Id Sustains \textit{Hes1} Expression to Inhibit Precocious Neurogenesis by Releasing Negative Autoregulation of \textit{Hes1}

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SUMMARY

Negative bHLH transcription factor \textit{Hes1} can inhibit neural stem cells (NSCs) from precocious neurogenesis through repressing proneural gene expression; therefore, sustenance of \textit{Hes1} expression is crucial for NSC pool maintenance. Here we find that \textit{Id}s, the dominant-negative regulators of proneural proteins, are expressed prior to proneural genes and share an overlapping expression pattern with \textit{Hes1} in the early neural tube of chick embryos. Overexpression of \textit{Id2} in the chick hindbrain upregulates \textit{Hes1} expression and inhibits proneural gene expression and neuronal differentiation. By contrast, \textit{Hes1} expression decreases, proneural gene expression expands, and neurogenesis occurs precociously in \textit{Id1;Id3} double knockout mice and in \textit{Id1–3} RNAi-electroporated chick embryos. Mechanistic studies show that \textit{Id} proteins interact directly with \textit{Hes1} and release the negative feedback autoregulation of \textit{Hes1} without interfering with its ability to affect other target genes. These results indicate that \textit{Id} proteins participate in NSC maintenance through sustaining \textit{Hes1} expression in early embryos.

INTRODUCTION

The vertebrate central nervous system derives from the neural tube, a tubular structure that arises from the neuroectoderm of the gastrula-stage embryo. Neural stem cells (NSCs) in the neural tube are self-renewing and multipotent cells which can generate intermediate and mature cells of both neuronal and glial lineages (Gage, 2000). During neural development, NSCs change their competences and developmental potential over time. In an early expansion stage, NSCs primarily undergo extensive self-renewal to enlarge their cell population (Alvarez-Buylla et al., 2001). As development proceeds, some NSCs progressively initiate the expression of proneural genes in response to region-specific neurogenic signals (Lillien, 1998). Proneural genes such as \textit{Mash1}, \textit{Ngns}, and \textit{Math1} can induce pan-neuronal gene expression and determine neuronal fate (Bertrand et al., 2002; Cai et al., 2000). With the initiation of proneural gene expression, cells in the neural tube become heterogeneous, including neuronal progenitors and NSCs which express and do not express proneural genes, respectively (Bertrand et al., 2002; Gage, 2000). Subsequently, neuronal progenitors exit the cell cycle and differentiate into postmitotic neurons, whereas NSCs remain in an undifferentiated state and give rise to later born cell types (Temple, 2001). Maintenance of the NSC pool is therefore essential for normal neural development, as precocious neurogenesis allows generation of early born cell types only and disorganizes the shape and cytoarchitecture of the brain (Kageyama et al., 2005). Although progress has been made in identifying some components of the genetic program involved in regulating NSC maintenance (Guillemot, 2005), the detailed underlying mechanisms still remain largely unknown.

The \textit{Hes} gene family of negative bHLH transcription factors plays crucial roles in maintaining the NSC population (Hatakeyama et al., 2004; Hirata et al., 2001; Kageyama et al., 2005; Sasai et al., 1992). \textit{Hes1}, the best-characterized family member, is highly expressed in NSCs (Kageyama et al., 2005). Through repressing proneural gene expression, it inhibits NSCs from differentiating along neuronal lineages (Nakamura et al., 2000). As development advances, \textit{Hes1} expression is downregulated in some NSCs, and proneural gene expression is activated (Sasai et al., 1992). Subsequently, proneural proteins promote these NSCs to commit to the neuronal fate and...
differentiate into neurons. In Hes gene mutant embryos, the proneural genes Mash1 and Math3α are upregulated together with premature neuronal differentiation (Hatakeyama et al., 2004; Ishibashi et al., 1995; Nakamura et al., 2000). In contrast, overexpression of Hes1 prevents cortical progenitors from migrating out of the ventricular zone and expressing neuronal markers (Ishibashi et al., 1994). Therefore, sustained Hes1 expression is essential for normal neural development. In NSCs, Notch signaling activates Hes1 expression and inhibits premature neuronal differentiation (Kageyama and Ohtsuka, 1999). However, it is difficult to explain how Notch-induced Hes1 expression is sustained at a relatively high level, considering its capacity for autorepression (Hirata et al., 2002; Takebayashi et al., 1994).

In contrast with Hes1, Id family members possess a highly conserved HLH domain but lack a DNA-binding domain (Perk et al., 2005; Ruzinova and Beneza, 2003). Id proteins act primarily by sequestering E proteins, preventing them from forming functional heterodimers with proneural proteins, and act as dominant-negative regulators that interfere with the transcriptional activities of proneural proteins in neuronal progenitors (Yokota, 2001). Similar to Hes1, Id expression is very important for proper neural development. Misexpression of Id in cortical progenitors inhibits neuron-specific gene expression (Cai et al., 2000). Conversely, progenitors in Id1/Id3 double mutant mice withdraw prematurely from the cell cycle and aberrantly express neuron-specific markers at an early embryonic stage (Lyden et al., 1999). Moreover, Id proteins are not only expressed in neuronal progenitors during neurogenesis but are also highly expressed in the early neural tubes prior to the onset of proneural gene expression (Kee and Bronner-Fraser, 2001a, 2001b; Martinsen and Bronner-Fraser, 1998). In contrast with the well-documented functions of Id proteins in neuronal progenitors, little is known of their earlier function in the neural tube.

