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Re-cycling paradigms: Cell cycle regulation in adult hippocampal neurogenesis and implications for depression

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Abstract:	Since adult neurogenesis became a widely accepted phenomenon, much effort has been put in trying to understand the mechanisms involved in its regulation. In addition, the pathophysiology of several neuropsychiatric disorders, such as depression, has been associated with imbalances in adult hippocampal neurogenesis. These imbalances may ultimately reflect alterations at the cell cycle level, as a common mechanism through which intrinsic and extrinsic stimuli interact with the neurogenic niche properties. Thus, the comprehension of these regulatory mechanisms has become of major importance to disclose novel therapeutic targets. In this review, we first present a comprehensive view on the cell cycle components and mechanisms that were identified in the context of the homeostatic adult hippocampal neurogenic niche. Then, we focus on recent work regarding the cell cycle changes and signaling pathways that are responsible for the neurogenesis imbalances observed in neuropathological conditions, with a particular emphasis on depression.

Dear Dr. Bazan,

Enclosed please find the revised version of the manuscript "Re-cycling paradigms: cell cycle regulation in adult hippocampal neurogenesis and implications for depression".

We hope you will find it suitable for publication in Molecular Neurobiology.

Sincerely,
Luísa Pinto

Below is an itemized list of alterations made, as well as clarifications in response to the previous peer-review round comments.

Reviewer #1:

Minor comments

1) *In this topic, the reviewer recommends that "since this review has a major emphasis on depression, the word should be mentioned in the title, thus distinguishing this work from other reviews discussing cell cycle/neurogenesis/pathology in general".*

We agree with the reviewer's point of view and therefore, we changed the more vague expression "pathology" by depression, in the title. The title of this revised manuscript is now: "Re-cycling paradigms: cell cycle regulation in adult hippocampal neurogenesis and implications for **depression**".

2) *In this topic the reviewer argues that when discussing the literature there is a "generally very broad use/abuse of the term "cell cycle effects", which reflects the lack of better assessments". Moreover, he suggests that since this is a very common problem of the field, we should "mention at least once the limitations of these "assessments" (e.g. BrdU incorporation after a short pulse)".*

According to the reviewer's comment, we included a short mention to the limitations of using BrdU incorporation studies to conclude about proliferation and cell cycle alterations **in page 12, first sentence**. Moreover, and upon this comment of the reviewer #1, we also found appropriate to discuss the need for alternative approaches to BrdU incorporation studies in the last section "Conclusions and further perspectives", **page 22, paragraph 1**.

Still in this comment, the reviewer suggests that we try to describe the "real experiments" rather than the "author's conclusions" when referring to previous works in order to critically re-evaluate the literature.

Regarding this suggestion of the reviewer we included some descriptions of the experiments and BrdU incorporation paradigms used in several studies discussed throughout the manuscript (e.g. **please see pages 10, 11, 12, 21**). Moreover, we further explain and discuss when appropriate, the results obtained with these experimental approaches in a more integrated perspective as suggested by the reviewer.

3) *In this point the reviewer mentions "there are short references to a few other neurological diseases beyond depression, but still the authors missed a nice and interesting link between injury (stroke) and cell cycle length".*

We followed the reviewer's suggestion and added now to the revised manuscript a short reference to stroke in the context of cell cycle deregulation in adult neurogenesis and its association with the pathogenesis of several disorders (**see page 15 of the revised manuscript**).

4) *In this topic, the reviewer suggests a reference to a review by Salomoni & Calegari 2010, regarding the "link between cell cycle (cdks/cyclins) and cell fate determinants (Notch, Shh, Wnt) in neural stem cells".*

In agreement with the reviewer's suggestion we added a small reference to the interplay between cell cycle components and cell fate determinants in neural stem cells (**first sentence of page 13 of the revised manuscript**) and cited the review paper by Salomoni & Calegari 2010. Additionally, and following this comment, we found appropriate to add a sentence further discussing the interplay between cell fate determinants and the length of the G1 phase of the cell cycle (in the context of the cell cycle length hypothesis) in **the last paragraph of page 9** of the revised manuscript.

5) *Here, the reviewer states that "Fig2 left is nearly identical to Fig1 so it would take very little to include Wnt and Notch in Fig1 and forget about that panel in Fig2. Similarly, Fig2 right will more nicely extend Fig3 when incorporated in it. As such Fig2 is entirely redundant".*

As suggested by the reviewer, both alterations in the Figures were performed in the revised manuscript. More specifically:

- **Fig. 1** of the revised manuscript: incorporates the cell cycle and signaling components identified in the adult hippocampal neurogenic niche (merge of the former Fig.1 and Fig.2 left panel);
- **Fig. 2a** of the revised manuscript: includes now the former Fig. 2 right panel (adult hippocampal neurogenesis process);
- **Fig. 2b** of the revised manuscript: includes the former **Fig. 3, panel a** (cellular and molecular alterations observed in the hippocampal neurogenic niche of animal models of depression) with some alterations also suggested by the reviewer #2 (please see the answer to the reviewer #2, major revisions, point 2).
- **Fig. 2c** of the revised manuscript: includes the former **Fig. 3, panel b** (cellular and molecular alterations observed in the hippocampal neurogenic niche after antidepressant treatment or other stimuli) with some alterations also suggested by the reviewer #2 (please see the answer to the reviewer #2, major revisions, point 2).

6) *This topic regards the Format and text of the manuscript. In here the reviewer states "wording and English can be improved". Moreover a problem in the formatting of the*

Bibliography is addressed. Finally, it is suggested that the "figure legends are more of a book chapter in themself rather than a caption of a synthetic sketch".

