation in neural progenitors. Ngn2 oscillation may be advantageous for maintenance/proliferation of neural progenitors at early stages, because it induces *Dll1* expression and activates Notch signaling without promoting neuronal differentiation.

proneural gene *Ngn2* and the Notch ligand *Dll1* also oscillates in neural progenitors (Figure 8A). Downregulation of *Hes1* expression by blockade of Notch signaling leads to sustained upregulation of *Ngn2* and *Dll1*, whereas sustained overexpression of *Hes1* downregulates *Ngn2* and *Dll1* expression. These data suggest that Hes1 regulates *Ngn2* and *Dll1* oscillations in neural progenitors by periodically repressing their expression. We then speculated that Hes1 directly represses *Ngn2* expression, as is the case for a related proneural gene *Mash1*, which Hes1 directly represses by binding to the *Mash1* promoter (Chen et al., 1997). However, regulation of *Dll1* expression by Hes1 could be indirect. It has been shown that Ngn2 upregulates *Dll1* expression by directly binding to the enhancer region (Castro et al., 2006). Thus, Ngn2 oscillation itself may induce periodic upregulation of *Dll1*. However, it is also possible that Hes1 directly represses *Dll1* expression by competing with Ngn2, because Hes1 can functionally antagonize proneural factors by forming a non-DNA-binding heterodimer complex (Sasai et al., 1992). Both mechanisms are not mutually exclusive and may be cooperative for precise regulation of *Dll1* expression.

It has been shown that Notch ligands are expressed by differentiating neurons (Henrique et al., 1995; Myat et al., 1996; Dunwoodie et al., 1997). However, it was previously reported that expression of Notch ligands and induction of the Notch effector *Hes5* occur before overt neuronal differentiation (Bettenhausen et al., 1995; Hatakeyama et al., 2004). We showed here that the Notch ligand *Dll1* is expressed in an oscillatory manner by neural progenitors. It is likely that *Dll1* oscillation mutually activates Notch signaling in neighboring neural progenitors, thereby maintaining *Hes1* oscillation and these cells (Figure 8B). At one time point, when the levels of Hes1 protein are high by activation of Notch signaling, those of *Ngn2* and *Dll1* expression are low (Figure 8B). About 1 hr later, the levels of Hes1 protein become low as a result of oscillation, leading to upregulation of *Ngn2* and *Dll1*, which activates Notch signaling of neighboring cells (Figure 8B). Our data also showed that persistent *Hes1* expression in subsets of neural progenitors represses *Dll1* expression and induces ectopic neuronal differentiation of the neighboring cells in the ventricular zone (Figure S10). These data suggest that *Hes1* oscillation is important for mutual activation of Notch signaling and maintenance of neural progenitors.

Apparently, *Ngn2* oscillation cannot induce neuronal differentiation, although *Ngn2* is known to have a neurogenic activity (Ma et al., 1996; Nieto et al., 2001; Sun et al., 2001). In differentiating neurons, *Ngn2* is expressed in a sustained manner (Figures 7C, 7E, and 7G). These results imply that oscillatory expression of *Ngn2* is not sufficient for but sustained upregulation is required for neuronal differentiation. *Ngn2* oscillation may be advantageous for maintenance/proliferation of neural progenitors at early stages, because it induces *Dll1* expression and activates Notch signaling without promoting neuronal differentiation. In agreement with this idea, various levels of Ngn2 and *Dll1* expression, which are indicative of oscillatory expression, are observed more frequently in neural progenitors at earlier stages (around E10.5 to E12.5), when many cells proliferate by symmetric cell division.

We showed that *Ngn2* and *Dll1* expression oscillates in neural progenitors (Figure 7). On immunohistochemical analysis, Ngn2 protein was expressed at various levels by neural progenitors (Figures 5 and 6), suggesting that expression of Ngn2 protein also oscillates in these cells. However, it remains to be determined whether Dll1 protein expression oscillates in neural progenitors. We did not show this, because it was technically difficult to measure the Dll1 protein levels on the cell surface. If the Dll1 protein is stable, *Dll1* mRNA oscillation does not lead to Dll1 protein oscillation; rather, it just maintains Dll1 expression at certain levels. Persistent expression of Dll1 protein would also induce Hes1 oscillation, because the addition of cells that persistently express Dll1 can induce Hes1 oscillation (Hirata et al., 2002).