Here we show that Ids have expression patterns that overlap with that of Hes1 in the early neural tube of chick embryos. Overexpression of Id2 increases Hes1 expression, whereas inhibition of Id expression downregulates Hes1 expression. We further show that Id proteins can directly interact with Hes1 and release the feedback autoinhibition of Hes1. These findings indicate that Id proteins participate in NSC maintenance through sustaining Hes1 expression in the NSCs of early embryos.

**RESULTS**

**Id Genes Are Expressed prior to Proneural Genes in Early Chick Embryos**

To better understand the function of Id proteins in early embryos, we first sought to examine the expression of Id1–3 in the chick neural tube. Consistent with previous observations (Kee and Bronner-Fraser, 2001a, 2001b; Martinsen and Bronner-Fraser, 1998), we found that Id1–3 exhibited broad expression throughout the neural tube at early stages (data not shown). In situ hybridization showed that Id1–3 mRNA primarily resided in the dorsal region of the metencephalon at HH (Hamburger and Hamilton, 1951) stage 11, when neurogenesis has not started in the metencephalon (Figures 1Aa–1Ac). Weak hybridization signals could also be detected in this region for Hes1 (Figure 1Ad). Hes5, however, was only expressed in a ventral domain (Figure 1Ae). At this stage, no expression of proneural genes such as Cash1, c-Ngn1, c-Ngn2, or Cath1 was detected, and no type III β-tubulin (Tuj1)-positive cells were found either (Figures 1Ba–1Bd and data not shown).

At HH stage 15, after the onset of neurogenesis in the metencephalon, Id1 mRNA could be detected in the dorsal-most region of the neural tube on either side of the midline, and weaker signals were present throughout the rest of the metencephalon except in the ventral-most region (Figure 1Aa'). Id2 was much more strongly expressed than Id1 in the dorsal-most region, and gradually decreased along the dorsal-ventral axis of the metencephalon. The ventral-most region was devoid of Id2 signals, except at the midline (Figure 1Ab'). Id3 was expressed in a pattern largely similar to that of Id2, except that significant signals were also detected in the ventral-most region (Figure 1Ac'). Hes1 expression was concentrated in the dorsal- and ventral-most regions of the neural tube, and only weaker expression was found in the intermediate region (Figure 1Ad'). Hes5 exhibited a pattern complementary to that of Hes1, being highly expressed in the intermediate region and absent in the dorsal- and ventral-most regions (Figure 1Ae'). At this stage, the expression of proneural genes except Cath1 was detectable in the intermediate region but not in the dorsal- and ventral-most regions (Figures 1Ba'–1Bc'). Similarly, Tuj1-positive cells were only detectable in the lateral aspects of the intermediate region (Figure 1Bd'). We observed similar expression patterns for Id, Hes, and proneural genes in the developing spinal cord of the chick embryo (data not shown).

Taken together, these data show that the onset of Id and Hes1 expression precedes that of proneural genes in the chick neural tube. In the dorsal- and ventral-most regions of HH stage 15 neural tubes, where both Id and Hes1 are highly expressed, the expression of proneural genes and the generation of Tuj1-positive neurons are both suppressed, suggesting that Id and Hes1 might play inhibitory roles in neuronal differentiation in early neural stem cells.

**Ectopic Expression of Id2 or Hes1 Inhibits Neuronal Differentiation**

To test this hypothesis, we expressed Id or Hes1 ectopically in the intermediate region of the metencephalon at HH stage 10–11 and assayed for neuronal differentiation at HH stage 15. Following electroporation of an Id2-IRES-GFP expression construct, the number of Tuj1-positive cells decreased significantly in regions of ectopic Id2 expression, and expression of Id2-GFP and type III β-tubulin appeared mutually exclusive (Figures 2A and 2B). As the reduction in Tuj1-positive cells might have been caused by cell death, we tested neuronal apoptosis by the TUNEL assay. No significant difference in level of apoptosis was observed between the Id2-electroporated...
side and the control side (data not shown). To assess the status of the Id2-expressing cells that were inhibited from neuronal differentiation, we examined the expression of the proliferative cell nuclear antigen (PCNA) and found that most of the Id2-GFP-expressing cells were PCNA immunoreactive (Figure 2C), suggesting that Id2 overexpression maintains two characteristics of neural stem cells: proliferative capacity and inhibition of differentiation. Similar results were obtained when electroporating a Hes1-IRES-GFP expression construct; the proportion of Tuj1-positive cells decreased significantly (Figures 2D and 2E) and most of the Hes1-GFP-expressing cells were PCNA immunoreactive (Figure 2F). Collectively, these data indicate that ectopic expression of Id2 or Hes1 maintains the self-renewal and inhibits the neuronal differentiation of NSCs.

