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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

A computational model for the coordination of neural progenitor self-renewal and differentiation through Hes1 dynamics

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ABSTRACT

Proper tissue development requires that stem/progenitor cells precisely coordinate cell division and differentiation in space and time. Notch-Hes1 intercellular signaling, which affects both differentiation and cell cycle progression and directs cell fate decisions at various developmental stages in many cell types, is central to this process. This study explored whether the pattern of connections among the cell cycle regulatory module, the Notch effector Hes1 and the proneural factor Ngn2 could explain salient aspects of cell fate determination in neural progenitors. A mathematical model that includes mutual interactions between Hes1, Ngn2 and G1-phase regulators was constructed and simulated at the single- and two-cell levels. By differentially regulating G1-phase progression, Hes1 and Ngn2 are shown to induce two contrasting cell cycle arrest states in early and late G1, respectively. Indeed, steady Hes1 overexpression promotes reversible quiescence by downregulating activators of G0/G1 exit and *Ngn2*. Ngn2 also downregulates activators of G0/G1 exit, but cooperates with Cip/Kip proteins to prevent G1/S transit, whereby it promotes G1-phase lengthening and, ultimately, contributes to reinforcing an irreversible late G1 arrest coincident with terminal differentiation. In this scheme, Hes1 oscillation in single cells is able to maintain a labile proliferation state in dynamic balance with two competing cell fate outputs associated with Hes1-mediated and Ngn2-mediated cell cycle arrest states. In Delta/Notch-connected cells, Hes1 oscillations and a lateral inhibition mechanism combine to establish heterogeneous Hes1, Ngn2 and cell cycle dynamics between proliferating neural progenitors, thereby increasing the chances of asymmetric cell fate decisions and improving the reliability of commitment to differentiation.

KEY WORDS: Cell fate decision, Cell cycle control, Delta-Notch signaling, Mathematical model, Neural stem cell, Regulatory network

INTRODUCTION

The maintenance of a pool of stem/progenitor cells with a differentiation capacity that evolves with time until late developmental stages is essential for the timely emergence of the innumerable cell types that constitute an adult tissue. Most fully developed organs also possess populations of stem cells in equilibrium between quiescence and proliferation that serve to replenish a wounded area and restore homeostasis. In the highly dynamic context of developing and regenerating tissues, the major challenge remains to uncover the core regulatory logic that ensures that right decisions are made at the cellular level to achieve tissue development and preserve homeostasis in adulthood.

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The G1 phase that occurs between nuclear division (M phase) and DNA synthesis (S phase) is a critical cell cycle period during which cells need to interpret a profusion of conflicting signals before deciding on entering S phase or exiting the cell cycle and making further choices regarding whether to return to quiescence, differentiate, senesce or die (Blomen and Boonstra, 2007). The selection of a particular cell fate following G1 arrest depends on the nature of the cell cycle inhibitory signal as well as the time in G1 at which cells receive such a signal (Wainwright et al., 2001; David-Pfeuty, 2006). Thus, mitogen withdrawal, contact inhibition and loss of adhesion typically settle a non-proliferative, low-metabolic, reversible state of quiescence in which cells can healthily reside indefinitely (Cheung and Rando, 2013). By contrast, overexpression of Cdk inhibitors (CKIs) of the Cip/Kip family resulting from the activation of diverse pathways – notably the stress and differentiation pathways – triggers irreversible cell cycle exit (Walsh and Perlman, 1997; Blagosklonny, 2003; Buttitta and Edgar, 2007). In order for the interplay between cell proliferation and cell differentiation to succeed in properly shaping a tissue, the early expression of differentiation traits and lineage-restricted identity in progenitor cells should not definitively block their proliferation, and irreversible cell cycle arrest should not occur before commitment to terminal differentiation. This requires the establishment of an exceedingly stringent spatiotemporal coupling between cell cycle progression and cell differentiation, which manifests itself in the fact that G1-phase duration tends to increase as stem cells gradually lose their proliferative potential and concurrently gain a higher differentiation capacity (Lange and Calegari, 2010).

Despite the apparently inextricable complexity of the developmental processes involved in the elaboration of a multicellular organism, only a few pathways have proved to cooperate in this huge task, notably the Notch, Ras/MAPK, Sonic hedgehog, Wnt/β-catenin, TGFβ/BMP and JAK/STAT pathways, which all are highly conserved throughout metazoa. Evidence of Notch involvement stems from the discovery that Notch-deficient *Drosophila* embryos succumb to hyperplasia of the nervous system due to an overproduction of neurons at the expense of epidermis by immature ectodermal cells (Poulson, 1940). The aberrant phenotype of Notch-deficient embryos highlights the crucial role that Notch signaling plays in organ patterning by controlling the ratio of two distinct cell types arising from a common precursor cell. In addition, Notch activation at the interface between two distinct cell groups is used to generate a so-called boundary cell population that insulates the two initial groups of cells from one another. Because of its dual function in controlling binary cell fate decisions and compartmental tissue organization, Notch signaling logically emerges as an essential determinant of spatial differentiation in virtually all metazoa, affecting cell fate outcome across a wide array of cell types within the same organism (Koch et al., 2013).

Notch signaling, in fact, has a relatively simple molecular design and its pleiotropic effects are likely to derive from its large-scale capacity for altering gene expression in response to paracrine

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cell-cell contact via the truncated Notch receptor intracellular domain that translocates to the nucleus to recruit co-activators and activate the transcription of Notch target genes (Andersson et al., 2011). The most widely expressed Notch targets are the Hairy/Enhancer of split (Hes1-7) bHLH transcriptional repressors, only three of which (Hes1, 5 and 7) are direct Notch targets and apparently mediate much of the developmental effects of Notch in many tissues and organs. Indeed, the Hes repressor genes, especially *Hes1*, function to prevent premature differentiation of stem/progenitor cells in early development and, thereby, safeguard the correct timing of cell differentiation (Hatakeyama et al., 2004). Major Hes1 targets are bHLH transcriptional activators that play prominent roles in the differentiation of various tissues from *Drosophila* to mammals. During neurogenesis, however, Hes1 not only represses the transcription of proneural genes, such as *neurogenin 2* (*Ngn2*, or *Neurog2*) and *Mash1* (also known as *Ascl1*), but also downregulates cell cycle regulators, many of which are involved in G1-phase control: e.g. the Cip/Kip genes that encode $p21^{Cip1}$ (Cdkn1a), $p27^{Kip1}$ (Cdkn1b) and $p57^{Kip2}$ (Cdkn1c) (Kabos et al., 2002; Murata et al., 2005), and cell cycle activators such as *cyclin D1*, *E2* and *A2* (Shimojo et al., 2008; Noda et al., 2011), *E2F1* and *Myc* (Hartman et al., 2004). Importantly, Hes1 also transcriptionally represses its own expression. This autoregulatory negative-feedback loop can induce oscillatory expression of *Hes1* mRNA and Hes1 protein, as has been observed in various cultured cells following serum treatment (Hirata et al., 2002), in mouse neural progenitors (Shimojo et al., 2008) and embryonic stem cells (Kobayashi et al., 2009). Yet, oscillatory Hes1 expression is not the rule. Indeed, Hes1 expression is kept low in most differentiated cells (Kageyama et al., 2007), whereas it is steadily expressed at high level in boundary areas of the developing nervous system (Baek et al., 2006), in quiescent fibroblasts (Sang et al., 2008), in contact-inhibited 3T3-L1 cells (Noda et al., 2011) and in some differentiated cells (Wu et al., 2003).