Although the periods of *Hes1* oscillation vary from cycle to cycle and from cell to cell, the average was 2–3 hr during E9.5–E14.5. Because there was some tendency for the period to be longer at earlier stages, different period lengths could be involved in different characteristics of neural progenitors. For example, symmetrically dividing early neural progenitors could have a longer period than asymmetrically dividing late progenitors. Further analysis will be required to reveal the relationship between the period lengths and characteristics of neural progenitors.

Oscillatory versus Sustained Hes1 Expression

The developing nervous system is partitioned into many compartments by such boundaries as the isthmus and zona limitans intrathalamica (Kiecker and Lumsden, 2005). Cells in

compartments proliferate efficiently and give rise to many neurons, whereas cells in boundary areas do not. In contrast to compartmental neural progenitors, boundary cells persistently express Hes1 at high levels (Baek et al., 2006). Furthermore, sustained overexpression of *Hes1* in telencephalic compartment cells represses expression of cell cycle regulators and proneural genes (Figure 4), suggesting that sustained Hes1 expression is involved in slow cell proliferation and inhibition of neurogenesis, two important features of boundary cells. Thus, it is likely that sustained expression of Hes1 is involved in the formation of boundary cells, whereas oscillatory expression of Hes1 causes formation of compartment cells in the embryonic brain.

We found that Hes1 expression is repressed at early G1 phase in compartmental neural progenitors and that sustained overexpression of *Hes1* in these cells downregulates expression of such cell cycle regulators as *cyclin D1* and *cyclin E2* and leads to G1 phase retardation. Thus, although Hes1 is required for maintenance of neural progenitors, it should be downregulated at certain points, such as in G1 phase, to allow cell cycle progression. Further analysis will be required to determine at which points Hes1 inhibits and promotes the cell cycle.

The precise mechanism underlying how persistent versus oscillatory Hes1 expression is regulated in neural progenitors is not known. We recently found that Hes1 oscillation is regulated by Stat3-Socs3 signaling in fibroblasts after serum stimulation (Yoshiura et al., 2007). Stat3-Socs3 signaling displays an oscillatory response that involves negative feedback: Jak activates Stat3 by phosphorylation, and phosphorylated Stat3 (p-Stat3) induces expression of Socs3, which antagonizes Jak-dependent activation of Stat3 (Levy and Darnell, 2002; Yu and Jove, 2004). As a result, formation of p-Stat3 and expression of Socs3 oscillate out of phase with each other (Yoshiura et al., 2007). Interestingly, this oscillation is important for Hes1 oscillation: in the absence of Stat3-Socs3 oscillations, Hes1 protein is expressed in a sustained manner, whereas *Hes1* mRNA expression is suppressed, although the precise mechanism remains to be analyzed (Yoshiura et al., 2007). Because Jak-Stat signaling is involved in maintenance of neural progenitors (Kamakura et al., 2004), it is likely that Hes1 oscillations are regulated by Jak-Stat signaling in these cells. Indeed, we found that blockade of this signaling abolished *Hes1* oscillation (Figure S12). It was also recently shown that Id factors are involved in sustained upregulation of *Hes1* in boundary regions of the developing nervous system (Bai et al., 2007). Id factors are highly expressed in boundary regions and inhibit Hes1 from negative autoregulation, thereby persistently upregulating *Hes1* expression. Interestingly, Id factors do not inhibit Hes1 from repressing proneural gene expression (Bai et al., 2007). Both Jak-Stat signaling and Id factors may be responsible for oscillatory versus persistent *Hes1* expression.

Biological Implications of Oscillatory Expression

It is well known that neural progenitors display a variety of responses to single signals. For example, although PDGF induces neuronal differentiation very efficiently, subsets of neural progenitors nevertheless become oligodendrocytes or astrocytes (Johe et al., 1996). We also noted that some neural progenitors start neuronal differentiation immediately, whereas others do not. Such nonresponding cells could adopt different cell phenotypes later. It is possible that Hes1 oscillation is involved in this different responsiveness. Cells expressing Hes1 at high levels probably do not respond, whereas cells expressing Hes1 at very low levels can respond immediately to differentiation signals. Cells expressing Hes1 at intermediate levels could have limited responsiveness to certain differentiation signals. Further analysis will be required to determine the relationship between the levels of Hes1 expression and the timing of cell differentiation.