Id2 Upregulates Hes1 Expression

Overlapping expression patterns and similar functions suggest a potential functional relationship between Id and Hes1 proteins. To address this possibility, we electroporated mouse Id2 (m-Id2) into the intermediate region of the metencephalon of HH stage 10–11 chick embryos and examined endogenous c-Hes1 and c-Hes5 expression by in situ hybridization (Figure 3A). We found that ectopic expression of m-Id2 upregulated c-Hes1 expression (Figures 3Aa and 3Ab), whereas no significant change was observed in c-Hes5 expression (Figure 3Ac). Interestingly, Hes1 upregulation was restricted to the ventral half of the m-Id2-electroporated region, suggesting that different regions of the metencephalon might have different levels of competence in Id expression. To confirm these in situ hybridization results, we dissected separately the
Figure 2. Inhibitory Effects of Id2 and Hes1 on Neuronal Differentiation

Immunostaining was performed on transverse sections of the chick metencephalon (HH stage 15), which had previously been electroporated on the right side of the neural tube with plasmids encoding GFP, Id2-IRES-GFP, or Hes1-IRES-GFP.

electroporated side and control side of the metencephalon, and used real-time PCR to determine gene expression levels. We found that Id2 increased c-Hes1 expression and inhibited Cash1 and p27 expression (Figure 3B). Cash1 and p27 mark neuronal commitment and neuronal differentiation, respectively, and are both known downstream targets of Hes1 (Chen et al., 1997; Murata et al., 2005; Stanke et al., 2004). These results suggest that up-regulation of c-Hes1 and the subsequent downregulation of c-Hes1 target genes might explain the inhibitory effect of Id2 on the neuronal differentiation of NSCs.

In the m-Hes1–electroporated region, no change was observed upon expression of c-Id1 and c-Id2, and c-Id3 expression was slightly repressed (see Figure S1 in the Supplemental Data available with this article online). However, the ectopically expressed m-Hes1 was found to repress expression of Cash1, p27, and endogenous c-Hes1 (Figure S1). This suggests that Hes1 cannot efficiently regulate Id expression. Together, these results indicate that Id proteins act as upstream regulators to sustain Hes1 expression in NSCs of the chick embryo.

**Id Proteins Are Required for Sustaining Hes1 Expression**

Given that ectopic expression of Id2 can increase Hes1 expression and inhibit neuronal differentiation, we wondered whether inhibition of Id1–3 expression could decrease Hes1 expression and promote precocious neurogenesis. We generated siRNAs directed against chick Id1, Id2, and Id3 and tested their efficiency and specificity by cotransfection with c-Id1–3 and c-Id3 expression vectors into HEK293 cells. Immunoblot analysis showed that knockdown of Id1–3 expression was efficient and gene specific (data not shown). In order to avoid functional redundancy of different Id proteins, we coelectroporated siRNAs against Id1, Id2, and Id3 into the dorsal-most region of the metencephalon at HH stage 10–11, and analyzed gene expression at HH stage 15 (Figure 3C). We found that the electroporation of Id1–3 siRNAs could inhibit endogenous Id1, Id2, and Id3 expression to moderate levels in the dorsal-most region (Figures 3Ca–3Cc), whereas control siRNAs had no effect (Figures 3Ca–3Cc). No apoptosis was detected by TUNEL assay (data not shown). As predicted, Id1–3 siRNAs caused Hes1 downregulation in the dorsal metencephalon, but had no effect on Hes1 expression in the unelectroporated ventral-most region (Figures 3Cd and 3Cd’). Thus, inhibition of Id expression in the dorsal metencephalon decreases Hes1 expression.

The dorsal patterning morphogen Wnt1 is almost missing in Hes1/Hes5 double knockout mice (Hatakeyama et al., 2004), and we wondered whether reduction of Hes1 expression by Id knockout could also affect Wnt1 expression. In the dorsal-most region, inhibition of Id expression indeed led to a dramatic reduction in Wnt1 expression (Figure 3Ce), whereas in control siRNA-treated embryos, Wnt1 expression could still be observed (Figure 3Ce). However, unlike Hes1 knockout mice (Hatakeyama et al., 2004), the transient and partial downregulation of Hes1 expression induced by Id1–3 knockdown did not result in the ectopic expression of Hes5. Therefore, Hes5 failed to compensate the Hes1 function of sustaining Wnt1 expression in this region (Figure S2). Together, these data further indicate that Id proteins are responsible for maintaining the Hes1-dependent expression of patterning morphogens such as Wnt1.

