

Expression dynamics and functions of Hes factors in development and diseases

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Abstract

Hes genes, encoding basic helix-loop-helix (HLH) transcriptional repressors, are mammalian homologues of *Drosophila hairy* and *Enhancer of split* genes, both of which are required for normal neurogenesis in *Drosophila*. There are seven members in the human *Hes* family, *Hes1* to *Hes7*, which are expressed in many tissues and play various roles mainly in development. All Hes proteins have three conserved domains: basic HLH (bHLH), Orange, and WRPW domains. The basic region binds to target DNA sequences while the HLH region forms homo- and hetero-dimers with other bHLH proteins, the Orange domain is responsible for the selection of partners during heterodimer formation, and the WRPW domain recruits co-repressors. *Hes1*, *Hes5*, and *Hes7* are known as downstream effectors of canonical Notch signaling, which regulates cell differentiation via cell-cell interaction. Hes factors regulate many events in development by repressing the expression of target genes, many of which encode transcriptional activators that promote cell differentiation. For example, *Hes1*, *Hes3*, and *Hes5* are highly expressed by neural stem cells, and inactivation of these genes results in insufficient maintenance of stem cell proliferation and prematurely promotes neuronal differentiation. Recently, it was shown that the expression dynamics of *Hes1* plays crucial roles in proper developmental timings and fate determination steps of embryonic stem cells and neural progenitor cells. Here we discuss some key features of Hes factors in development and diseases.

Key words: Hes, stem cells, differentiation, *Hes1* oscillation, embryonic stem cells, neural stem cells

1. Introduction

Development is a highly organized process that depends on the timely proliferation of stem cells and their differentiation into multiple cell types. The balance between cell proliferation and cell fate determination is critical to forming organs with the right shape and size and the right cell composition. Hes factors (hairy and Enhancer of split homologs) play important roles in the development of many organs to expand progenitor cell pools and regulate cell fate decisions. Some Hes factors, Hes1, Hes5, and Hes7, are dynamically expressed by auto-negative feedback loops, and also function as effectors of the Notch signaling pathway, which coordinates cell proliferation and differentiation via cell-cell interaction. Recent studies have shown that the expression dynamics of Hes1 contribute to its multiple functions. In this review, we describe the features of Hes factors, their functions in development and diseases, and the significance of Hes1 oscillation, including its expression dynamics in embryonic stem cells and neural progenitor/stem cells.

2. Hes family members and protein structures

The protein products of the Hes gene family comprise seven members in human, Hes1 to Hes7. Hes1, Hes2, and Hes4 are more similar to *Drosophila* hairy in their amino acid sequences than are the other members (Fig. 1A). Hes4 is the most similar to Hes1 and is not present in mouse or rat. *Hes3* encodes two isoforms due to alternative splicing: Hes3 variant 1 lacks the N-terminal half of the basic region for DNA binding, while Hes3 variant 2 has a complete basic region (Hirata, et al., 2000; Katoh & Katoh, 2007). Hes factors have three conserved domains: the basic helix-loop-helix (bHLH) domain, the Orange domain, and the WRPW domain, harboring a WRPW motif (Trp-Arg-Pro-Trp) (Fig.1B) (Kageyama, et al., 2007). There are also other Hes-related bHLH factors such as Hey1, Hey2, HeyL, and HELT (Hes/Hey-like), that do not have the WRPW motif.

The bHLH domain is essential for DNA binding and homo- and heterodimer formation. The N-terminal basic region plays an important role in the specific binding to target DNA sequences, that is, the N box (CACNAG) and the class C site (CACG(C/A)G) (Sasai, et al., 1992). It has been suggested that a proline residue in the middle of the basic region (Fig. 1B), which is conserved in all Hes factors as well as *Drosophila* hairy and Enhancer of split (E(spl)) proteins, may be involved in the target sequence specificity, providing a higher affinity for the N box and the class C site than

for the E box (CANNTG), a consensus binding sequence for activator-type bHLH factors. The HLH domain is characterized by two alpha-helix motifs linked by a flexible loop structure, and interacts with other HLH motifs of binding partner proteins to form homo- or hetero-dimers. Hes factors can form homo-dimers and hetero-dimers with Hes-related bHLH repressors, such as Hey factors, as well as with other bHLH factors, such as Mash1, E47, and Id. The Orange domain has two amphipathic helices that are involved in the selection of partners to form hetero-dimers. The WRPW domain, located at the C-terminal end, is essential for the repressor activity via recruitment of the co-repressor Transducin-like E(spl) (TLE1-4)/ Groucho-related gene (Grg), a homologue of *Drosophila* Groucho. The Hey family factors contain a YRPW (Tyr-Arg-Pro-Trp) motif instead of the WRPW motif, which contributes to the weaker binding of Hey factors to TLE than the Hes factors (Iso et al., 2001; Fischer & Gessler, 2007).

3. Hes general functions

Hes factors repress transcription by two different mechanisms, active repression and passive repression (Fig. 1C) (Kageyama et al., 2007). During active repression, Hes factors form homodimers or heterodimers with other Hes/Hey factors and bind to the N-box or the class C site sequences in the promoter region of target genes. In the case of the heterodimers between Hes1 and Hey1 or Hey2, binding to the class C site exhibits a higher affinity than Hes1 or Hey1 homodimers, and such heterodimers repress transcription more efficiently than the homodimers. The C-terminal WRPW domain in Hes factors interacts with TLE/Grg co-repressors (Buscarlet et al., 2008), which recruit histone deacetylase and repress the target gene expression by inducing changes in the chromatin structures. Hes1 can also repress target genes in a TLE-independent manner, by recruiting Sirt1, a homologue of silent information regulator 1 and a class III histone deacetylase, via its bHLH domain, and thereby repressing target genes (Takata & Ishikawa, 2003). Although Hes family genes encode transcriptional repressors, there is one exceptional gene, *Hes6*. The Hes6 protein has no DNA binding ability and inhibits Hes1 function by forming heterodimers with it.

During passive repression, Hes factors form heterodimers with bHLH-type activators, such as Mash1 and E47, and inhibit their binding to the E box in the promoter region of target genes (Akazawa, et al., 1992). Thus, Hes factors function as dominant negative regulators to these bHLH activators. Id proteins, which lack the basic DNA binding domain, also negatively regulate bHLH activators by inhibiting their

DNA binding through heterodimerization. Hes factors can tightly repress the expression of target genes via these two types of repression. For example, Hes1 represses Mash1, a mouse homologue of *Drosophila achate-scute* genes that encode transcriptional activators and promote neural differentiation, both via active repression by directly binding to the class C-site on the *Mash1* promoter and via passive repression by capturing E-protein activators such as E47 (Fig. 1C).