The molecular strategies developed by stem cells to coordinate self-renewal and differentiation have been most thoroughly investigated in the context of neurogenesis, throughout which Notch signaling plays a prominent role (Louvi and Artavanis-Tsakonas, 2006; Kageyama et al., 2009; Pierfelice et al., 2011). Neurogenesis in vertebrates primarily relies on the activity of proneural bHLH transcription factors, such as Ngn1, Ngn2 and Mash1, which not only are direct Notch targets but also directly regulate G1-phase progression (Farah et al., 2000; Nguyen et al., 2006; Castro et al., 2011; Lacomme et al., 2012) and which exhibit oscillatory expression that inversely correlates with that of Hes1 in mouse neural progenitors (Shimojo et al., 2008; Imayoshi et al., 2013). The neurogenic phase of cortical development therefore appears as the most propitious system in which to study how the interactions between Notch-Hes1 signaling, G1-phase regulation and differentiation signals may succeed in dictating proper cell fate decisions.

The mathematical modeling approach has proved itself in the identification of key topological and dynamical features of regulatory pathways, including Notch signaling and cell cycle control. Notably, computational models of Notch signaling have clarified how intracellular feedbacks and intercellular coupling cooperate in regulating spatial differentiation and organ patterning during development (Lewis, 2008; Shaya and Sprinzak, 2011; Morelli et al., 2012). Computational models of the mammalian cell cycle have characterized regulatory pathways and checkpoint mechanisms that regulate sequential progression through the G1/S/G2/M cell cycle phases (Novak and Tyson, 2004; Gérard and Goldbeter, 2009) and that promote cell cycle arrest in response to stress conditions (Toettcher et al., 2009). The mathematical model used here to explore the connection between cell cycle dynamics and Notch signaling in neural progenitors consists of a regulatory network that encompasses cell cycle regulators, the transcription factor Ngn2, the Notch effector Hes1 and the Delta-like Notch ligand Dll1. A thorough computational analysis revealed two features to be of utmost importance: first, the biphasic organization of the G1 phase enables neural progenitors to experience two distinct types of cell cycle arrest characterized by either Hes1-high/Ngn2-low or Hes1-low/Ngn2-high levels, coincident with two opposing cell fates; second, Hes1 oscillation generated by the combination of intracellular and intercellular regulations maintains Delta/Notch-connected neural progenitors in a labile proliferation state, in which they are prone to shift toward divergent cell fate outputs upon signals stabilizing Hes1 and Ngn2 expression at opposite levels.

RESULTS

Model of the cell cycle and differentiation regulatory networks

The present model, which integrates selected signaling and regulatory pathways involved in the coordination of cell cycle progression and neural differentiation (Fig. 1), can be translated into a set of mathematical equations using the specific procedures and assumptions described in the Materials and Methods. Differential equations, reaction rate functions and parameter values are recapitulated in supplementary material Tables S1-S4.

Cell cycle regulatory pathway

Because G1-phase lengthening and arrest are two critical features of neural progenitor differentiation, the cell cycle regulatory module

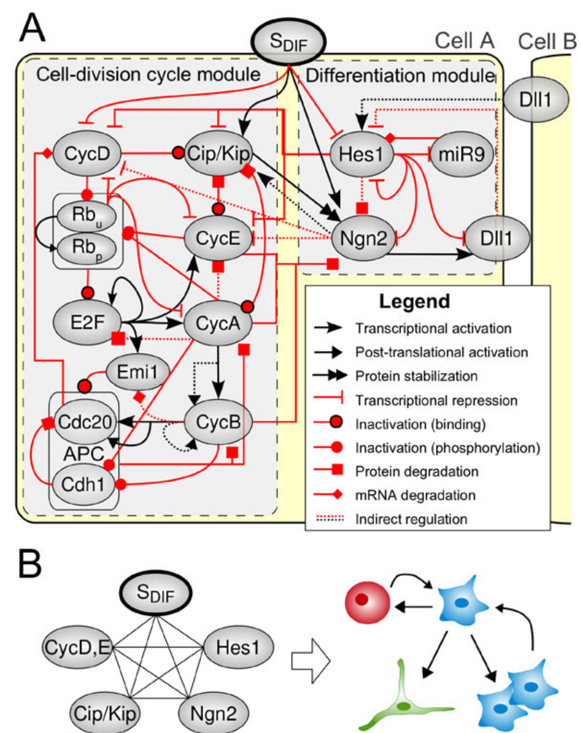

Fig. 1. Regulatory network model of neural progenitor fate decision. (A) Single- and two-cell network models of the coupling between the cell cycle and neural differentiation regulatory modules. CycD, CycE, CycA and CycB refer to the cyclin(s) complexed with Cdks. (B) Core and densely connected regulators that direct the neural stem cell fate decision between self-renewal (blue), quiescence (red) and Ngn2-mediated neural differentiation (green).

lays a greater emphasis on G1-phase control than on the other cell cycle phases. The cell cycle regulators included in the model are: cyclin D/E/A/B-Cdk complexes, the CKIs of the Cip/Kip family, the retinoblastoma (Rb) proteins, the E2F transcriptional factors, the anaphase-promoting complex (APC) and its positive (Cdh1, Cdc20) and negative (Emi1) regulators, the mutual interactions and temporal activity patterns of which are well characterized (Morgan, 2007).

Neural differentiation regulatory pathway

A crucial component of the neurogenic pathway is the proneural protein Ngn2 that not only regulates neuronal differentiation, migration and maturation, but also contributes to cell cycle exit by indirectly repressing a subset of cyclins, notably *cyclin D1*, *E1* and *E2*, and by activating, directly or indirectly, the Cip/Kip genes (Farah et al., 2000; Lacomme et al., 2012). Multi-site Ngn2 phosphorylation by cyclin-Cdk1,2, in turn, leads to its inactivation as a transcriptional activator and its destabilization (Ali et al., 2011), whereas Ngn2 binding to Cip/Kip proteins (e.g. $p27^{Kip1}$) induces its stabilization (Nguyen et al., 2006).