To confirm the results obtained with siRNAs in chick embryos, we examined Hes1 expression in Id1;Id3 double mutant mice. Id1<sup>−/−</sup>/Id3<sup>−/−</sup> mutant embryos have been shown to display aberrant neurogenesis, with premature neuronal differentiation and extensive expression of proneural genes (Lyden et al., 1999). We found that E11.5 Id1<sup>−/−</sup>/Id3<sup>−/−</sup> embryos present a significant reduction in Hes1 expression in the dorsal-most region of the metencephalon and developing spinal cord (Figures 4Aa’ and 4Ca’). Noticeably, downregulation of Hes1 expression was also evident in the intermediate region of the hindbrain and developing spinal cord (Figures 4Ab’ and 4Ca’). These results were confirmed by real-time PCR (Figure 4D). As previously reported (Lyden et al., 1999), the expression of proneural genes, such as Math1, expanded significantly in regions with reduced Hes1 expression (Figures 4Ac’ and 4Cb’). MAP2-positive neurons were prematurely generated from normally neuron-free regions, such as the dorsal-most region and the ventricular zone, in Id1;Id3 double knockout embryos (Figures 4B and 4E).

Taken together, these results show that inhibition of Id expression by siRNA electroporation in chick embryos or knockout in mice causes Hes1 downregulation, proneural gene expansion, and precocious neurogenesis, supporting the view that Id proteins are required for sustaining Hes1 expression in NSCs.
Hes1 Mediates the Inhibitory Effect of Id Proteins on Neuronal Differentiation

Having established the roles of Id proteins in sustaining Hes1 expression, we set out to demonstrate that Hes1 is involved in mediating the inhibitory effect of Id proteins on neuronal differentiation. For this, we electroporated an Id2-IRES-GFP expression construct together with a control siRNA or a Hes1 siRNA into the intermediate region of the metencephalon of an HH stage 10–11 chick embryo, and examined Cash1 expression and Tuj1-positive neurons at HH stage 15. We reasoned that if the inhibitory effect on neuronal differentiation by Id proteins requires upregulation of endogenous Hes1 expression, coelectroporation of a Hes1 siRNA should rescue the Id2-induced inhibition of Cash1 expression and neuronal differentiation. Consistent with this hypothesis, we found...
Figure 4. Decreased Hes1 Expression in Id1−/− Id3−/− Mouse Embryos

(A) In situ hybridization in transverse sections of E11.5 mouse hindbrain. In Id1−/− Id3−/− embryos, Hes1 mRNA expression decreased in the dorsal-most region of the metencephalon (a′) and the intermediate region of the hindbrain (b′), compared with control Id1+/+ Id3+/+ embryos. Math1 expression was expanded in the intermediate region of Id1−/− Id3−/− hindbrain (c). The scale bars represent 100 μm for (a) and (a′), and 200 μm for (b), (b′), (c), and (c′).

(B) Immunostaining of MAP2 in transverse sections of E11.5 metencephalon. The boxed region is enlarged in the insets. The scale bar represents 200 μm.

(C) In situ hybridization in transverse sections of E11.5 spinal cord. In Id1−/− Id3−/− embryos, Hes1 mRNA expression was barely detectable in the dorsal and intermediate regions of the spinal cord (a′), compared with control Id1+/+ Id3+/+ embryos (a). Math1 was ectopically expressed in the dorsal-most region of Id1−/− Id3−/− spinal cord (b). The scale bar represents 100 μm.

(D) The neural tube was dissected from E11.5 Id1−/− Id3+/+ or Id1−/− Id3−/− embryos, and total RNA was extracted. Hes1 mRNA expression was analyzed by real-time PCR. Data are presented as the mean ± SD of triplicate quantifications.

(E) Immunostaining of MAP2 in transverse sections of E11.5 spinal cord. Note that MAP2-positive neurons were prematurely generated from the dorsal-most region and the ventricular zone of Id1−/− Id3−/− embryos (arrows). The scale bar represents 100 μm.
that the Hes1 siRNA could not only inhibit Id-induced Hes1 upregulation but also partially rescue both Cash1 repression and neuronal inhibition imposed by ectopic expression of Id2 (Figures 5Aa–5Ad and 5B), whereas the control siRNA was not effective (Figures 5Aa–5Ad). These results suggest that Hes1 is a downstream effector of Id proteins in the inhibition of proneural gene expression and neuronal differentiation. However, the observation that Hes1 knockdown failed to fully rescue the inhibitory effect of Id on neuronal differentiation (Figure 5B) suggests that there might be an additional Hes1-independent mechanism(s) involved in mediating the functions of Id proteins.

Taken together, gain- and loss-of-function analysis of Id proteins has allowed us to identify a functional relationship between Id and Hes1; Id proteins act as upstream regulators of Hes1 to sustain Hes1 expression, and Hes1 mediates, at least partially, Id’s inhibitory effect on neuronal differentiation through repressing its target genes, such as Cash1 and p27.