4. Roles of Hes factors in various tissues

Hes gene expression is regulated by the Notch-dependent signaling pathway (Fig. 2). Notch signaling is activated when ligands such as Delta or Jagged expressed on the surface of neighboring cells bind to Notch receptors. Upon activation of Notch signaling, the Notch intracellular domain (ICD) is released from the cell surface and is translocated into the nucleus, where it forms a complex with the DNA-binding protein RBPj/CSL. The RBPj-ICD complex recruits additional transcriptional co-activators and induces the expression of downstream genes, including *Hes* and *Hey*. This pathway is often called the canonical pathway of Notch signaling. Mouse *Hes1*, *Hes5*, and *Hes7*, which are all activated by Notch signaling, have RBPj binding sites on their promoters (Bessho, et al., 2001a; Katoh & Katoh, 2007; Ohtsuka et al., 1999). On the other hand, other signaling pathways are also reported to induce Hes expression. For example, Hes1 expression is induced by many signaling pathways, including bone morphogenetic protein (BMP), fibroblast growth factor (FGF), leukemia inhibitory factor (LIF), NF- κ B, Sonic hedgehog (Shh), and Wnt signaling pathways (Kageyama, et al., 2006; Nakayama, et al., 2008). Hes1 regulates the proliferation of neural stem/progenitor (NS) cells in the central (see below) and peripheral nervous systems under the control of these pathways. *Hes7*, a segmentation clock gene involved in somitogenesis, is regulated by the Notch and FGF pathways during somite segmentation (Niwa et al., 2007). Thus, many pathways regulate Hes expression in a tissue-specific manner.

Hes genes are involved in regulation of many different cell types, including hematopoietic stem cells in embryos (Kageyama et al., 2007), digestive organs, lung, cochlea, skeletal tissues, testis and vascular endothelial cells, as discussed below. In digestive organs, Hes1 and Hes5 are expressed by proliferating progenitor/stem cells, and play an important role in the maintenance of these cells by preventing their differentiation. Hes1 is detected in the developing pancreas, where it represses the expression of the bHLH-type activators Neurogenin3 (Ngn3) and pancreas transcription factor 1 (Ptf1a), which enhance the differentiation of endocrine and exocrine cells,

respectively. *Hes1* maintains progenitors by inhibiting the expression of these bHLH activators. Genetic inactivation of *Hes1* up-regulates the expression of these bHLH activators, resulting in, for example, severe pancreatic hypoplasia and the formation of an ectopic pancreas (Fukuda et al., 2006; Jensen et al., 2000; Sumazaki et al., 2004). *Hes1* is also expressed in the intestinal crypt, where stem cells and proliferating progenitors are located, and represses the expression of bHLH-type activator genes, *Math1* and *Ngn3*, whose translational products promote the differentiation of secretory cells. *Math1* regulates the development of all types of secretory cells, while *Ngn3* regulates enteroendocrine cell development. *Hes1* knockout (KO) mice showed an overproduction of secretory cells (Jensen et al., 2000). Importantly, *Hes1* inactivation markedly reduces tumor cell proliferation and induces their differentiation into intestinal epithelial cells without affecting the homeostasis of normal intestinal crypts in adenomatous polyposus coli (*Apc*) mutation-induced intestinal tumors, suggesting that *Hes1* is a promising target for intestinal cancer therapy (Ueo et al., 2012).

In sensory organs, it has been reported that *Hes* and *Hes*-related genes are expressed in the developing inner ear and the developing retina. *Hes1*, *Hes5*, and *Hey1* are expressed in the developing cochlea of the inner ear, and they cooperatively inhibit the formation of hair cells, which are sensory neurons. Inactivation of these genes leads to disorganized cell alignment and polarity and to hearing loss due to hair cell overproduction (Tateya, et al., 2011). In the developing retina, *Hes1* and *Hes5* function downstream of both *Shh* and *Notch* signaling, inhibit the activity of proneural bHLH genes such as *Mash1* and *Math3*, and maintain progenitor cell populations (Wall, et al., 2009; Ohtsuka, et al., 2001). *Hes6* is exceptionally expressed in the differentiated neural retina and functions as an inhibitor of *Hes1*, thereby promoting neuronal differentiation (Bae, et al., 2000).

In hematopoietic stem cells (HSC), both *Hes1* and *Hes5* are induced by *Notch* signaling and regulate several blood lineages, including T cells, B cells, and stem cells. Forced expression of *Hes1* results in increased numbers of quiescent HSCs, and *Hes1*-deficient HSCs are unable to differentiate into T cells, suggesting that *Hes1* is an important regulator for HSC functions (Tomita et al., 1999). It has been shown that, although *Hes1*-deficient mice do not show clear abnormalities in their hematopoietic system, the depletion of both *Hes1* and *Hes5* induces overproduction of nonfunctional HSCs, in which *Hes* target genes such as *Gata2* are abnormally up-regulated (Guiu et al., 2013). *Hes1* is also reported to be an important regulator of the development of T cell acute lymphoblastic leukemia (Espinosa et al., 2010; Wendorff et al., 2010).

Hes7 is a segmentation clock gene involved in somitogenesis of embryos (Bessho, et al., 2001a; Bessho, et al., 2001b). Somites are metameric structures periodically segmented from the anterior parts of the presomitic mesoderm. These segmented, paired blocks of mesoderm form vertebrae, ribs, skeletal muscles, and subcutaneous tissues. During somitogenesis, the oscillatory expression of *Hes7* regulates the periodic segmentation events of the presomitic mesoderm. *Hes7* is a causative gene in the inheritable diseases, spondylocostal dysostoses type 4 and congenital scoliosis, which exhibit developmental bone and vertebral defects (Sparrow, et al., 2008; Sparrow et al., 2012).

5. Hes functions in the central nervous system

5.1 Hes genes in neural stem cells

Hes genes play crucial roles in inhibiting neuronal differentiation and promoting the proliferation of neural stem (NS) cells in the developing central nervous system (Ohtsuka et al., 2001). During neural development, neuroepithelial cells appear initially and undergo extensive self-renewal. As development proceeds, neuroepithelial cells are gradually elongated and become radial glial cells, which have a cell body in the inner region, called the ventricular zone, and a radial fiber extending from the internal surface to the pial (outer) surface of the brain. Both neuroepithelial cells and radial glial cells are considered to be NS cells. NS cells are known to change their competency over time to generate distinct types of neurons. Radial glial cells divide asymmetrically, forming one radial glial cell and one neuron or basal progenitor from each cell division. Basal progenitors proliferate and produce more neurons in the subventricular zone. After producing neurons, NS cells give rise to oligodendrocytes and astrocytes (Fig. 3A). *Hes1* and *Hes3* are widely expressed by neuroepithelial cells, whereas *Hes1* and *Hes5* are mainly expressed by radial glial cells (Hatakeyama et al., 2004).