The Notch-Hes1 signaling pathway

Several computational models have explored how Notch-Hes1 intercellular signaling coordinates the fates of adjacent cells (Agrawal et al., 2009; Wang et al., 2011). Here, emphasis is laid on the Notch effector Hes1, which is known to repress the transcription of many cell cycle and differentiation regulatory genes, including *cyclin D1* and *E2*, *Ngn2*, *Dll1* and *Hes1* (Kageyama et al., 2007). By competing with Ngn2 for binding to E-box DNA, Hes1 further promotes Ngn2 destabilization, thus preventing Ngn2-mediated transcriptional activation of proneural factors (Hindley et al., 2012). Hes1 expression is assumed to be regulated at two main levels: through the regulation of *Hes1* mRNA stability by microRNA 9 (miR9), which in turn is transcriptionally repressed by Hes1 (Bonev et al., 2012; Tan et al., 2012); and, importantly, through Dll1-ligand-induced trans-activation and cis-inhibition of Notch signaling (Kageyama et al., 2007; Wang et al., 2011).

Prodifferentiation signaling pathways

A number of pathways besides Notch, including Fgf, Shh and Wnt, display a specific spatiotemporal activation pattern during neural development and contribute to neural progenitor fate decision and diversity (Guillemot, 2007). In fact, because the processes of cell division and terminal differentiation are essentially mutually exclusive, an extensive signaling crosstalk between the two is expected to occur throughout the course of development as stem cells gradually lose their innate predisposition to divide and concurrently acquire differentiation traits. Thus, prodifferentiation pathways are expected to exert antimitogenic effects (e.g. upregulate Cip/Kip and downregulate cyclin D genes), to promote the timely expression of prodifferentiation factors (e.g. Ngn2) and to disrupt Notch-Hes1 signaling. In neural progenitors, many of these effects arise in great part from inactivation of Fgf signaling and activation of TGFβ and Wnt signaling (Falk et al., 2008; Sato et al., 2010; Guillemot and Zimmer, 2011; Qu et al., 2013). Accordingly, the interweaving pathways that ultimately cooperate to promote differentiation are taken into account in the model by assuming a global differentiation signal [S_{DIF}], which, in the main, upregulates Cip/Kip and Ngn2 proteins and downregulates cyclin D genes and *Hes1*.

Control of G1-phase progression, lengthening and arrest

Before exploring the mechanism of coordination between cell cycle progression and neural differentiation, a preliminary step is to analyze the dynamical behavior of the cell cycle regulatory module alone (Fig. 2A). For an appropriate choice of parameters (see Materials and Methods), the concentrations of the cell cycle regulators display a typical evolution with time during progression throughout the cell division cycle (Fig. 2B). Orderly progression throughout G1, S, G2 and M phases depends on the balance between mitogenic signals, which promote cell division, and antimitogenic signals, which can delay and possibly arrest cell cycle progression at various stages. Extracellular signals impinge on cell cycle progression at two main levels by regulating the rates of cyclin D and Cip/Kip protein synthesis (s_{cD} and s_{CK}, respectively). In order for cell cycle progression to occur, s_{cD} needs be high enough and s_{CK} low enough (Fig. 2C). If s_{cD} values are too small, cyclin D-Cdk levels may remain too low to efficiently relieve Rb-dependent transcriptional repression of cyclin E genes, which prevent G0/G1

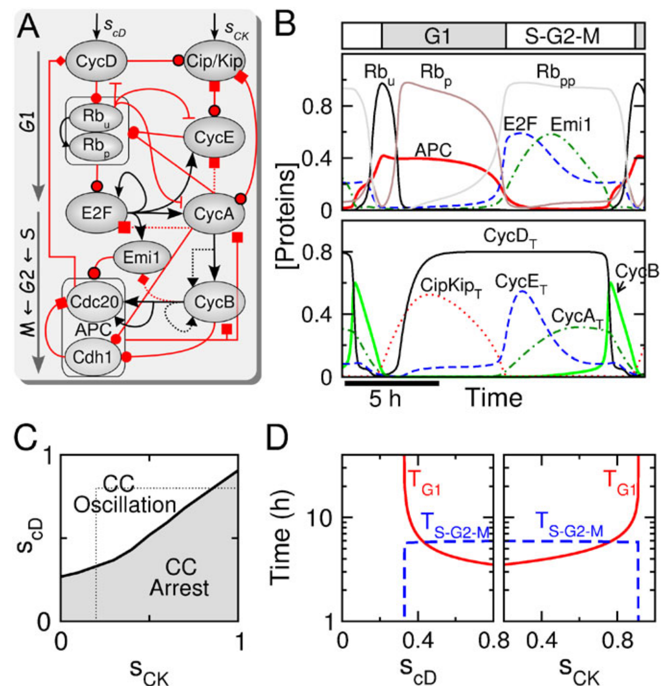

Fig. 2. Dynamics of cell cycle progression lengthening and arrest. (A) The cell cycle regulatory module (see Fig. 1A for legend). (B) Typical oscillatory behavior of cell cycle regulatory proteins during G1-to-M-phase progression obtained for s_{cD}=0.8 and s_{CK}=0.8 ($CycD_T$, E_T, A_T include free and Cip/Kip-bound cyclin-Cdks). Postmitotic cells start their division cycle with very low levels of active cyclin-Cdks and high levels of unphosphorylated E2F-bound Rb and of Cdh1-bound APC. Mitogen-dependent accumulation of cyclin D-Cdk4,6 complexes initiates Rb phosphorylation and sequesters the Cip/Kip proteins, thereby allowing cyclin E-Cdk2 to accumulate and hyperphosphorylate Rb, freeing the E2F factors, which activate many cell cycle regulatory genes including E2F members, cyclin E and A and *Emi1*, and trigger S-phase entry. Cyclin E-Cdk2 and Emi1 cooperate to inactivate Cdh1-APC, thus stabilizing and augmenting the pool of cyclin A-Cdk1,2, which drives progression throughout the S/G2 phases and facilitates the degradation of cyclin E-Cdk2, the inactivation of E2Fs, the synthesis and activation of cyclin B-Cdk1 and keeps APC inactive. M-phase entry coincides with a burst of active cyclin B-Cdk1 followed by the reappearance of APC activity (linked to Emi1 degradation and Cdc20 synthesis and activation). Cdc20-bound APC initiates the degradation of cyclins A and B, relieving Cdh1 inactivation and restoring Cdh1-APC-dependent degradation of cyclins A and B, which precipitates the birth of daughter cells and marks the beginning of new division cycles. (C) Cell cycle (CC) oscillations versus arrest as a function of s_{cD} and s_{CK}. Dotted lines indicate parameter variations in D. (D) Variations in length of the G1 phase (T_{G1}) and of S-to-M phase ($T_{S\text{-}G2\text{-}M}$) as a function of s_{cD} (for s_{CK}=0.8) and s_{CK} (for s_{cD}=0.2).