Figure 5. Hes1 Mediates the Inhibitory Effect of Id Proteins on Neuronal Differentiation

(A) Transverse sections through HH stage 15 chick metencephalon, with the right side electroporated with a mouse Id2-IRES-GFP expression construct and Hes1 or control siRNA. In situ hybridization was performed with mouse Id2 (a–a’), chick Hes1 (b–b’), and chick Cash1 (c–c’) as probes. Immunostaining was performed with anti-Tuj1 antibody (d–d’). The scale bar represents 250 μm.

(B) The percentage of Tuj1+ cells among GFP+ cells in GFP-electroporated (n = 6), Id2-GFP–plus control siRNA-electroporated (n = 6), or plus Hes1 siRNA-electroporated chick metencephalon (n = 6). Data are presented as the mean ± SD.
Id Proteins Activate Hes1 Expression by Releasing Its Negative Autoregulation

Previous studies have shown that a dominant-negative form of Hes1 (DN-Hes1), in which the DNA-binding domain was mutated, could form a non-DNA-binding heterodimer with wild-type Hes1 protein and increase Hes1 expression through suppressing the negative autoregulation of the Hes1 gene (Hirata et al., 2002; Strom et al., 1997). As Id proteins also lack the DNA-binding domain (Benezra et al., 1990), we speculated that they might use a similar mechanism to regulate Hes1 expression. To validate this hypothesis, we performed a luciferase assay with the 2.5 kb mouse Hes1 promoter (Takebayashi et al., 1994), cotransfecting it with increasing amounts of either DN-Hes1 or Id2 expression constructs into P19 cells. We found that Id2 was indeed as efficient as DN-Hes1 at increasing reporter construct activity (Figure 6A). To confirm that Id2 promotes Hes1 transcription through the Hes1-binding N box in the Hes1 promoter (Takebayashi et al., 1994), we generated a luciferase reporter construct containing a mutant Hes1 promoter in which all three N boxes were inactivated (Takebayashi et al., 1994). We cotransfected this construct with DN-Hes1 or Id2 expression plasmids, and found that the basal transcriptional activity of the mutant Hes1 promoter was higher than that of wild-type in P19 cells, which might be due to the mutant Hes1 promoter failing to respond to the inhibition of endogenous Hes1 in P19 cells (Figure 6A). And, increasing amounts of DN-Hes1 or Id2 had no effect on reporter activity, suggesting that the N box was required for DN-Hes1 or Id2 to increase Hes1 gene transcription. To further confirm this issue, we generated a luciferase reporter plasmid containing six repeats of the N box and cotransfected the construct with DN-Hes1 or Id2. Similarly, DN-Hes1 or Id2 also increased luciferase reporter activity (Figure 6B). Similar results were obtained with Id1 and Id3 (Figure S3). As P19 cells express endogenous Hes1 protein (Sasai et al., 1992), we could not determine whether Id proteins actually act through Hes1. Therefore, HeLa cells which do not express endogenous Hes1 (Murata et al., 2005) were chosen to perform the same luciferase assay. Cotransfection of DN-Hes1 or Id2 with the N box-luciferase reporter in HeLa cells did not increase reporter gene expression (data not shown). However, a Hes1 expression vector could repress luciferase reporter construct activity, and both DN-Hes1 and Id2 could rescue such repression (Figure 6C). Taken together, these results support our model whereby Id proteins sustain Hes1 expression by releasing the inhibitory effect of Hes1 on its own promoter.

Id Proteins Interact with Hes1 and Suppress Its N Box Binding Activity

To determine whether Id proteins release the inhibitory effect of Hes1 by directly interacting with Hes1 proteins, GST pull-down and coimmunoprecipitation assays were performed. In vitro translated Hes1 protein was pulled down by a GST-Id2 fusion protein, but not by GST alone (data not shown). To clarify whether Id2 interacted with Hes1 through direct physical binding, Myc-tagged Id2 and Flag-tagged Hes1 were cotransfected into HEK293T cells. A coimmunoprecipitation assay showed that Hes1 could be coprecipitated with Id2 and vice versa (Figure 6D). Id1 and Id3 proteins could also be coprecipitated with Hes1 (Figure S4). Similar results have been previously reported (Jogi et al., 2002). To confirm these results obtained in transfected cells, coimmunoprecipitation experiments were repeated in P19 cells and E10.5 mouse brain tissue. Endogenous Hes1 protein was found in immunoprecipitates with anti-Id2 antibody but not with control IgG (anti-Hes1 antibody was not available for immunoprecipitation) (Figure 6E), indicating that endogenous Id2 and Hes1 proteins indeed form a complex in P19 cells and in the developing mouse brain.