In addition to *Hes1*, *Ngn2*, and *Dll1*, more and more genes have been shown to display oscillatory expression. For example, more than 30 genes, including *Hes1*, *Hes7*, and *Dll1*, are cyclically expressed during somite segmentation (Jouve et al., 2000; Bessho et al., 2001; Maruhashi et al., 2005; Dequéant et al., 2006). Furthermore, expression of NF-κB signaling, p53, Socs3, and Smad6 also oscillates (Lev Bar-Or et al., 2000; Hoffmann et al., 2002; Nelson et al., 2004; Yoshiura et al., 2007). These data suggest that oscillatory expression is more general than was previously thought. There seem to be at least three different modes of response of downstream target genes to Hes1 oscillation. If downstream target gene products are very stable, transcriptional induction by activators and periodic repression by Hes1 oscillation could lead to upregulation of the products in a stepwise manner. When the expression of downstream genes reaches a certain level, the next event could happen. In this case, the number of oscillation cycles can be converted into the timing information of the next event, suggesting that Hes1 functions as a cellular clock. If the downstream target gene products are relatively unstable, they do not accumulate. Expression of such factors can be maintained within a certain range by periodic downregulation by Hes1 oscillation. If the downstream target gene products are very unstable, their expression should be periodically downregulated by Hes1 oscillation, thus responding in an oscillatory manner, like Ngn2. Further analysis of the expression of downstream target genes will be required to reveal the full significance of Hes1 oscillations.

EXPERIMENTAL PROCEDURES

Reporter Vectors

pHes1-Ub1-Luc was described elsewhere (Masamizu et al., 2006). For pNgn2-Ub1-Luc, 1.8 kb E1 enhancer (Scardigli et al., 2001) and 1.7 kb of *Ngn2* promoter were placed upstream, and 3′-untranslated region of *Ngn2* was placed downstream of Ub1-Luc. For pDll1-Ub1-Luc, 4.3 kb of *Dll1* promoter (Castro et al., 2006) was placed upstream, and 3′-untranslated region of *Dll1* was placed downstream of Ub1-Luc.

Mice

pHes1-Ub1-Luc transgenic mice were described elsewhere (Masamizu et al., 2006). All mice used for this study were maintained and handled according to the protocols approved by Kyoto University.

In Utero Electroporation

In utero electroporation was performed as described elsewhere (Ohtsuka et al., 2001). Embryos were harvested 18–24 hr after electroporation.

BrdU Labeling

For cell cycle phase labeling, pregnant mice were injected intraperitoneally with 50 μg BrdU/g of body weight. By changing BrdU exposure time, different cell cycle phases were labeled. For analysis of E14.5 mouse embryos, brain sections were examined 30 min, 90 min, 8 hr, and 14 hr after BrdU administration, which labeled cells in the S phase, S-G2 phase, early G1 phase, and late G1 phase, respectively (Takahashi et al., 1995).

For analysis of cell cycle progression of Hes1-overexpressing cells, in utero electroporation of pEF-Hes1 or pEF control vector with pEF-EGFP was performed, and embryos were examined 24 hr later. BrdU was injected 2 hr before harvesting.

Tissue Processing

After electroporation or BrdU labeling, embryos were harvested, and brains were excised in phosphate buffered saline (PBS). For immunohistochemistry, brains were fixed in 2% paraformaldehyde (PFA) for 30 min or in 4% PFA for 6 hr or overnight at 4°C. For in situ hybridization, brains were fixed in 4% PFA for 6 hr or overnight at 4°C. Fixed brains were cryoprotected overnight in 25% sucrose/PBS at 4°C, embedded in OCT compound, and sectioned at 15 μm by cryostat.