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transit and establish a robust G0 arrest state. For large s_{cD} values also, high enough s_{CK} values might enable overexpressed Cip/Kip proteins to evade sequestration by cyclin D-Cdks, allowing them to bind and inactivate the emerging cyclin E-Cdks. Kinase-inactive cyclin E-Cdks then fail to hyperphosphorylate Rb and liberate the E2F factors required for crossing the G1/S border. Here, the transition from cell cycle oscillation to cell cycle arrest for decreasing s_{cD} or increasing s_{CK} values is fully reversible. Before cell cycle arrest is induced, decreasing s_{cD} or increasing s_{CK} is shown to slow down cell cycle progression, exclusively during G1 phase (Fig. 2D). Numerical simulations of cell cycle progression for changing levels of s_{cD} and s_{CK} indicate that the G1-phase duration (T_{G1}) gradually increases from 3 to about 15 h, whereas the S-G2-M-phase duration ($T_{S\text{-}G2\text{-}M}$) remains nearly constant at ~5 h, which are typical durations in mouse (Takahashi et al., 1993).

Hes1 and Ngn2 promote distinct cell cycle arrest states associated with quiescence and differentiation, respectively

The expression of G1-specific cyclins and CKIs in stem/progenitor cells is tightly regulated by intercellular signaling and intrinsic differentiation pathways. In the context of neurogenesis, both the Notch effector Hes1 and the proneural transcription factor Ngn2 can oppose G1-phase progression by repressing the transcription of cell cycle activators such as *cyclin D1* and *E2*. Yet, the precise way in which each restrains G1-phase progression might differ given that Hes1 also represses Cip/Kip and *Ngn2* transcription, and that Ngn2 protein is stabilized by binding the Cip/Kip proteins and, possibly, activates Cip/Kip gene transcription whereas its phosphorylation by cyclin-Cdk1,2 promotes its inactivation as a transcriptional activator and its destabilization.

In order to explore how these contrasting features of Hes1 and Ngn2 combine to regulate cell cycle progression and arrest, cell cycle progression was simulated in the presence of static Hes1 levels (omitting its regulation by miR9, Ngn2 and its autoregulation), of differentiation signal (Fig. 3A) and of elevated mitogen levels (s_{cD}=1). Numerical simulations revealed the existence of three distinct stable states associated with proliferation, quiescence and differentiation, depending on specific combinations of [*Hes1*] and [S_{DIF}] and on the initial conditions (Fig. 3B). (1) For low [*Hes1*] and [S_{DIF}] levels that are insufficient to overcome the mitogenic activity of the cell cycle activators, the cell cycle regulatory network displays an oscillatory behavior similar to that shown in Fig. 2B and associated with cell cycle progression through the G1/S/G2/M phases. As in Fig. 2D, the proliferation state exhibits a lengthening of the cell cycle duration as [S_{DIF}] or [*Hes*1] increases, which is due to G1-phase elongation. (2) High enough [*Hes1*] and low enough [S_{DIF}] levels lead to a cell cycle arrest reminiscent of quiescence, which is characterized by low levels of both Ngn2 and G1-specific cyclin-Cdks owing to Hes1-dependent transcriptional repression of *Ngn2* and cyclin genes. (3) High enough [S_{DIF}] levels induce a cell cycle arrest state characterized by high Ngn2 and Cip/Kip protein levels, which cooperate in preventing S-phase entry: Ngn2 represses *cyclin D1* and *E2* transcription and the Cip/Kip proteins inhibit activity of the cyclin E-Cdk2 complexes, which is associated with commitment to neural differentiation.

Whether the stability domains of the three cell fate states overlap is of strategic importance to determine whether the phenotypic transition will be reversible or irreversible and inducible or not by transient signals. The lack of overlap between the quiescence and proliferation domains indicates that G0/G1 and G1/G0 transits are fully reversible upon slow variations of *Hes1* levels. By contrast, the existence of an overlapping (i.e. bistable) domain between the proliferation and differentiation states means that the transition toward a terminal differentiation state is irreversible and then robust to an extent defined by the size of the overlapping domain. The occurrence of bistability between the proliferation and terminal differentiation states also implies that a transient pulse of [S_{DIF}] is able to induce a cell to differentiate. The bifurcation analysis of this elaborate cell fate landscape is performed in supplementary material Fig. S1.