To determine whether Id proteins could interfere with the DNA binding activity of Hes1, in vitro purified Hes1 protein was subjected to an electrophoresis mobility-shift assay (EMSA) using a 32P-labeled N box-containing oligonucleotide as a probe. A prominent band appeared when the probe was mixed with Hes1 protein (Figure 6F, lane 2) but not with BSA (Figure 6F, lane 1), and the band disappeared in the presence of an excess of unlabeled wild-type (WT) but not N box-mutated (Mut) oligonucleotide (Figure 6F, lanes 3 and 4). To examine the effect of Id2 on the DNA binding activity of Hes1, we preincubated the same amount of Hes1 protein with increasing amounts (50, 150, and 450 ng) of GST-Id2 fusion protein or GST protein as a control. The binding activity of Hes1 to the N box probe decreased gradually with increasing concentrations of Id2 protein (Figure 6F, lanes 5–7) but not of control GST protein (Figure 6F, lanes 8–10). These results show that Id proteins can interact with Hes1 and suppress its N box binding activity.

To determine which domain of Id2 protein was required to interact with Hes1, we generated GFP-Id2 fusion proteins with deletions of the N-terminal (ΔN), HLH (ΔHLH), or C-terminal (ΔC) domains and cotransfected these constructs with Flag-Hes1 into HEK293T cells. Coimmunoprecipitation experiments showed that Hes1 could interact with GFP-Id2 fusion proteins with the deletion of the N-terminal or C-terminal domain, but not with the deletion of the HLH domain. The luciferase reporter assay further showed that the GFP-Id2-ΔHLH fusion protein could not release the inhibitory effect of Hes1 on the N box (Figure S5). These results indicate that the HLH domain of Id2 protein is the region that interacts with Hes1, and that Id2 requires this interaction to activate the Hes1 promoter.

Taken together, these results show that Id proteins interact directly with Hes1 through their HLH domain and suppress the DNA binding activity of Hes1, thereby releasing the negative feedback loop of Hes1 on its own promoter.

Id2 Releases the Negative Autoregulation of Hes1 in the Developing Chick Brain

In the previous section, we described in vitro experiments to establish the mechanism through which Id2 proteins interact with Hes1 and release its negative feedback loop.
Figure 6. Id2 Interacts with Hes1 and Suppresses Its Negative Autoregulation

(A and B) The wild-type/mutant Hes1 promoter-luc (A) or pN6-IA-luc plasmid (B) was transfected into P19 cells with increasing amounts of DN-Hes1 or Id2 expression construct. Each experiment was repeated at least three times. The results are presented as mean ± SD.

(C) pN6-IA-luc plasmid was transfected into HeLa cells with Hes1 expression vector and increasing amounts of DN-Hes1 or Id2 expression construct as indicated. Each experiment was repeated at least three times. The results are presented as mean ± SD.

(D) Interaction of Hes1 and Id2 proteins in transfected cells. HEK293T cells were transfected with plasmids encoding Flag-Hes1, Id2-Myc, or the corresponding empty vectors as indicated. The cell lysates were subjected to immunoprecipitation (IP) with antibodies to Myc or Flag, and the resultant immunoprecipitates were analyzed by immunoblot (IB).
Id Sustains Hes1 Expression in Early Neural Tube

To confirm that this mechanism operates in vivo, an EGFP reporter plasmid driven by the 2.5 kb Hes1 promoter (Hes1 promoter-EGFP) was electroporated into the intermediate region of the metencephalon of HH stage 10–11 chick embryos. EGFP expression was detected at HH stage 15 (Figures 7Aa and 7Ab), probably due to endogenous Notch signaling, which is known to activate the Hes1 promoter. When the reporter plasmid was coelectroporated with a Hes1 expression construct, EGFP expression was abolished (Figures 7Ac–7Ae). Moreover, when the reporter plasmid was coelectroporated with both Hes1 and Id2-myc expression plasmids, EGFP expression was rescued (Figures 7Af–7Ai). These results indicate that Id2 protein can release the autorepression of Hes1 in the developing chick brain.

**Id2 Protein Fails to Release the Inhibitory Effect of Hes1 from the Class C Site of the Hash1 Promoter**

Hes1 protein can bind to the promoters of proneural genes to inhibit proneural gene expression (Chen et al., 1997). Therefore, it is possible that Id proteins can also release the inhibition of Hes1 on proneural genes. To validate this possibility, the proneural gene Hash1 promoter, to which Hes1 protein bound and suppressed its expression (Chen et al., 1997), was chosen to perform the reporter assay. We cotransfected a luciferase reporter plasmid containing five repeats of the class C site of the Hash1 promoter with the Hes1 expression vector in HeLa cells and found that Hes1 could repress the expression of the reporter gene, whereas DN-Hes1 could release this repression. Interestingly, Id2 expression could not release the inhibition of Hes1 to the class C site of the Hash1 promoter (Figure 7B). Similar effects were also observed in P19 cells (Figure 7C). These results suggest that Id proteins can only release the inhibitory effect of Hes1 protein from the N box of its own promoter (Figures 6B and 6C) but not from the class C site of the proneural gene promoter.