Immunohistochemistry

Frozen sections of fixed brains were treated as described elsewhere (Ohtsuka et al., 2006). For immunostaining of BrdU, HCl treatment was performed as follows. After washing in PBS, sections were incubated in 2N HCl for 30 min at 37°C and then were neutralized in 0.1 M sodium tetraborate for 10 min at room temperature. Primary antibodies used are as follows: guinea pig anti-Hes1 (1:500 dilution, Baek et al., 2006), goat anti-Ngn2 (1:500; Santa Cruz Biotechnology), mouse anti-BrdU (1:500; Sigma), mouse anti-Ki67 (1:100; BD PharMingen), mouse antiphosphorylated histone H3 (1:500; Sigma), rabbit anti-GFP (1:500; Molecular Probes), mouse anti-TuJ1 (1:1000, Covance), and mouse anti-cyclinD1 (1:100; Santa Cruz Biotechnology). Sections were incubated with primary antibody overnight or for 2 days at 4°C and then were incubated with secondary antibody for 1–3 hr at room temperature. Secondary antibodies used were as follows: biotinylated donkey anti-guinea pig IgG (1:200; Jackson Immunoresearch), Alexa488-conjugated avidinD (1:1000; Molecular Probes), Alexa594-conjugated anti-goat IgG (1:200; Molecular Probes), Alexa594-conjugated anti-mouse IgG (1:200; Molecular Probes), and Cy3-conjugated anti-mouse IgG (1:200; Jackson Immunoresearch).

In Situ Hybridization

Preparation of DIG-labeled antisense RNA probes and in situ hybridization using NBT/BCIP detection were performed as described elsewhere (Ohtsuka et al., 2006). For fluorescent in situ hybridization (FISH), Fast Red (Roche) was used as substrates instead of NBT/BCIP. For double staining of FISH and immunohistochemistry, FISH was performed first. After antigen retrieval was done in 0.01 M citrate buffer (pH 6.0) using autoclave (105°C for 10 min), immunohistochemistry was performed, as described above.

Bioluminescence Imaging of Dissociation Culture

Dissociation cultures of neural progenitors were prepared from E9.5 to E14.5 pHes1-Ub1-Luc transgenic mice or mice whose brain had been transfected with pEF-EGFP and pNgn2-Ub1-Luc or pDll1-Ub1-Luc 20 hr before by in utero electroporation, as described elsewhere (Ohtsuka et al., 2001). Neural progenitors were plated into glass-based dishes with 1 mM luciferin in neurosphere culture medium (DMEM/F-12 supplemented with 100 μg/ml transferrin, 25 μg/ml insulin, 20 nM progesterone, 30 nM sodium selenite, 60 μM putrescine, 20 ng/ml EGF, and 20 ng/ml bFGF). For measurement of bioluminescence, the dish was placed on the stage of inverted microscope (Olympus IX81) and was maintained at 37°C in 5% CO_2. Bioluminescence was collected by an Olympus ×40 UPlanApo objective and was transmitted directly to a cooled charge-coupled device (CCD) camera (Princeton Instruments, VersArray 1kb), as described elsewhere (Masamizu et al., 2006). The signal-to-noise ratio was increased by 2 × 2 or 4 × 4 binning and 20 min exposure.

Bioluminescence Imaging of Slice Culture

About 20 hr after cotransfection of GFP expression vector and pHes1-Ub1-Luc reporter vector by in utero electroporation, embryos were harvested in PBS, and brains were isolated in DMEM/F-12. Brain tissue was immediately transferred into a silicon rubber-coated dish with DMEM/F-12, which was previously conditioned by a mixture of 5% CO_2 and 95% O_2 for 10–15 min on ice. Meninges were removed, and brains were coronally sliced manually using microknives (100–200 μm thick). Sliced cortex was transferred to a dish containing enriched slice culture medium (DMEM/F-12 supplemented with 100 μg/ml transferrin, 25 μg/ml insulin, 20 nM progesterone, 30 nM sodium selenite, 60 μM putrescine, 20 ng/ml EGF, 20 ng/ml bFGF, 5% horse serum, and 5% fetal bovine serum). Slices were transferred onto slice culture inserts (Millipore) in a glass-based dish with 1 mM luciferin in enriched slice culture medium. The dish was placed on the stage of inverted microscope and was maintained at 37°C in 5% CO_2 and 40% O_2. Bioluminescence from the sample was measured using the CCD camera, as described above.