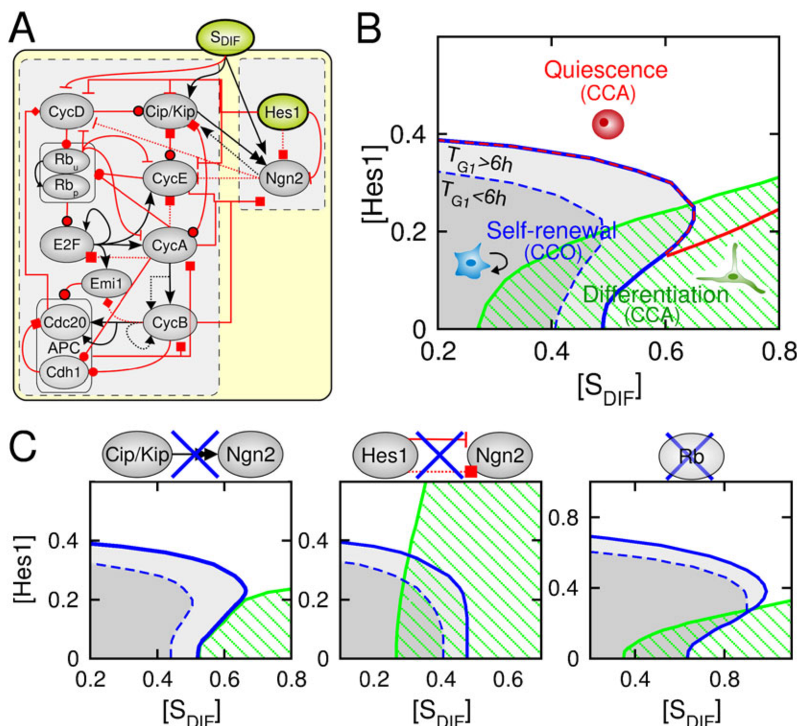

Fig. 3. Cell fate landscapes shaped by the interplay between Hes1 and Ngn2. (A) Single-cell model without the Hes1 regulations (see Fig. 1A for legend). (B) Cell fate landscape as a function of [*Hes1*] and [S_{DIF}]. The gray domain with blue boundary represents a proliferation cell cycle oscillations (CCO) state: dark-gray versus light-gray areas correspond to short (<6 h) and long (>6 h) G1 lengths, respectively. The white domain above the red boundary represents a quiescent cell cycle arrest (CCA) state with [*Ngn2*]<0.1. The green hatched domain represents a differentiation-associated CCA state with [*Ngn2*]>0.1. (C) Alteration of the cell fate landscape induced in various conditions: (left) failure to stabilize Ngn2 by Cip/Kip binding ($d_{CK\rightarrow Ngn}$=0 instead of $d_{CK\rightarrow Ngn}$=2) destroys the bistability domain between proliferation and differentiation; (middle) failure of Hes1 to repress *Ngn2* transcription ($\theta_{Hes\rightarrow Ngn}$=2 and $d_{Hes\rightarrow Ngn}$=0 instead of $\theta_{Hes\rightarrow Ngn}$=0.5 and $d_{Hes\rightarrow Ngn}$=30) compromises the ability of inducing CCA toward differentiation via Hes1 downregulation; (right) defective Rb module ([Rb_T]=0.4 instead of [Rb_T]=1) prevents G1-phase elongation and extends the bistability domain.

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The next step was to identify the key regulatory features in Fig. 3A that shape the cell fate landscape depicted in Fig. 3B. Most alterations favor or oppose one of the three phenotypic states by promoting an expansion or shrinkage of the domain associated with it. Thus, downregulation of G1-specific cyclins or upregulation of Ngn2 and Cip/Kip proteins induces a narrowing of the proliferation domain, a reduced proliferation rate and an expansion of the differentiation domain. However, particular types of alterations also produce more subtle effects. First, the size of the bistability domain between the proliferation and differentiation states is highly contingent upon the strength of positive-feedback loops arising from the mutual activation between Ngn2 and Cip/Kip proteins and from the mutual inhibition between Ngn2 and G1- and G1/S-specific cyclin-Cdks. For instance, preventing Cip/Kip-mediated Ngn2 stabilization not only augments the $[S_{DIF}]$ threshold needed for differentiation, but also destroys the bistability domain between the proliferation and differentiation states (Fig. 3C, left). Second, because Hes1 behaves as a general transcriptional repressor that indiscriminately represses activators and inhibitors of the cell cycle and differentiation factors, Hes1 expression can, at intermediate levels, induce proliferation whereas, at high or low levels, it can block proliferation. Mitigating Hes1-dependent transcriptional and post-translational regulation of Ngn2 abrogates such a sophisticated cell fate control by Hes1 levels (Fig. 3C, middle). Third, the early G1-phase elongation by the antimitogenic S_{DIF} signal is subject to tight control by cyclin D-Cdk-dependent Rb phosphorylation, and bypassing the Rb/cyclin D-Cdk module – for instance by *Rb* loss (or cyclin E gene upregulation) – would lead to a short and poorly tunable G1 phase and significantly augment the $[S_{DIF}]$ threshold needed for commitment to differentiation (Fig. 3C, right).

Altogether, these computational data provide a strong hint that the timely coordination between G1-phase lengthening, cell cycle exit and commitment to differentiation, which occurs during neural development, is rooted in the particular pattern of connections among components of the G1-phase regulatory network, prodifferentiation factors and *Hes1*-dependent signaling.

Hes1 oscillation sustains a dynamic proliferation state that is well adapted for versatile fate decisions in both single- and two-cell systems

The most striking property of the Hes1 protein is that it exhibits an oscillatory pattern of expression owing to its ability to repress its own transcription. Yet, Hes1 is also found steadily expressed in certain conditions, notably at low levels in most differentiated cells and at elevated levels in many other conditions. How division can proceed efficiently in cells in which Hes1 levels oscillate, but not in cells with stationary, high or low Hes1 levels, remains enigmatic. An examination of the cell fate landscape depicted in Fig. 3B as a function of steady Hes1 levels provides a hint as to the putative role of Hes1 oscillations. Indeed, for intermediate $[S_{DIF}]$ levels cells divide only for intermediate $[Hes1]$ levels, whereas for higher or lower $[Hes1]$ levels they stop dividing while expressing either low or high Ngn2 levels, respectively (Fig. 4A). Thus, the model predicts that the cyclic expression of high and low Hes1 levels, each of which separately is incompatible with cell division, could nevertheless cause efficient cell cycle progression through fluctuating levels of prodifferentiation factors and G1-phase regulators (Fig. 4B). This suggests that the oscillatory Hes1 dynamics is prone to establishing a proliferative state that is dynamically sensitive to signals capable of triggering divergent cell fates, notably by stabilizing Hes1 expression at either high or low levels.

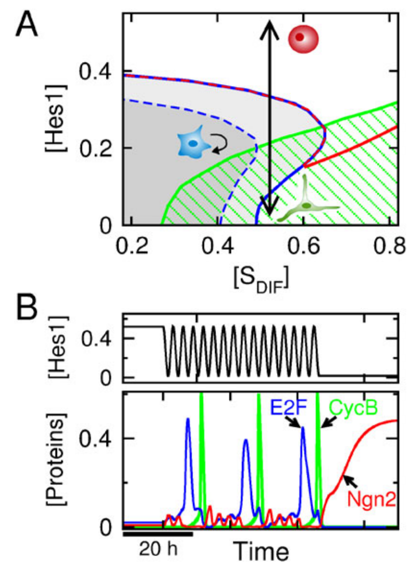

Fig. 4. Hes1 oscillation promotes a proliferation state suitable for binary cell fate decisions. A typical cell trajectory induced by a given temporal change in Hes1 levels, represented in the cell fate landscape by the double-headed arrow (A) and through a timecourse simulation of $[CycB]$, $[E2F]$ and $[Ngn2]$ (B). Hes1 oscillation between $[Hes1]_{min}$=0.02 and $[Hes1]_{max}$=0.52 induces division, whereas steady $[Hes1]_{min}$ and $[Hes1]_{max}$ levels induce CCA states characterized by high and low Ngn2 levels, respectively.