Hes1, *Hes3*, and *Hes5* are important for maintaining NS cells. Inactivation of these Hes genes leads to failure of maintenance of the undifferentiated state, accelerating neuronal differentiation. As a result, virtually all NS cells become neurons and are therefore depleted prematurely (Hatakeyama et al., 2004). In the absence of these Hes genes, proneural bHLH genes, such as *Mash1*, *Neurogenin2* (*Ngn2*), and others, are highly up-regulated, leading to premature neuronal differentiation. On the other hand, miss-expression of *Hes1* or *Hes5* inhibits neuronal differentiation and maintains NS cells (Ohtsuka et al., 2001). Based on their expression patterns, *Hes1* and

Hes3 are important for the maintenance of neuroepithelial cells, while Hes1 and Hes5 are required for most radial glial cells (Hatakeyama et al., 2004), although these Hes factors are mostly redundant and compensate each other.

5.2 Hes genes in brain morphogenesis

Hes genes play important roles in brain morphogenesis (Kageyama et al., 2007). The developing nervous system is partitioned into many compartments by boundary structures, such as the *zona limitans intrathalamica*, the isthmus, the floor plate and the roof plates (Fig. 3B). The *zona limitans intrathalamica* is the boundary between the thalamus and the prethalamus; the isthmus is the boundary between the midbrain and the hindbrain; and the floor and roof plates are the boundaries between the right and the left parts of the neural tube. Cells in these boundary structures proliferate poorly and do not give rise to neurons, suggesting that the boundary cells are quiescent, whereas cells in compartments between boundaries proliferate extensively, maintain the NS state, and differentiate into neurons. Several knockout mouse studies showed that the *zona limitans intrathalamica* is lost in the absence of *Hes1* and *Hes5*, and that the isthmus and the floor and roof plates are not formed in *Hes1/3/5* triple knockout mice. In the absence of Hes genes, ectopic neuronal differentiation occurs in these defective boundary regions, collapsing the boundary structures (Hirata, et al., 2001). Forced expression of *Hes1* in compartment cells inhibits both cell proliferation and neuronal differentiation by repressing cell cycle regulator genes and proneural genes (Baek, et al., 2006; Shimojo, et al., 2008). These results demonstrate that Hes genes are crucial to maintaining these boundary structures in the developing brain by inhibiting differentiation and proliferation of their cells.

Hes1 regulates the maintenance of both quiescent boundary cells and proliferating compartment NS cells, but how does it control two opposite cell cycle states (quiescent versus proliferating)? It has been shown that the expression of *Hes1* is different in these two regions (Baek et al., 2006). *Hes1* protein levels are persistent and high in boundary cells, whereas they are variable in individual compartment cells (Fig. 3B). It was recently found that this variability in *Hes1* levels in compartments is due to oscillatory expression (see below) (Shimojo et al., 2008). These different expression patterns of *Hes1* may be responsible for its different regulatory influences in compartment and boundary cells: repressed proliferation and differentiation in boundary cells, and intense proliferation and neuronal differentiation in compartment cells.

6. Hes1 oscillation and cell proliferation in cultured cells

Hes1 expression oscillates in many types of cultured cells, including fibroblasts, myoblasts, and neuroblasts, and the oscillatory expression can be induced following serum stimulation or Notch activation (Hirata et al., 2002; Masamizu et al., 2006). The period of Hes1 oscillation is about 2 h in many mouse cell lines. Hes7, which also oscillates with a 2-h periodicity in the presomitic mesoderm of mouse embryos, acts as a molecular clock to regulate the periodic somite segmentation (Pourquié, 2011). This oscillatory expression is regulated by a negative feedback loop (Fig. 4A). After the activation of *Hes1* transcription by Notch signaling or others, translated Hes1 protein represses its own expression by directly binding to N-box sequences in its promoter. Hes1 gene products, both mRNA and protein, are very unstable, with a very short half-life of about 20 min and therefore disappear rapidly, indicating that this auto-repression is short and transient. Disappearance of Hes1 protein allows the next round of expression. In this way, Hes1 autonomously initiates oscillatory expression with a short periodicity. Real-time imaging analyses of Hes1 expression in single cells revealed that the oscillatory expression of Hes1 is intrinsic and surely occurs in normal culture conditions even in the absence of special stimulations such as serum shock, which synchronizes Hes1 induction in cell populations (Masamizu et al., 2006).

It has been reported that the oscillatory expression of Hes1 is regulated by the Jak-Stat signaling pathway in mouse fibroblast cells (Fig. 4B) (Yoshiura et al., 2007). Jak2 kinase activates Stat3 by phosphorylation, and activated Stat3 induces the expression of downstream genes, including *Socs3*, an inhibitor of Jak2 kinase. Stat3 phosphorylation oscillates in an approximately 2-h cycle, depending on the negative-feedback regulation of Jak2 through *Socs3* expression. The blockade of Jak2-Stat3-*Socs3* oscillation inhibits Hes1 oscillation and stabilizes Hes1 protein levels by a still unknown mechanism.

Results of several studies suggest that Hes1 oscillation and cell proliferation are correlated with each other. Sustained Hes1 expression leads to G1 phase retardation of the cell cycle (Yoshiura et al., 2007). Hes1 can directly or indirectly repress the transcription of cell cycle-related genes, including *E2f1*, *p21Cip1*, *p27Kip1*, and *p57Kip2* (Castella, et al., 2000; Georgia, et al., 2006; Hartman et al., 2004; Murata et al., 2005). Hes1 oscillation may be important for efficient cell proliferation. Hes1 is identified as one of the factors that protects the quiescent state in fibroblast cells by preventing these cells from undergoing differentiation or senescence (Sang, Collier, & Roberts, 2008). High and sustained expression of Hes1 protects fibroblasts against

senescence or differentiation, but the expression of dominant negative-type Hes1 mutants allows quiescent fibroblasts to undergo differentiation. Increased expression of Hes1 has been reported in malignant tumors, including ovarian carcinomas and breast cancers (Wang et al., 2010; Sang, et al., 2010), but the patterns of its expression in these cells remains unknown. It is important to understand the more detailed relationships between Hes1 expression patterns or levels and cell proliferation or quiescent state.

The molecular mechanism by which cells exhibit different (oscillatory versus sustained) expression modes of Hes1 is still unknown. One candidate is the regulation of microRNA-9 (miR-9) (Bonev, et al., 2012; Tan, et al., 2012). MiR-9 negatively regulates Hes1 mRNA stability by interacting with the 3'-UTR of Hes1 mRNA, enabling oscillatory expression of Hes1. MiR-9 expression is absent in the roof plate and floor plate where Hes1 expression is high and sustained. Over-expression of miR-9 induces cell cycle exit and neuronal differentiation, while miR-9 knockdown inhibits neuronal differentiation of NS cells and dampens Hes1 oscillation.