Image Analysis

Images collected from the CCD camera were analyzed with IMAGE-PRO PLUS (Media Cybernetics), as described elsewhere (Masamizu et al., 2006).

Cell Sorting

Eighteen hours after electroporation of pEF-EGFP and pEF-Hes1, or pEF control vector, embryos were harvested, and brains were excised. Cells were prepared as described elsewhere (Ohtsuka et al., 2006). Cell sorting was performed using a FACSAria cell sorter (BD Biosciences). Dead cells were excluded by gating on forward and side scatter and by elimination of cells stained with propidium iodide (PI). Cells in the GFP+ fraction were sorted and collected into culture medium. A fluorescence intensity of $>5 \times 10^2$ was used to define cells as GFP positive.

Microarray Analysis

Total RNA was prepared from sorted cells. Microarray analysis using GeneChip Mouse Genome 430 2.0 Array (Affymetrix) was performed, and data were analyzed by using GCOS (Affymetrix) and Gene Spring (Agilent Technologies), as described elsewhere (Yoshiura et al., 2007). To explore the candidates for Hes1 targets, two criteria were set. First, the candidates have two-fold changes in signal value between the control and Hes1 overexpression. Second, the signal intensities of higher one should be flagged "Present" and higher than 300. The microarray data have been deposited in the Genome Network Platform (http://genomenetwork.nig.ac.jp/index_e.html).

ACCESSION NUMBERS

The microarray data have been deposited in the Genome Network Platform http://genomenetwork.nig.ac.jp/index_e.html under the code names control_1, control_2, Hes1_1, and Hes1_2 of H1elepo.

SUPPLEMENTAL DATA

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/58/1/52/DC1/.

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REFERENCES

Alvarez-Buylla, A., Garcia-Verdugo, J.M., and Tramontin, A.D. (2001). A unified hypothesis on the lineage of neural stem cells. Nat. Rev. Neurosci. *2*, 287–293.

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770–776.

Baek, J.H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T., and Kageyama, R. (2006). Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. Development *133*, 2467–2476.

Bai, G., Sheng, N., Xie, Z., Bian, W., Yokota, Y., Benezra, R., Kageyama, R., Guillemot, F., and Jing, N. (2007). Id sustains *Hes1* expression to inhibit precocious neurogenesis by releasing negative autoregulation of *Hes1*. Dev. Cell *13*, 283–297.

Bertrand, N., Castro, D.S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat. Rev. Neurosci. *3*, 517–530.

Bessho, Y., Sakata, R., Komatsu, S., Shiota, K., Yamada, S., and Kageyama, R. (2001). Dynamic expression and essential functions of *Hes7* in somite segmentation. Genes Dev. *15*, 2642–2647.

Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J.L., and Gossler, A. (1995). Transient and restricted expression during mouse embryogenesis of *Dll1*, a murine gene closely related to *Drosophila Delta*. Development *121*, 2407–2418.

Castella, P., Sawai, S., Nakao, K., Wagner, J.A., and Caudy, M. (2000). HES-1 repression of differentiation and proliferation in PC12 cells: role for the helix 3-helix 4 domain in transcription repression. Mol. Cell. Biol. *20*, 6170–6183.

Castro, D.S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I.J., Parras, C., Hunt, C., Critchley, J.A., Nguyen, L., Gossler, A., Göttgens, B., et al. (2006). Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev. Cell *11*, 831–844.

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.

Chen, J., Moloney, D.J., and Stanley, P. (2001). Fringe modulation of Jagged1-induced Notch signaling requires the action of β4galactosyltransferase-1. Proc. Natl. Acad. Sci. USA *98*, 13716–13721.

Dequéant, M.-L., Glynn, E., Gaudenz, K., Wahl, M., Chen, J., Mushegian, A., and Pourquié, O. (2006). A complex oscillating network of signaling genes underlies the mouse segmentation clock. Science *314*, 1595–1598.

Dunwoodie, S.L., Henrique, D., Harrison, S.M., and Beddington, R.S.P. (1997). Mouse *Dll3*: a novel divergent *Delta* gene which may complement the function of other *Delta* homologues during early pattern formation in the mouse embryo. Development *124*, 3065–3076.