How Hes1 expression oscillates in proliferating cells and how these oscillations readily halt coincidentally with cell cycle exit and commitment to differentiation have been addressed by investigating the complete two-cell model incorporating the dynamic regulation of Hes1 expression (Fig. 1A). The upregulation of Hes1 expression by Delta-Notch (DN) signaling and its downregulation through self-repression and miR9-dependent inhibition of mRNA stability and translation combine to generate three distinct dynamical regimes characterized by different Hes1 expression patterns depending on the DN signaling level. At low (respectively, high) DN signaling, Hes1 expression is stationary and low (respectively, high), whereas at intermediate DN signaling, Hes1 expression oscillates (supplementary material Fig. S2). Because transcription of *Dll1*, which is an essential component of DN signaling, is repressed by Hes1 and activated by Ngn2, the Hes1 dynamics and *Hes1*-dependent cell fate decisions are expected to be tightly coupled between DN-connected cells. Simulations of the Hes1, Ngn2 and cell cycle dynamics in the two-cell model indeed show sophisticated modes of coupling (Fig. 5; supplementary material Fig. S3). First, for relatively low $[S_{DIF}]$ levels, the DN-connected cells tend to efficiently proliferate while their Hes1 levels oscillate, either synchronously or asynchronously, depending on whether the initial conditions are the same (e.g. in identical sister cells) or different (Fig. 5A). However, when such proliferating cells are exposed to a transient pulse Δ of $[S_{DIF}]$ ($[S_{DIF}]=[S_{DIF,0}]+\Delta$ during time τ_{Δ}), they have the possibility to arrest cell cycle progression by stabilizing Ngn2 at high levels and Hes1 at low levels, which is likely to occur in one cell whereas the other cell keeps dividing with oscillating Hes1 levels (Fig. 5B) or, possibly, arrests cell cycling due to high Hes1 levels (supplementary material Fig. S3A). The probability that each cell differentiates naturally increases with the absolute levels of transient differentiation signal Δ received, but it also depends on the relative level of Δ between the two DN-connected cells, although differently according to whether the two cells display synchronous or asynchronous Hes1, Ngn2 and cell cycle dynamics (Fig. 5C;

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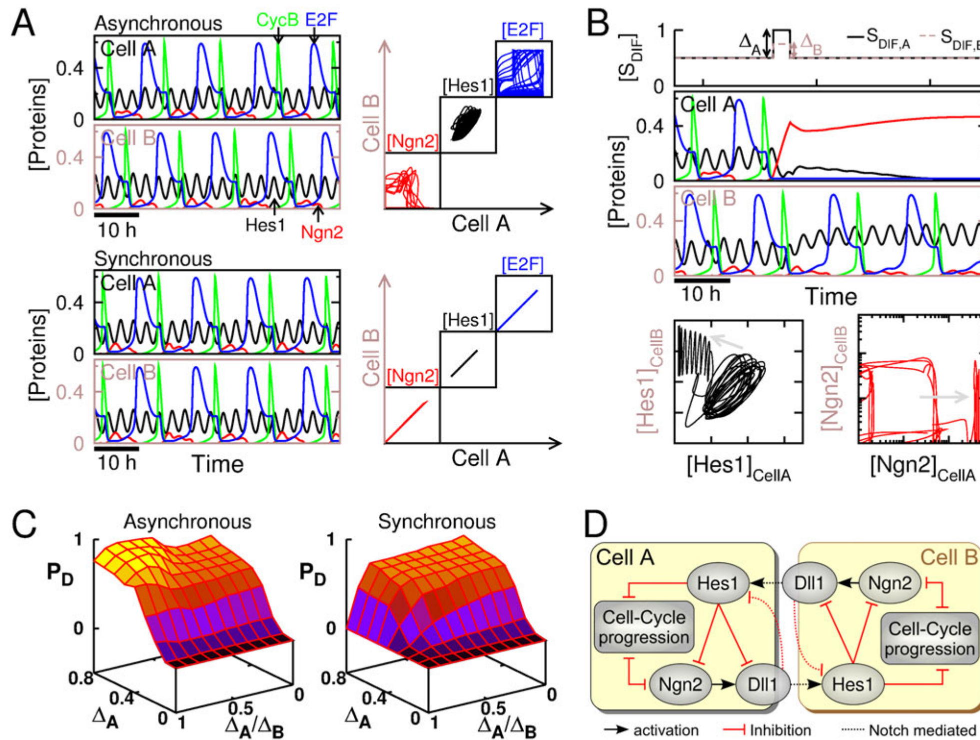

Fig. 5. Coordination between Hes1 dynamics, cell cycle progression and commitment to differentiation in Delta/Notch (DN)-connected cells. Simulations were performed with the two-cell model (Fig. 1A). (A) Simulations of protein concentration dynamics for two proliferating DN-connected cells ($[S_{DIF}]$=0.5 for both cells) represented as a time plot (left) or as a two-cell correlation plot (right). The upper and lower panels show two examples in which the cellular dynamics are asynchronous and synchronous, respectively, depending on whether the initial conditions are the same or very different. (B) Simulations of protein concentration dynamics during asymmetric fate decision of two proliferating DN-coupled cells, represented as a time plot (top) or as a two-cell correlation plot (bottom): cell A receiving a pulse Δ_A commits to differentiation, whereas cell B receiving a pulse Δ_B keeps proliferating. (C) Differentiation probability P_D for dynamically asynchronous cells (left) versus dynamically synchronous cells (right) as a function of the $[S_{DIF}]$ pulse amplitude Δ_A and the ratio of pulse amplitude Δ_A/Δ_B. Probability is measured over 100 trials characterized by an uniform random distribution of basal $[S_{DIF,0}]$ (between 0.45 and 0.55) and of signal timing over one cell cycle and with τ_Δ=3 h. (D) Schematic representation of the lateral inhibition mechanism mediated by DN signaling. Whereas Hes1 inhibits Ngn2 and Dll1 expression in one cell, Dll1 upregulates Hes1 expression in the adjacent cell by activation of Notch signaling, which implements mutual antagonisms between Hes1, Ngn2 and cell cycle dynamics in the two DN-connected cells, and between Ngn2 and Hes1 in the same cell.

supplementary material Fig. S3C). When the two cells display asynchronous dynamics, they are indeed much more likely to differentiate in response to similar levels of Δ ($\Delta_\mathbf{A}\sim\Delta_\mathbf{B}$) than when their dynamics are synchronous. Both the stabilization of Ngn2 and Hes1 at opposite levels in differentiating cells and the propensity of two Hes1-asynchronous DN-connected cells to undergo efficient asymmetric fate decision can be explained by a lateral inhibition mechanism mediated by DN signaling (Fig. 5D). Whereas Hes1 inhibits Ngn2 and Dll1 expression in one cell, Dll1 upregulates Hes1 expression in the adjacent cell by activation of Notch signaling, which implements a mutual antagonism between the Hes1 dynamics in the two DN-connected cells and between Hes1 and Ngn2 in the same cell. This dual mutual antagonism, owing to which commitment to differentiation in one cell represses Ngn2-dependent differentiation in the adjacent DN-connected cell, is responsible for asymmetric cell fate decision provided, however, that the two DN-connected cells have a different initial inclination for differentiation. If indeed the two DN-connected cells display synchronous Hes1, Ngn2 and cell cycle dynamics and receive the same level of differentiation signals, both cells might start committing to differentiation while struggling to inhibit the differentiation of the other cell, which would result in a slowing down or failure of differentiation in both cells (supplementary material Fig. S3C). Such a lateral inhibition mechanism does not preclude symmetric differentiation, which remains possible if the two cells are exposed to strong and persistent $[S_{DIF}]$ signals that disrupt DN signaling between the two cells (supplementary material Fig. S3B).