Next, we wondered whether this was caused by the different abilities of Id2 to interfere with the Hes1 DNA binding activity between the N box and the C site. As previously reported (Jogi et al., 2002), Hes1 could also bind to a 32P-labeled class C site probe (Figure 7D, lanes 1 and 2), and the binding was specific for the class C site (Figure 7D, lanes 3 and 4). The high concentration of Id2 protein (300 and 450 ng) could efficiently compete with Hes1 binding to this probe (Figure 7D, lanes 7 and 8), whereas the effect of lower concentrations (50 and 150 ng) was not as significant (Figure 7D, lanes 5 and 6). This was different from our previous data that even relatively low doses of Id2 protein (150 ng) could significantly interfere with Hes1 binding to the N box (Figure 6F, lane 6). Moreover, the high-dose Id2 protein (300 ng) could completely block the binding activity of Hes1 to the N box (Figure 7D, lane 14), whereas this concentration was insufficient to completely block Hes1 binding to the class C site (Figure 7D, lane 7). Together, these results show that Id2 has a lower threshold to inhibit the N box binding activity of Hes1 than to inhibit that of the class C site. This might provide an explanation for the differing abilities of Id2 to release the inhibition of Hes1 on itself and on its target genes.

**DISCUSSION**

In this study, we showed that in the neural tube of the early chick embryo, Id genes shared an overlapping expression pattern with Hes1 and that ectopic expression of Id2 induced Hes1 expression and repressed proneural gene expression and normal neuronal differentiation. Conversely, inhibition of Id expression led to decreased Hes1 expression, expanded proneural gene expression, and premature neuronal differentiation in many regions of the central nervous system. As a result, RNAi-electroporated chick embryos and Id1;Id3 double mutant mice had a reduced hindbrain size (data not shown). Taken together, these results strongly support the notion that sustained Hes1 expression by Id proteins is a critical mechanism for maintenance of the NSC pool in early embryos.

Previous studies showed that Id proteins could act as dominant-negative regulators to interfere with the transcriptional activities of proneural proteins and inhibit premature differentiation of neuronal progenitors (Yokota, 2001). In this study, we found a novel function of Id proteins to sustain Hes1 expression and prevent precocious neuronal differentiation of NSCs in the early embryo. How do the two activities of Id proteins relate to each other? We showed that Id2 overexpression could strongly inhibit neuronal differentiation of NSCs, and that Hes1 knockdown could not fully rescue the inhibitory effect of Id2 protein. Based on this observation and the fact that the neural tube at this stage is composed of heterogeneous cell types including NSCs and neuronal progenitors, we propose that Id proteins inhibit neuronal differentiation possibly through two different mechanisms: they repress proneural gene expression through sustaining Hes1 expression in NSCs, or act as dominant-negative regulators to block the function of proneural proteins in neuronal progenitors. Therefore, the observed inhibition of neuronal differentiation of Id2 protein in early embryos should be the synergistic effect of these two mechanisms. The Hes1 expression inhibition by siRNA could only interfere with the former mechanism and leave the latter intact. Thus, Hes1 knockdown could only partially rescue the...
Figure 7. Effect of Id2 on the Negative Autoregulation of Hes1 in the Developing Chick Brain

(A) Hes1 promoter-EGFP and tracer construct pHcRed1 were coelectroporated into chick metencephalon with plasmids encoding Hes1, Myc-tagged Id2, or the corresponding empty vector. Tracer (a, c, and f), EGFP (b, d, and g), Hes1 (anti-Hes1; [e and h]), and Id2 (anti-Myc; [i]) expressions were detected 20 hr later. The scale bar represents 500 μm.

B

C

D

E

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inhibitory effect of Id2 protein. Of course, we cannot rule out another unknown Hes1-independent mechanism(s) involved in this process.

Feedback inhibition is an important mechanism to regulate Hes1 expression during embryonic development (Baek et al., 2006; Hirata et al., 2002; Takebayashi et al., 1994). In the neural tube, for example, Hes1 exhibits two different modes of expression. In compartments regions such as the intermediate aspects of the neural tube, Hes1 is expressed at variable levels in different cells, probably due to the activity of the Hes1 negative feedback loop. In boundary cells, such as the dorsal-most region of the neural tube, however, Hes1 has a persistent and high-level expression, and it seems that the negative feedback loop does not work there (Baek et al., 2006). Moreover, expression of proneural genes is inhibited and neuronal differentiation is delayed or does not occur in these boundary regions (Guthrie and Lumsden, 1991; Kahane and Kalcheim, 1998). Cells in the dorsal-most region of the neural tube express Wnt and BMP family members and act as an organizing center to regulate the dorsal-ventral patterning of the neural tube. Persistent, high-level Hes1 expression in these regions is crucial for maintenance of these NSC populations as well as maintenance of organizer activities (Baek et al., 2006). However, it remains unclear how this high and persistent Hes1 expression is sustained in these boundary regions. We found that the inhibition of Id expression reduced Hes1 expression in the dorsal-most region of the metencephalon and the developing spinal cord in Id1–3 RNAi-electroporated chick and Id1;Id3 double mutant mouse embryos. We also found that Id proteins can interact directly with Hes1 and inhibit its DNA binding activity, thereby suppressing the feedback repression of its own promoter. These results suggest that in these boundary regions, the highly expressed Id proteins act as suppressors of the Hes1 negative feedback loop and sustain the persistent and high expression of Hes1.