Fujita, S. (2003). The discovery of the matrix cell, the identification of the multipotent neural stem cell and the development of the central nervous system. Cell Struct. Funct. *28*, 205–228.

Fishell, G., and Kriegstein, A.R. (2003). Neurons from radial glia: the consequences of asymmetric inheritance. Curr. Opin. Neurobiol. *13*, 34–41.

Gaiano, N., and Fishell, G. (2002). The role of Notch in promoting glial and neural stem cell fates. Annu. Rev. Neurosci. *25*, 471–490.

Götz, M., and Huttner, W.B. (2005). The cell biology of neurogenesis. Nat. Rev. Mol. Cell Biol. *6*, 777–788.

Hartman, J., Müller, P., Foster, J.S., Wimalasena, J., Gustafsson, J.-Å., and Ström, A. (2004). HES-1 inhibits 17β-estradiol and heregulin-β1-mediated upregulation of E2F–1. Oncogene *23*, 8826–8833.

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.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995). Expression of a *Delta* homologue in prospective neurons in the chick. Nature *375*, 787–790.

Hoffmann, A., Levchenko, A., Scott, M.L., and Baltimore, D. (2002). The IkB-NF-kB signaling module: temporal control and selective gene activation. Science *298*, 1241–1245.

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., and Kageyama, R. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science *298*, 840–843.

Honjo, T. (1996). The shortest path from the surface to the nucleus: RBP-J κ/Su(H) transcription factor. Genes Cells *1*, 1–9.

Ishibashi, M., Moriyoshi, K., Sasai, Y., Shiota, K., Nakanishi, S., and Kageyama, R. (1994). Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J. *13*, 1799–1805.

Ishibashi, M., Ang, S.L., Shiota, K., Nakanishi, S., Kageyama, R., and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. *9*, 3136–3148.

Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., and McKay, R.D.G. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. *10*, 3129–3140.

Jouve, C., Palmeirim, I., Henrique, D., Beckers, J., Gossler, A., Ish-Horowicz, D., and Pourquié, O. (2000). Notch signalling is required for cyclic expression of the hairy-like gene *HES1* in the presomitic mesoderm. Development *127*, 1421–1429.

Kageyama, R., Ohtsuka, T., and Kobayashi, T. (2007). The Hes gene family: repressors and oscillators that orchestrate embryogenesis. Development *134*, 1243–1251.

Kamakura, S., Oishi, K., Yoshimatsu, T., Nakafuku, M., Masuyama, N., and Gotoh, Y. (2004). Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signaling. Nat. Cell Biol. *6*, 547–554.

Kiecker, C., and Lumsden, A. (2005). Compartments and their boundaries in vertebrate brain development. Nat. Rev. Neurosci. *6*, 553–564.

Lev Bar-Or, R., Maya, R., Segel, L.A., Alon, U., Levine, A.J., and Oren, M. (2000). Generation of oscillations by the p53-Mdm2 feedback loop: a theoretical and experimental study. Proc. Natl. Acad. Sci. USA *97*, 11250–11255.

Levy, D.E., and Darnell, J.E., Jr. (2002). STATS: transcriptional control and biological impact. Nat. Rev. Mol. Cell Biol. *3*, 651–662.

Ma, Q., Kintner, C., and Anderson, D.J. (1996). Identification of *neurogenin*, a vertebrate neuronal determination gene. Cell *87*, 43–52.

Masamizu, Y., Ohtsuka, T., Takashima, Y., Nagahara, H., Takenaka, Y., Yoshikawa, K., Okamura, H., and Kageyama, R. (2006). Real-time imaging of the somite segmentation clock: revelation of unstable oscillators in the individual presomitic mesoderm cells. Proc. Natl. Acad. Sci. USA *103*, 1313–1318.

Maruhashi, M., De Putte, T.V., Huylebroeck, D., Kondoh, H., and Higashi, Y. (2005). Involvement of SIP1 in positioning of somite boundaries in the mouse embryo. Dev. Dyn. *234*, 332–338.

Miller, F.D., and Gauthier, A.S. (2007). Timing is everything: making neurons versus glia in the developing cortex. Neuron *54*, 357–369.