Overall, these results suggest that Hes1 oscillations and Notch-mediated lateral inhibition cooperate to settle heterogeneous Hes1 and cell cycle dynamics between proliferating neural progenitors and to promote asymmetric cell fate decisions, but also to improve the reliability of commitment to differentiation.

DISCUSSION

The primary challenge of this work was to attempt to extract the pattern of connections among the cell cycle machinery, Notch-Hes1 signaling and differentiation signals that multipotent stem cells need to develop in order to acquire the capacity to generate a plethora of differentiated cell types. The present theoretical approach reveals regulatory principles that could explain many aspects of cell fate determination in neural progenitors, including the tight coordination between cell cycle events (progression, lengthening and exit) and commitment to differentiation (Dehay and Kennedy, 2007; Lange and Calegari, 2010; Hardwick and Philpott, 2014) and the implication of oscillatory Notch-Hes1 signaling in cell fate decisions (Kageyama et al., 2009; Imayoshi et al., 2013).

It emerged that three main rules need be respected: (1) Notch-Hes1 signaling and prodifferentiation factors should differentially control G1-phase progression such as to promote two distinct cell

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cycle arrest states in early and late G1, respectively; (2) G1-phase regulators and differentiation factors should mutually interact relatively specifically so that G1-phase lengthening and the accumulation of differentiation markers, on the one hand, and irreversible cell cycle exit and terminal differentiation, on the other hand, would coordinately take place; (3) Hes1 expression should oscillate in order for mitogen-stimulated progenitor cells to progress through the cell cycle, although with the possibility to switch from the proliferative state to either one of the two competing (*Hes1*-mediated and *Ngn2*-mediated) cell cycle arrest states upon context-specific cues, notably from DN intercellular signaling.

The detailed structure of the model strictly applies to the case of *Ngn2*-dependent neurogenesis. However, many other tissue-specific differentiation factors also regulate cell cycle progression and are repressed by Hes1 (Walsh and Perlman, 1997; Kuroda et al., 1999; Ishiko et al., 2005; Papetti et al., 2010), suggesting that the above rules might apply more generally. In addition, the pattern of connections among components of the cell cycle machinery and differentiation pathways could conceivably differ from one differentiation factor to another even within the same tissue, as evidenced by the fact that the proneural factor Mash1 does not impede cell cycling in exactly the same way as Ngn2 (Castro et al., 2011; Imayoshi et al., 2013). This ability of multipotent progenitor cells to develop many subtly differing patterns of connections between the cell cycle machinery and a selected differentiation pathway could allow for the generation of the numerous cell subtypes that characterize a mature tissue.

Biphasic G1-phase organization is a requisite for binary cell fate decisions

Proliferating cells can withdraw from the cell cycle, generally during G1 phase, in response to a wealth of antimitogenic cues, although they may use different strategies to stop cycling and commit to diverse cell fates. This ability of mammalian cells to retire into different G1 arrest states has been proposed to take root in the biphasic organization of their G1-phase regulatory network, which consists of two flexibly coupled cyclin D-Cdk and cyclin E-Cdk modules (Pfeuty et al., 2008; Pfeuty, 2012) that are differentially regulated by the Ink4 (Cdkn2a) and Cip/Kip CKI families, respectively (Sherr and Roberts, 1999). Remarkably, during neurogenesis the products of the Notch target gene *Hes1* and of the proneural gene *Ngn2* preferentially restrain the activity of the upstream and downstream G1-phase regulatory module to induce an early and late G1-phase arrest, respectively.

Both Hes1 and Ngn2 can delay and even stop cell cycle progression by downregulating cell cycle activators such as *cyclin D1*, *E2* and *A2*, *E2F1* and *Myc*. Yet, because Hes1 also represses Cip/Kip transcription and Cip/Kip proteins restrain G1-phase progression by inactivating cyclin E-Cdk2, Hes1-overexpressing cells are expected to undergo an early G1/G0-like arrest, owing to *cyclin D1* repression and the ensuing inactivation of the cyclin D-Cdk module. Furthermore, because Hes1 also represses the transcription of prodifferentiation factors, Hes1-mediated cell cycle arrest should be incompatible with differentiation (Baek et al., 2006).

Intriguingly, however, steady Hes1 overexpression promotes differentiation in certain conditions, e.g. in cultured mouse embryonic stem cells (ESCs) (Kobayashi et al., 2009) or in glial-restricted progenitor cells (Wu et al., 2003). In the first case, Hes1 oscillation was driven by LIF and BMP signals, but not by Notch signaling. The authors showed that Hes1-high and Hes1-low ESCs were prone to differentiate into mesodermal and neuroectodermal lineage-restricted cells, respectively. Thus, neural progenitors that derive from ectodermal cells and have lost pluripotency would be induced to quiescence instead of adopting a mesodermal fate upon steady Hes1 overexpression. Upon incorporation of a distinctive Hes1-dependent regulation of mutually antagonistic prodifferentiation factors, the present model could be adapted to account for the selection of two distinct partially or terminally differentiated fates by low and high Hes1 expression levels, respectively. The model predicts that the Hes1-high and Hes1-low differentiation fates would be associated with two distinct G1 arrest states, in early and late G1. In line with this hypothesis, the capacity of human ESCs to select between endoderm and neuroectoderm specification has been shown to depend on the exact G1 period at which they initiate differentiation, although the role of Hes1 in this context has not been examined (Pauklin and Vallier, 2013). In the second example, Notch-mediated Hes1 overexpression drove preferential differentiation to astrocytes over oligodendrocytes from tripotent glial progenitors but not from multipotent neuroepithelial cells. These two examples suggest that the outcome of Hes1 overexpression on cell fate determination depends on developmental stage-specific epigenetic programs and signaling contexts.

In contrast to Hes1, Ngn2 does not oppose the Cip/Kip proteins, but is stabilized by their binding (Nguyen et al., 2006) and may activate their transcription (Farah et al., 2000). Furthermore, Ngn2 has a short half-life and its stabilization by DNA binding is inhibited following its phosphorylation by cyclin-Cdk2/1 (Ali et al., 2011). Although Ngn2 alone can exert a brake on early G1-phase progression by repressing *cyclin D*, *E1* and *E2* transcription (Lacomme et al., 2012), concomitant Ngn2 and Cip/Kip overexpression coincident with irreversible cell cycle exit is only observed at a later differentiation stage, in nascent neurons (Gui et al., 2007). The present model accounts for such an intricate collaboration between Cip/Kip and Ngn2, in which early Ngn2 accumulation promotes the expression of neural differentiation markers and early G1-phase lengthening before activating a positive-feedback loop with the emerging Cip/Kip proteins, which sets up an irreversible and robust cell cycle exit associated with terminal differentiation.