Lateral inhibition is a well-characterized Notch-mediated mechanism involved in NSC maintenance (Bertrand et al., 2002). Newly formed neurons upregulate the Notch ligand Delta, thereupon activating Notch signaling in adjacent NSCs. Upon activation, the intracellular domain of Notch is translocated to the nucleus and forms a complex with the intracellular molecule RBP-J. This complex directly binds to the Hes1 promoter and activates Hes1 expression, thereby inhibiting the precocious neuronal differentiation of NSCs (Selkoe and Kopan, 2003). However, in Id1;Id3 double knockout embryos, Hes1 expression decreases to a very low level and premature neurogenesis occurs, suggesting that Notch signaling alone is not sufficient to sustain Hes1 expression and prevent premature neuronal differentiation of NSCs. Therefore, Id proteins are required to repress the negative feedback of Hes1 and maintain Notch signal-induced Hes1 expression (Figure 7E). Moreover, given that secreted factors, such as Wnts and BMPs, can regulate Id expression in different tissues (data not shown; Hollnagel et al., 1999; Nakashima et al., 2001; Rockman et al., 2001), this novel functional relationship between Id and Hes1 proteins provides a molecular basis to integrate extrinsic signals with the intrinsic program of NSC maintenance (Figure 7E).

The function of Hes1 is to inhibit neuronal differentiation of NSCs through repressing expression of its target genes, such as Mash1 and p27. In contrast with the self-inhibition that involves binding to N boxes (CACNAG) in its own promoter, Hes1 represses target gene transcription through binding to the class C site (CACCGA) in the promoter of the target gene (Chen et al., 1997). However, little is known about the effects of different binding activities of Hes1 on these different binding sites. It is also unclear whether Id proteins interfere differentially with Hes1 binding activities to the N box and C site. We found that Id2 could not liberate the inhibition of Hes1 on C site-driven reporter gene expression in P19 and HeLa cells. We also found that only high concentrations of Id2 fusion protein could effectively compete with Hes1 binding to a C site probe, and that the effect of lower concentrations was not as significant. These results were different from our observations on the N box, in which Id expression could release the inhibition of Hes1 on N box-driven reporter gene expression, and that even relatively low doses of Id2 protein could efficiently interfere with Hes1 binding to the N box. One possible explanation for this discrepancy is that Hes1 protein has different binding affinities for the N box and C site, with Hes1 binding to the C site with higher affinity than to the N box (data not shown; Ohsako et al., 1994; Van Doren et al., 1994). Due to rapid degradation or interaction with other proteins (Bounpheng et al., 1999; Jogi et al., 2002), it is difficult for Id proteins to accumulate to a very high concentration in the developing nervous system. Thus, Id proteins could only release...
Hes1 feedback repression on its own promoter, but not on Hes1’s downstream targets. Of course, we cannot rule out the possibility that unknown cofactors might exist, and that different complexes between Id proteins and cofactors might determine their interaction with Hes1 and affect the DNA binding activity of Hes1 to the N box or C site in NSCs.

Experimental Procedures

Animals
The generation of Id1/Id3 double mutant mice has been described previously (Lyden et al., 1999). All experiments were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In Ovo Chick Embryo Electroporation
Fertilized eggs were obtained from the Shanghai Academy of Agricultural Sciences. The chick embryo in ovo electroporation was performed as previously described (Yan et al., 2004).

In Situ Hybridization
Section in situ hybridization was performed as described previously (Birren et al., 1993). Detailed protocols are available upon request. The following chick in situ probes were used: Id1, Id2, Id3, Hes1, Hes5, Cash1, Ngn1, Ngn2, and Wnt1. The following mouse in situ probes were used: Id1, Id2, Id3, Hes1, and Math1.

Commmunoprecipitation Assay
Immunoprecipitations were performed as described previously (Jogi et al., 2002). The following antibodies were used: anti-Flag (Sigma), anti-Myc (Covance), anti-GFP (Covance), anti-Id2 (Santa Cruz), and anti-Hes1 (kindly provided by T. Sudo).

Luciferase Assay
The luciferase plasmid containing the N box mutant version of the Hes1 promoter was constructed as described previously (Takebayashi et al., 1994). Luciferase assays were performed as described previously (Cheng et al., 2004).

Electrophoretic Mobility-Shift Assay
Protein-DNA complexes were examined by EMSA as previously described (Yan et al., 2004).

Statistics
Each experiment was repeated at least three times, and similar results were obtained. Data were expressed as mean ± SD. Student’s t tests were used to compare the effects of all treatments. Differences were considered statistically significant at p < 0.05.

Supplemental Data
Supplemental Data include five figures, Supplemental Experimental Procedures, and Supplemental References and are available at http://www.developmentalcell.com/cgi/content/full/13/2/283/DC1/.

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