7. Hes1 oscillation in embryonic stem (ES) and NS cells

7.1 Hes1 oscillation in ES cells.

ES cells are pluripotent stem cells derived from the inner cell mass of blastocyst-stage embryos. Due to their pluripotency, these cells have the ability to differentiate into various cell types belonging to all three germ layers: ectoderm, mesoderm and endoderm (Smith, 2001). ES cells are known to exhibit heterogeneous gene inductions in response to the same differentiation cues, but the basic mechanism governing such heterogeneity in ES cells is not fully understood. Hes1 oscillation is one of the candidates for regulating heterogeneous differentiation of ES cells (Kobayashi et al., 2009; Kobayashi & Kageyama, 2010a). Hes1 is highly expressed in mouse ES cells under the control of leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP), two essential factors for mouse ES cell culture, and the expression oscillates in individual mouse ES cells with a 3-5-h periodicity, a longer period than that observed in fibroblast cells. This Hes1 oscillation contributes to the heterogeneous properties of ES cell differentiation: Hes1 protein-low cells are prone to differentiate into neuroectodermal cells, while Hes1 protein-high cells differentiate into early mesodermal cells (Fig. 5). Although Hes1 oscillation is dispensable for ES cell pluripotency (*Hes1*-null ES cells maintain their pluripotent states in cultured ES cells), Hes1 oscillation regulates the fate determination step during ES cell differentiation. In

ES cells, Hes1 regulates Notch signaling molecules and cell cycle-related molecules, both of which are expressed in an oscillating manner in ES cells, suggesting that Hes1 oscillation sets the heterogeneous activation of Notch signaling and the variable timing of cell cycle exit in genetically identical ES cells (Kobayashi & Kageyama, 2010b). Other fluctuating molecules, including Nanog, Rex1, and Stella, have been identified in ES cells, so they may also contribute more diverse responses in cells to produce various kinds of cells at once even in the same environment (Chambers et al., 2007; Toyooka, et al., 2008; Hayashi, et al., 2008; Kobayashi & Kageyama, 2011). One attractive hypothesis is that such fluctuating and oscillatory expression of genes is a common feature of stem cells to endow them with their specific properties (Chang, et al., 2008; Furusawa & Kaneko, 2001, 2012).

7.2 Hes1 oscillation in developing mouse brain

Hes1 expression oscillates dynamically in NS cells both in mouse embryonic brain and in *in vitro* cultured mouse NS cells with a periodicity of about about 2-3 h (Shimojo et al., 2008). The period and amplitude are variable from cell to cell and from cycle to cycle, suggesting that this oscillation does not serve as an accurate molecular clock but rather enables various (not synchronizing) responses to be individually timed. In NS cells, Hes1 represses the expression of proneural genes, Notch signaling molecules, and cell cycle regulators. Interestingly, time-lapse imaging analyses show that both the proneural gene *Ngn2* and the gene for the Notch ligand *Delta-like 1 (Dll1)* are expressed in an oscillatory manner in NS cells (Fig. 6A). These oscillations are specific to undifferentiated NS cells and are not observed in differentiating neurons. It was previously thought that *Ngn2* and *Dll1* expression did not occur in NS cells, but were specific to differentiating neurons. *Ngn2* up-regulates the expression of *Dll1*, which activates Hes1 via Notch signaling in neighboring cells to repress *Ngn2* and *Dll1* expression and maintain these cells in an undifferentiated state. Thus, Notch signaling is unidirectional, from neurons to NS cells, in this context. However, *Ngn2* and *Dll1* are actually expressed by NS cells in an oscillatory manner, which is probably dependent on periodic repression by Hes1 oscillation. This oscillatory expression provides another interesting strategy to maintain NS cells in their undifferentiated state. Oscillatory expression of *Ngn2* activates only early-response genes such as *Dll1* without activation of the neuronal differentiation program. The oscillatory expressions of *Dll1* and *Ngn2* maintain proliferating NS cell populations by mutual activation of Notch signaling (Fig. 6B, left). The expression of *Ngn2* becomes sustained when Hes1 expression is repressed,

thereby promoting neuronal differentiation (Fig. 6B, right).

7.3 Hes1 oscillation in NS cells and the optogenetic control

NS cells are multipotent, generating neurons, oligodendrocytes and astrocytes. NS cells, derived from the ventral telencephalon of mouse embryos, express *Hes1*, *Mash1*, and *Olig2*. *Mash1* promotes cell cycle exit and neuronal fate determination; *Olig2* regulates oligodendrocyte specification, and *Hes1* regulates the maintenance and proliferation of NS cells. Importantly, these three factors are also reported to function in other events: *Mash1* upregulates the gene expression for cell cycle progression (Castro et al., 2011), *Olig2* functions in NS cell proliferation (Ligon et al., 2007), and *Hes1* promotes astrocyte differentiation (Ohtsuka et al., 2001). Thus, each of these factors has two contradictory functions: maintenance and proliferation of NS cells and determination of a specific cell fate. Time-lapse imaging of these proteins in single NS cells revealed that their gene expressions oscillate: the levels of both *Hes1* and *Mash1* proteins oscillate with a periodicity of 2-3 h, and that of *Olig2* oscillates with a longer periodicity (5-8 h) (Imayoshi et al., 2013). Moreover, these gene expressions change dynamically when NS cells are differentiating. During neuronal differentiation, *Hes1* expression is repressed before asymmetric cell division, when one NS cell produces one neuron and one NS cells. *Mash1* protein gradually accumulates and expression becomes sustained in differentiating neurons, whereas *Hes1* and *Mash1* oscillations resume in daughter NS cells. During astrocyte differentiation, *Hes1* protein continues to oscillate but the expression level becomes higher than in NS cells, and then *Mash1* and *Olig2* expressions become undetectable. During oligodendrocyte differentiation, *Olig2* protein expression oscillates at high levels, and *Mash1* and *Hes1* are down-regulated. These observations reveal that one of the bHLH factors, which are fluctuating in NS cells, becomes dominant and sustained during differentiation (Fig. 6C).

A light-switchable gene expression system is able to generate the oscillatory gene expression with short periodicity. The system makes use of the *Neurospora crassa* photoreceptor Vivid that is fused with the Gal4 DNA binding domain and the p65 activation domain (GAVPO) (Wang, et al., 2012). Blue light illumination induces the formation of GAVPO homodimers, which bind to UAS sequences and activate transcription of target genes under the control of UAS sequences. A *Mash1*-inducible system with both *Mash1* coding region and its 3'-UTR under the control of UAS sequences in *Mash1*-null NS cells reveals the importance of gene expression modes (Imayoshi et al., 2013). Repeated exposure to blue light at 3-h intervals successfully

generates oscillatory expression of Mash1 with a 3-h period, which enhances the cell cycle progression of NS cells. On the other hand, repeated exposure at 30-min intervals, which generates sustained expression of Mash1, increases neuronal differentiation. These results clearly demonstrate that the differences in expression modes of these factors are important for a cell's switch between proliferation and differentiation (Imayoshi et al., 2013). The detailed molecular mechanism by which the oscillatory expression up-regulates the cell cycle progression genes remains unclear.