The present results, together with the aforementioned work, challenge the cell cycle length hypothesis which claims that G1-phase lengthening is a cause rather than a consequence of differentiation (Lange and Calegari, 2010), as they show that cell cycle lengthening and withdrawal and neuronal differentiation are separable processes that proceed coordinately as developmental programs unfold because of the peculiar connection pattern among components of their respective regulatory networks. Nevertheless, G1-phase lengthening can certainly provide undetermined cells with a broader temporal window for sensing and integrating a greater number and variety of transient differentiation-inducing cues, thereby expanding their repertoire of attainable fates.

Developmental cell fate decisions through oscillatory dynamics and lateral inhibition

The periodic formation of somites from the presomitic mesoderm (PSM) in the vertebrate embryo is given rhythm by the transcriptional oscillations of Hes/Her family members (Dequéant and Pourquié, 2008; Oates et al., 2012). This finding originally led to the proposal that periodic *Hes1* transcription would function as a molecular oscillator (or segmentation clock) during somitogenesis (Palmeirim et al., 1997). Subsequently, cyclic expression of *Hes1* mRNA and Hes1 protein was also shown to occur in non-PSM cells, notably in neural progenitors (Hirata et al., 2002; Shimojo et al., 2008), although the exact function of *Hes1* oscillations in the latter

systems was not as apparent. The prevailing hypothesis in the developing nervous system was that *Hes1* oscillation maintains a pool of proliferating neural progenitors by mutual activation of Notch signaling between neighboring cells via the ability of Hes1 to induce oscillations of the proneural gene *Ngn2* and of the Notch ligand *Dll1*. The present theoretical approach brings to light that Hes1 oscillation actually keeps in balance a labile proliferation state and two competing cell fate outputs associated with distinct Hes1-high and Hes1-low cell cycle arrest states, the outcome of which depends on the relative sensitivity of neighboring cells to extracellular signaling cues stabilizing Hes1 expression at either high or low levels. Hes1 oscillation thus maintains a highly dynamic precommitment state that is well-equipped with a wealth of decision-making properties, such as decision gates through signal timing sensitivity (Schultz et al., 2013; Pfeuty and Kaneko, 2014) or population-level decisions finely tuned by synchronization/desynchronization mechanisms (Lewis, 2003; Suzuki et al., 2011; Wang et al., 2011). For the latter, lateral inhibition constitutes a powerful intercellular coupling mechanism for promoting reliable cell fate decisions while maintaining cell type diversity (Kageyama et al., 2008).

In conclusion, this work supports the notion that the multipotent stem cell state is characterized by the oscillatory expression of synergistically and antagonistically acting transcription factors and that commitment to a given differentiation state entails the selective stabilization of a limited set of these factors by well-defined locally provided cues, in compliance with a predetermined developmental program. Hes1 oscillatory activity would conduct the timely orchestration of this program by dynamically controlling the balance between self-renewal and binary cell fate decisions at both single- and multi-cell levels.

MATERIALS AND METHODS

Model outline

The network diagram depicted in Fig. 1A translates into a deterministic mathematical model comprising: (1) a set of 22 differential and steady-state equations for the temporal evolution of species concentrations in a single cell (see supplementary material Table S1); (2) a set of reaction rates of synthesis, degradation, activation and inactivation processes governed by mass action law kinetics with eventual cooperativity (supplementary material Table S2); and (3) a set of 98 kinetic parameters (supplementary material Tables S3 and S4). Numerical integration of the differential equation system is performed using a Runge-Kutta 4 integration scheme.

Selection of model variables and their interactions

The model is based on the coupling between cell cycle regulatory pathways and Notch-Hes1 signaling pathways. Several assumptions have been made: (1) the model incorporates the main cell cycle regulators and their direct interactions, whereas the participation of components such as Ink4, Skp2, Plk1, Wee1 or Cdc25 in cell cycle regulation is taken into account through the use of effective interactions, e.g. the self-activation of cyclin B-Cdk1 or the degradation of E2F, cyclin E and Cdh1 by cyclin A-Cdks (which display an activity profile similar to SCF-Skp2 ubiquitin ligase); (2) no discrimination is made between the different members of the E2F, Rb or Cip/Kip families of cell cycle regulators, which are pooled into a single entity; (3) variables for mRNA species, the regulation of which is supposed to have limited impact, are omitted, except for *Hes1* mRNA, for which the timescales of transcription and degradation are crucial for Hes1 oscillatory dynamics; (4) no discrimination is made between nuclear and cytoplasmic concentrations; (5) macroscopic variables for cell size or growth rate are neglected since cell growth in multicellular systems is supposedly driven by signaling and cell cycle dynamics. Compared with other influential models of the mammalian cell cycle (Novak and Tyson, 2004; Gérard and Goldbeter, 2009), the specificity of the present model resides in the detailed description of G1-phase regulation that takes into consideration the peculiar biphasic organization of the G1 phase (relying on the two-step phosphorylation of Rb), while retaining a relatively simple description of the other phases. In the context of neural progenitors, the G1 phase is indeed a sensitive period of the cell cycle during which cells make important fate decisions in response to manifold cell division and cell differentiation signals.

Selection of model parameter values

The lack of experimental data regarding the reaction kinetics raises the difficult problem of estimating reasonable parameter values for model design. However, the number of parameters to be estimated could be reduced from 98 to 53 using normalization techniques (see caption of supplementary material Table S4), experimentally provided protein half-lives (supplementary material Table S3) and assuming similar kinetics rates for (1) the phosphorylation of Ngn2 and Cip/Kip proteins by cyclin-Cdk1,2, (2) the hyperphosphorylation of Rb by cyclin E,A-Cdks and (3) the binding and unbinding between cyclin D,E,A-Cdks with Cip/Kip proteins. The remaining parameters values (supplementary material Table S4) have been chosen based on qualitative properties of cell cycle progression, such as the specific profile of accumulation and activity of each regulator in the distinct phases of the cell cycle, as well as the duration of each phase in agreement with observations in mouse neural stem cells (Takahashi et al., 1993). Furthermore, all cell cycle variables must reach some maximal and minimal values on both sides of a threshold $\theta \sim 0.2$ in normalized units. Finally, the arbitrary choices of certain model parameters are *a posteriori* validated through parameter sensitivity analysis of the modeling results (supplementary material Fig. S4).

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Competing interests

The author declares no competing or financial interests.

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Supplementary material

Supplementary material available online at
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