Mueller, P.R., Coleman, T.R., Kumagai, A., and Dunphy, W.G. (1995). Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. Science *270*, 86–90.

Myat, A., Henrique, D., Ish-Horowicz, D., and Lewis, J. (1996). A chick homologue of *Serrate* and its relationship with *Notch* and *Delta* homologues during central neurogenesis. Dev. Biol. *174*, 233–247.

Nelson, D.E., Ihekwaba, A.E., Elliott, M., Johnson, J.R., Gibney, C.A., Foreman, B.E., Nelson, G., See, V., Horton, C.A., Spiller, D.G., et al. (2004). Oscillations in NF-κB signaling control the dynamics of gene expression. Science *306*, 704–708.

Nieto, M., Schuurmans, S., Britz, O., and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron *29*, 401–413.

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.

Ohtsuka, T., Sakamoto, M., Guillemot, F., and Kageyama, R. (2001). Roles of the basic helix-loop-helix genes *Hes1* and *Hes5* in expansion of neural stem cells of the developing brain. J. Biol. Chem. *276*, 30467–30474.

Ohtsuka, T., Imayoshi, I., Shimojo, H., Nishi, E., Kageyama, R., and McConnell, S.K. (2006). Visualization of embryonic neural stem cells using Hes promoters in transgenic mice. Mol. Cell. Neurosci. *31*, 109–122.

Ross, S.E., Greenberg, M.E., and Stiles, C.D. (2003). Basic helix-loop-helix factors in cortical development. Neuron *39*, 13–25.

Rouault, J.-P., Falette, N., Guéhenneux, F., Guillot, C., Rimokh, R., Wang, Q., Berthet, C., Moyret-Lalle, C., Savatier, P., Pain, B., et al. (1996). Identification of *BTG2*, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. Nat. Genet. *14*, 482–486.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to *Drosophila hairy* and *Enhancer of split*. Genes Dev. *6*, 2620–2634.

Scardigli, R., Schuurmans, C., Gradwohl, G., and Guillemot, F. (2001). Crossregulation between *Neurogenin2* and pathways specifying neuronal identity in the spinal cord. Neuron *31*, 203–217.

Selkoe, D., and Kopan, R. (2003). Notch and presenilin: regulated intramembrane proteolysis links development and degeneration. Annu. Rev. Neurosci. *26*, 565–597.

Ström, A., Arai, N., Leers, J., and Gustafsson, J.-Å. (2000). The *Hairy* and *Enhancer of Split* homologue-1 (HES-1) mediates the proliferative effect of 17β-estradiol on breast cancer cell lines. Oncogene *19*, 5951–5953.

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M.Z., Zubiaga, A., Hua, X., Fan, G., and Greenberg, M.E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell *104*, 365–376.

Takahashi, T., Nowakowski, R.S., and Caviness, V.S., Jr. (1993). Cell cycle parameters and patterns of nuclear movement in the neocortical proliferative zone of the fetal mouse. J. Neurosci. *13*, 820–833.

Takahashi, T., Nowakowski, R.S., and Caviness, V.S., Jr. (1995). The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J. Neurosci. *15*, 6046–6057.

Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S., and Kageyama, R. (1994). Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1, Negative autoregulation through the multiple N box elements. J. Biol. Chem. *269*, 5150–5156.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.-L., Nakanishi, S., Guillemot, F., and Kageyama, R. (1996). Mammalian *hairy* and *Enhancer of split* homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron *16*, 723–734.

Vairapandi, M., Balliet, A.G., Hoffman, B., and Liebermann, D.A. (2002). GADD45b and GADD45g are cdc2/cyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress. J. Cell. Physiol. *192*, 327–338.

Yoshiura, S., Ohtsuka, T., Takenaka, Y., Nagahara, H., Yoshikawa, K., and Kageyama, R. (2007). Ultradian oscillations of Stat, Smad, and Hes1 expression in response to serum. Proc. Natl. Acad. Sci. USA *104*, 11292–11297.

Yu, H., and Jove, R. (2004). The STATS of cancer—new molecular targets come of age. Nat. Rev. Cancer *4*, 97–105.