8. Conclusions and Perspectives

Hes factors regulate the maintenance of stem cells and the cell fate decision into multiple cell types in various tissues under multiple signaling pathways, including Notch signaling as described here. These functions are sometimes contradictory to one another. Different expression dynamics is clearly one of the biological strategies to resolve such complex regulations. We mainly focused on Hes1 oscillation in cultured cells, ES cells, and NS cells. It will be interesting to study the role of Hes factor oscillations in other (periodic) developmental processes using imaging techniques. It is also important to address the detailed molecular mechanisms maintaining Hes1 oscillation with biochemical studies and those that switch the expression modes between oscillatory and sustained expressions.

Oscillations were also reported in some signaling pathways regulating Hes factors, such as Notch, Jak-Stat, and Fgf signalings (Kageyama, et al., 2010; Niwa et al., 2007; Yoshiura et al., 2007). For example, Notch and Fgf signaling cooperatively regulate Hes7 oscillation and achieve a spatiotemporal regulation during somite segmentation events (Niwa et al., 2011). Oscillatory Hes7 expression is essential for maintaining periodic somite segmentation. Fgf signaling is important for initiating periodic Hes7 expression in the posterior end of the presomitic mesoderm, and Notch signaling synchronizes and amplifies Hes7 expression from the posterior to the anterior end, thereby inducing periodic somite segmentation at the anterior end. At the same time, Hes7 connects two signaling pathways by regulating the expression of signaling factors involved in both pathways, leading to coupled oscillation of the two pathways. Such oscillatory coupling between multiple signals via Hes factors may be important for other kinds of tissue development as well.

In mouse embryos, Hes7 expression oscillates with a 2-h cycle, and somite segmentation events also occur with a 2-h cycle. It is known that the period of somite segmentation is different among different species: 30 min in zebrafish, 90 min in chick,

and 8 h in human. In the case of Hes1 oscillation in cultured cells, the period is variable between cell types derived from the same species. It is 2-3 hours in NS cells and 3-5 hours in ES cells. It is not known how different cells or cell types set the lengths of these cycles and what the importance of the differential cycle lengths may be. Reconstitution and regulation of the oscillatory expressions using light-inducible systems such as the GAVPO system is an attractive tool not only to reveal the function of oscillation itself but also to understand the significance of the oscillation period.

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Figure legends

Figure 1. Features of Hes genes and their functions. (A) Comparison of protein homology in human Hes factors (Hes1-7, black) and their *Drosophila* homologues (hairy and E(spl), gray). Phylogenetic tree was generated by ClustalW software from the amino acid sequence alignment. (B) Structure of Hes1 protein. (C) Dual repressor functions of Hes factors: active repression and passive repression. During active repression, Hes factors bind to the N-box or the class C site in the promoter regions of target genes and repress their transcription. During passive repression, Hes factors form heterodimers with bHLH activators and inhibit their binding to the E box in the promoter regions of target genes.

Figure 2. Notch signaling pathway. The Notch receptor is a transmembrane protein activated by the binding of Notch ligands expressed on the surface of neighboring cells (signal sender). Upon ligand binding, the Notch receptor on the signal receiver cell is sequentially cleaved by the ADAM protease and the gamma-secretase, releasing the intracellular domain of Notch (ICD) from the plasma membrane. ICD translocates into the nucleus and forms a complex with the DNA-binding transcription factor RBP-J. Without the ICD, RBP-J represses target gene expression, but is converted to a transcriptional activator when complexed with the ICD and the co-activators (Co-A). Finally, Notch signaling activation induces the expression of target genes such as *Hes* and *Hey*.

Figure 3. Hes functions in the central nervous system. (A) Neural stem cells and their differentiation during neural development (upper panel). Neuroepithelial cells initially proliferate by symmetric cell division, and then become radial glial cells, which sequentially generate neurons by asymmetric cell division. Both neuroepithelial cells and radial glial cells are considered to be neural stem (NS) cells. Radial glial cells eventually differentiate into astrocytes, oligodendrocytes, and ependymal cells. Hes factors, expressed in these NS cells, maintain these NS cells and then promote astrocyte differentiation. Proneural bHLH genes, such as *Mash1* and *Ngn2*, are activated in differentiating neurons, leading to the promotion of neurogenesis. (lower panel) Expression pattern of *Hes* genes in the ventricular zone.

(B) The structure of the developing central nervous system (CNS) and differential expression of *Hes1* gene. The CNS in the mouse at E10.5 (left) is partitioned into two regions, compartments (a) and boundaries (b). Boundary cells function as organizing centers by expressing several signaling ligands, including Shh, Wnt1, or Fgf8. The temporal expression patterns of *Hes1* and *Mash1* (right panels) are different in compartment and boundary cells. *Hes1* expression is variable and oscillating in NS cells in compartments, but is sustained in boundary cells.

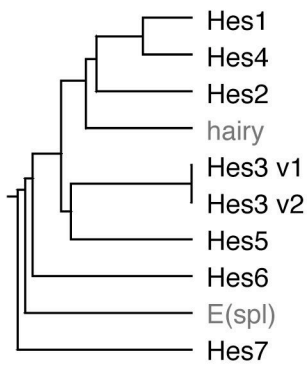
Figure 4. Oscillatory expression of Hes1. (A) *Hes1* oscillation is regulated by negative feedback: *Hes1* protein represses its own expression by directly binding to N-box sequences in its promoter. Both *Hes1* mRNA and protein are unstable and disappear quickly when the expression is repressed. By this mechanism, *Hes1* autonomously initiates oscillatory expression with a periodicity of about 2 h. (B) Jak-Stat signaling regulates *Hes1* oscillation. Jak2 activity is regulated via negative feedback by *Socs3*, a downstream gene of Jak-Stat signaling. The levels of phosphorylated Stat3 (Stat3-P)

and *Socs3* are oscillating, which regulate *Hes1* oscillation and cell proliferation.

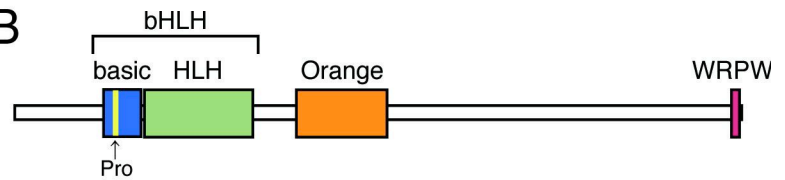
Figure 5. *Hes1* oscillation in ES cells. *Hes1* expression oscillates in embryonic stem (ES) cells via LIF and BMP stimulation. When differentiation signals activate ES cell differentiation, cells low in *Hes1* protein levels differentiate into neuroectodermal cells (neuronal fate), and those high in *Hes1* protein levels tend to differentiate into early mesodermal cells.

Figure 6. *Hes1* oscillation in NS cells. (A) The levels of both *Hes1* and *Ngn2*, encoded by a downstream gene of *Hes1*, oscillate in neural stem (NS) cells. When *Hes1* expression is repressed in differentiating neurons, *Ngn2* expression becomes sustained. (B) *Hes1* oscillation in NS cells induces the oscillatory expression of *Ngn2* and *Dll1* by periodic repression. *Dll1* oscillation mutually activates Notch signaling between neighboring NS cells, which maintain their proliferation without any help from neurons (Left). When *Hes1* is repressed, *Ngn2* and *Dll1* expressions become sustained in differentiating neurons (Right). Notch signaling is unidirectional from differentiating neurons to NS cells. Sustained *Dll1* expression might prevent neurons from receiving Notch signaling by the cis-inhibitory function. (C) In NS cells, oscillatory expression of *Hes1*, *Mash1*, and *Olig2* are important for cell proliferation and stem cell maintenance: oscillatory expression of several fate-determination factors contributes to the multipotent state of NS cells. When differentiation signals arrive, the expression of a single factor becomes sustained or high and determines the cell fate.

A



B



C

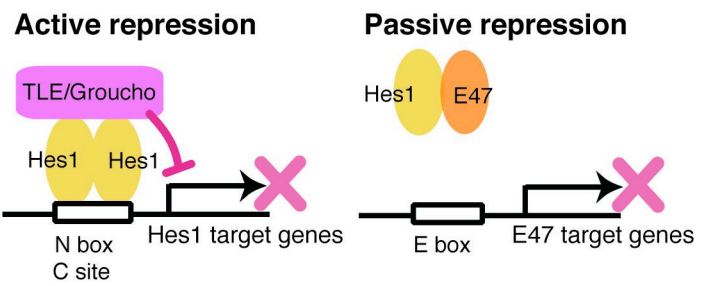


Figure 1

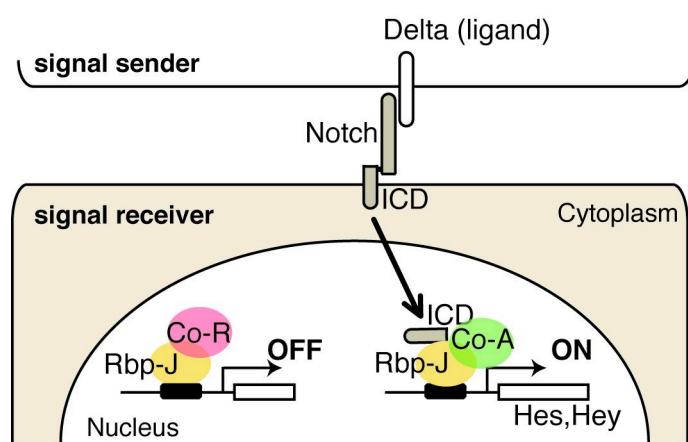


Figure 2

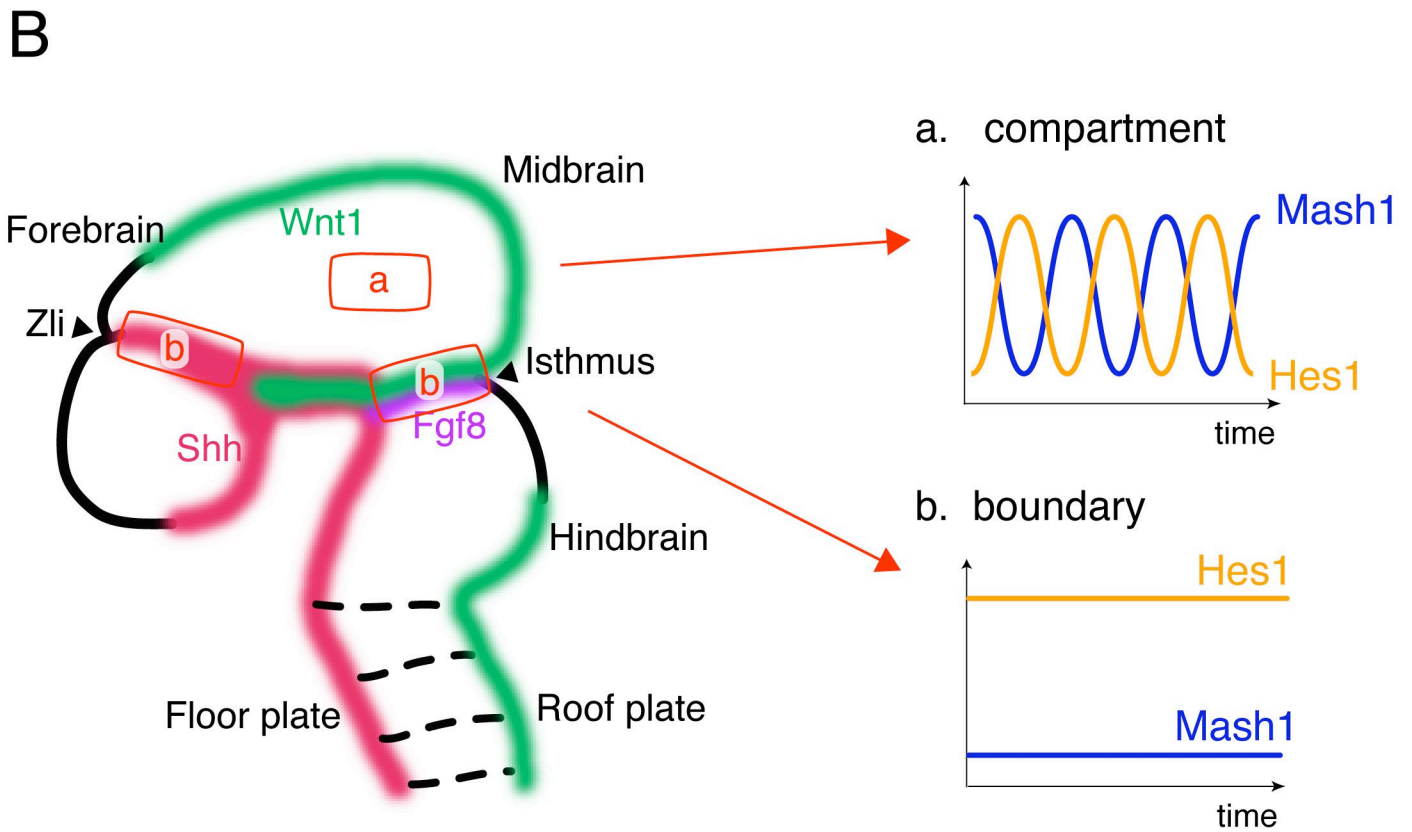
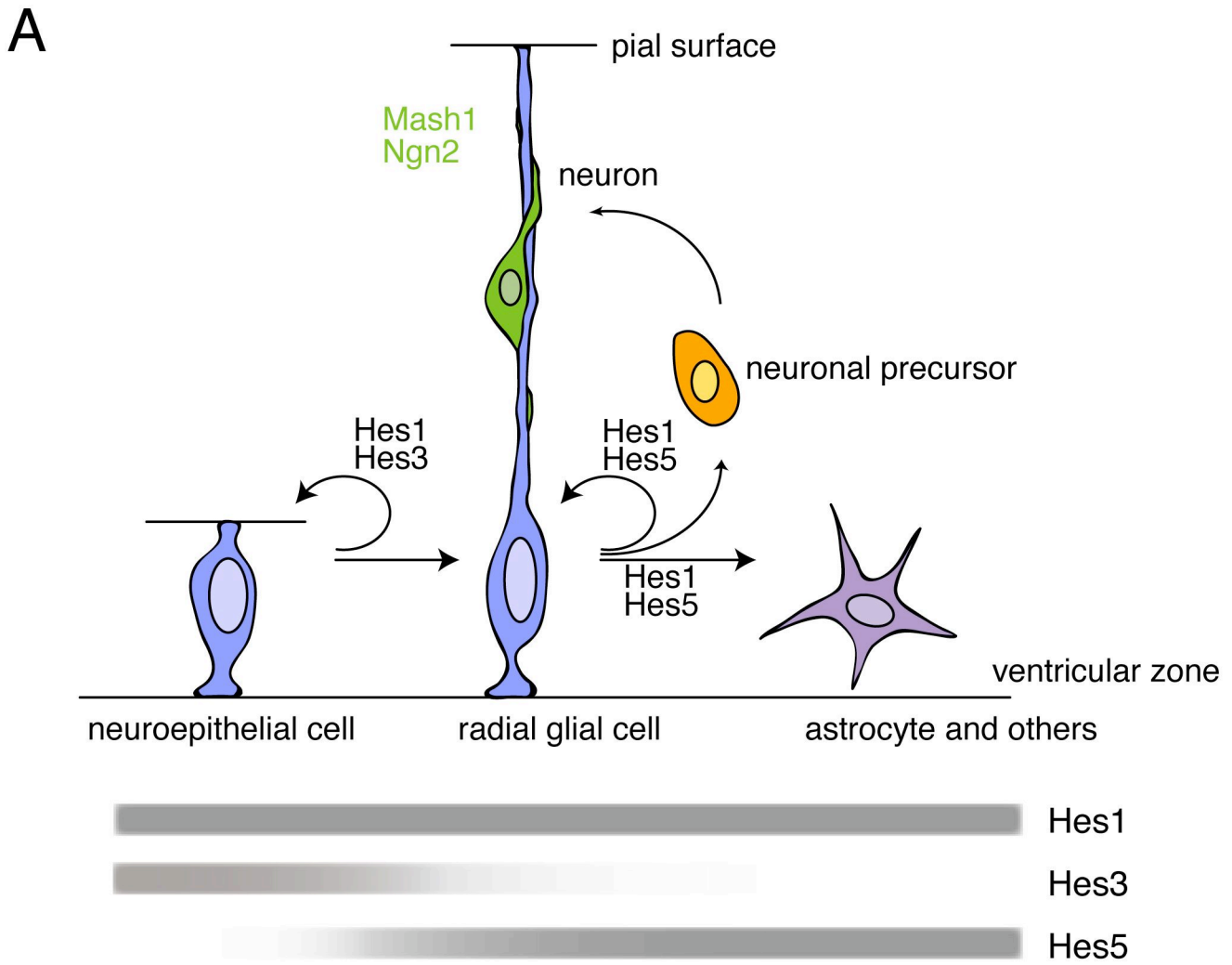


Figure 3

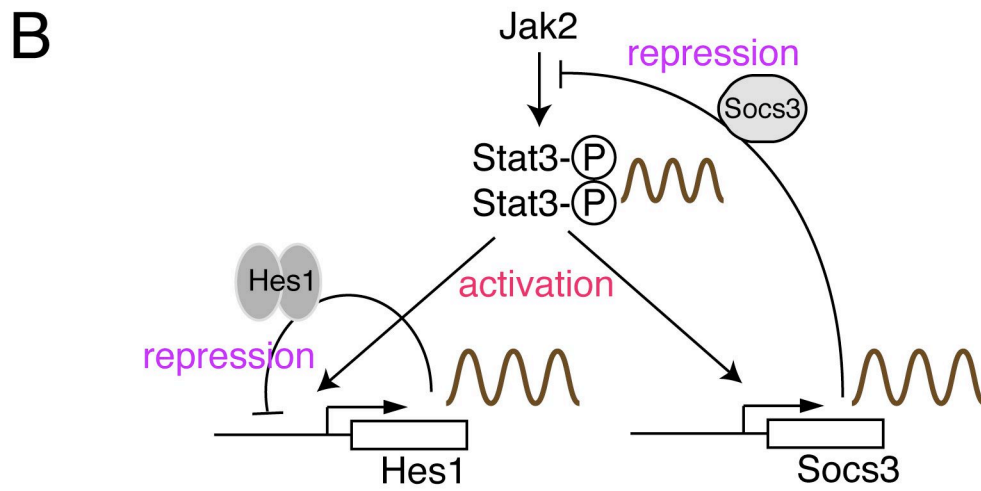
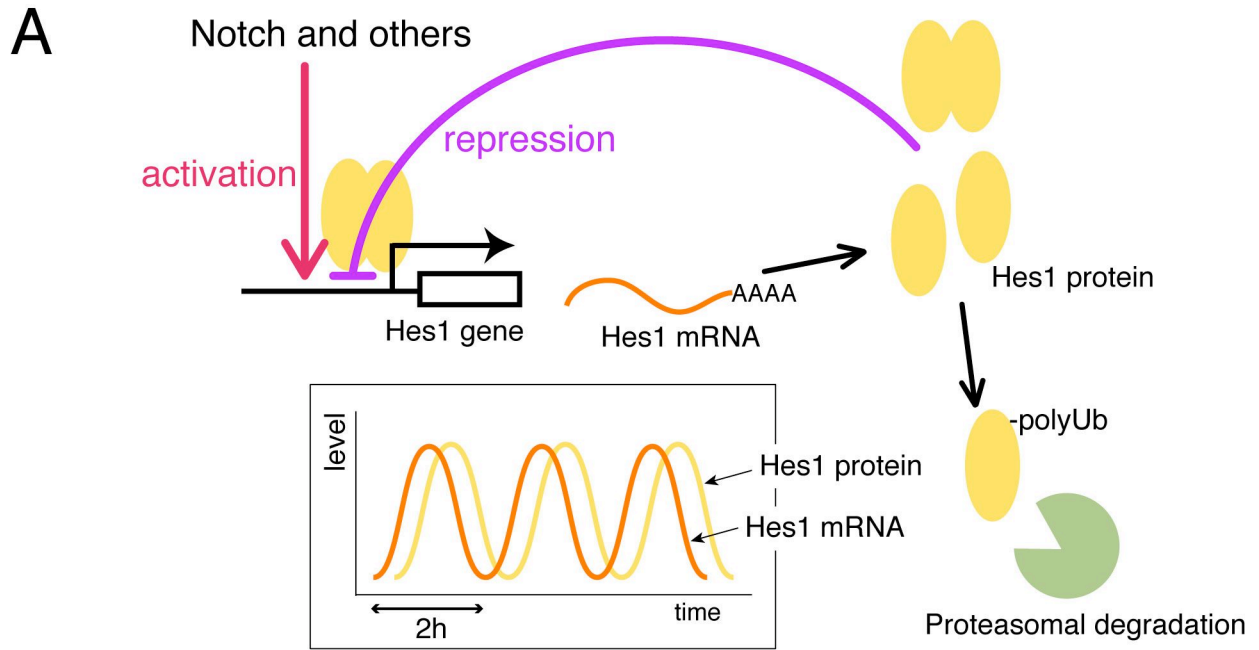


Figure 4

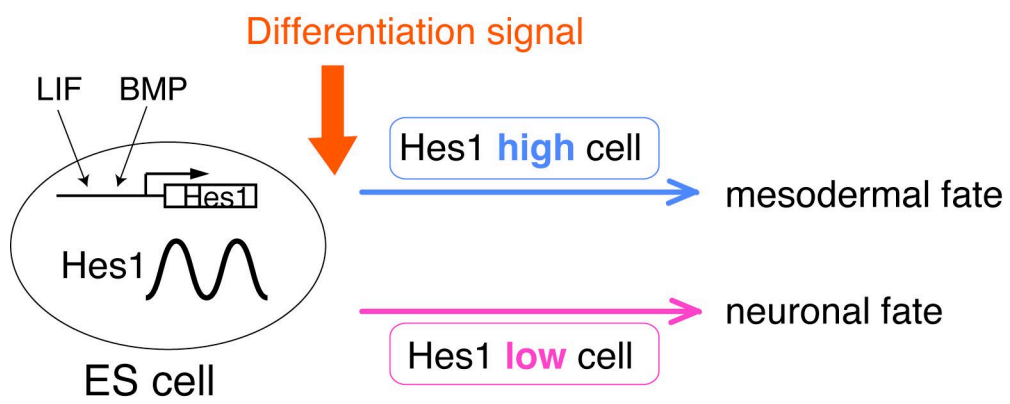


Figure 5

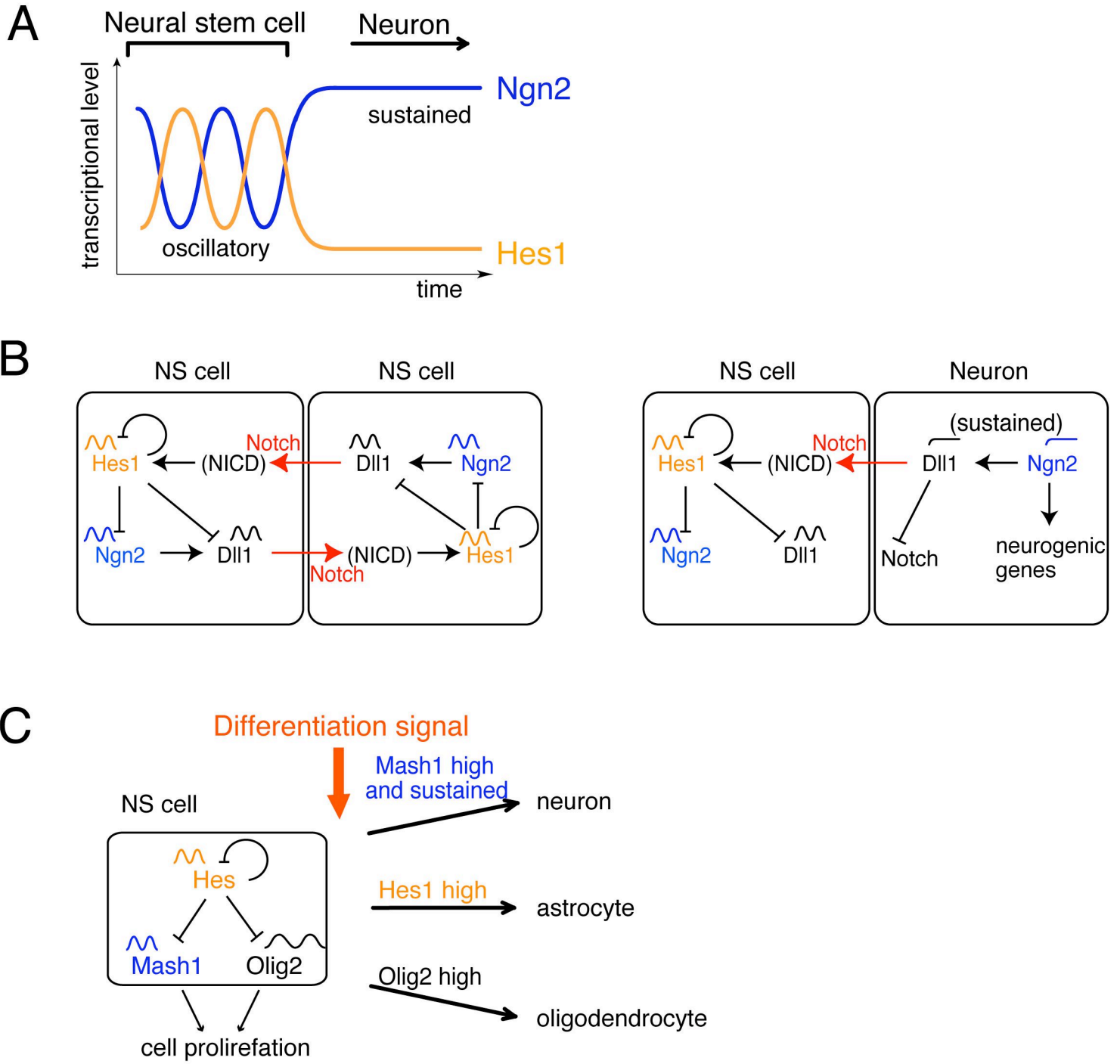


Figure 6