Regarding the first part of the reviewer's comment we went through the entire text and improved the wording and English.

In what concerns the bibliography problem, we corrected the formatting manually, according to the "Instructions for authors" online file, as we were facing some problems with the endnote file.

Finally, as suggested by the reviewer, the Figure legends were shortened and made clearer for the readers in the revised manuscript.

Reviewer #2:

Major comments:

1) *In this topic the reviewer states that "the text under "Cell cycle regulators" and "Signaling pathways" seems to be a list of related facts" and recommends that we "try to develop the text into a logical story", making it clearer from the start why this information is important. Still in this topic, we are suggested to "shorten the more general part of the manuscript and elaborate the latter part about depression and neurogenesis".*

We think that the suggestion made by the reviewer was valuable, and thus we included a paragraph that we hope will serve to better explain why it is important to introduce the factors and pathways that have been described to regulate the adult hippocampal neurogenic niche (please see **page 8, last paragraph** of the revised manuscript). Moreover, and in agreement with the reviewer's recommendation, we shorten this more descriptive part of the text and made it more appealing to the readers. Additionally, we further elaborated the latter part about depression and neurogenesis in the revised manuscript.

2) *In this comment the reviewer recommends that, "in addition to the figures or perhaps to replace some of them", we add a "summarizing table of the different factors affecting the cell cycle". Moreover, he refers that "the figures are somewhat hard to read because of the coloring and small size".*

According to the reviewer's suggestions, we added a summarizing Table of the different cell cycle molecules/signaling pathways implicated in the regulation of adult hippocampal neurogenesis in the context of depression and antidepressant drugs/stimuli.

Additionally, in the revised manuscript, the Figures were changed in agreement to the suggestions made by the reviewer #1 (please see the answer to comment 5 of the reviewer #1). We also improved the Figures coloring (e.g. consistency of the arrows coloring in both Figures) and size (e.g. the vertical disposition of the panels in Figure 2 allowed us to resize each of the elements), to make them more perceptible to the readers, as suggested by the reviewer #2.

3) *In this topic the reviewer states that in page 18 (first version of the manuscript) the reference made to unpublished results from our lab is vague, further soliciting that we elaborate more. Moreover, he asks for a reference to published work.*

Regarding the reviewer's comment, at the time we first submitted this manuscript, these results from our lab were not yet published. However, they were recently published (January 2013), and thus we added this reference to the revised manuscript (please see the first paragraph of **page 18** of the revised manuscript). This work focuses on the role of new fully mature and incorporated neural cells in the hippocampal neurogenic niche, and its relevance for the spontaneous recovery from depression and for the actions of antidepressants, further consolidating the so-called "Neurogenic hypothesis of depression". Therefore, and regarding the pertinent comment of the reviewer, we further explored this part of the manuscript (please see the first paragraph of **page 18** of the revised manuscript).

4) *In this comment, the reviewer requests that we "discuss animal models of depression in more detail", and explain "how depression is manifested in humans vs. rodents" (Section Major Depression).*

Concerning this reviewer's comment, we introduced in the section entitled "Major Depression" more detailed information about animal models of depression (**page 16** of the revised manuscript). We also added a brief sentence in which we describe the major behavioral manifestations of the human disease (**page 15, first paragraph of the "Major Depression" section** of the revised manuscript). Additionally, as suggested by the reviewer, we discussed some of the behavioral manifestations of depressive-like behavior in rodents and approached their correlation with the human disease (**page 16, first paragraph** of the revised manuscript).

5) *In this comment, the reviewer suggests that we "elaborate on the effects of voluntary exercise on cell cycle regulation related to adult neurogenesis".*

According to the suggestion of the reviewer, we explored now in the revised manuscript the role of voluntary exercise as a pro-neurogenic and antidepressant stimulus, focusing on the changes it induces at the cell cycle and signaling level (please see **page 21, second paragraph** of the revised manuscript).

6) *In this topic the reviewer states that the manuscript "language is hard to understand, mostly because of the structure of the sentences". Therefore, he suggests we "try simplifying the expressions avoiding overly long and complicated sentences". Finally, he suggests that the manuscript needs to be proof-read by a native English speaker.*

We followed the reviewer's advice, and revised all the text to make it clearer to the readers, namely concerning the structure of the sentences and their size.

Moreover, the text was proof-read as suggested by the reviewer, and the appropriate changes were performed.

Minor comments:

1) *The reviewer asked us to add the reference by Shors et al 2012 " Use I or lose it: how neurogenesis keeps the brain fit for learning".*

As requested by the reviewer we included in the revised manuscript the reference of *Shors et al 2012* in the corresponding sentence (please see **page 4** of the revised manuscript).

2) *The reviewer asked us to add the reference by Gu et al 2012 "Optical controlling reveals time-dependent roles for adult-born dentate granule cells".*

As requested by the reviewer we included in the revised manuscript the reference of *Gu et al 2012* in the corresponding sentence (please see **page 6** of the revised manuscript).

3) *The reviewer asked us to add the reference by Nokia et al 2012 "Learning to learn: theta oscillations predict new learning, which enhances related learning and neurogenesis".*

As requested by the reviewer we included in the revised manuscript the reference of *Nokia et al 2012* in the corresponding sentence (please see **page 6** of the revised manuscript).

4) *The reviewer suggested changing the expression "physiological and pathological conditions" by another less vague expression.*

We followed the reviewer's suggestion and replaced the expression "physiological and pathological conditions" by others more suitable (e.g. Physiological: basal conditions, homeostatic; pathological: disease throughout the revised manuscript. Please see **pages 3 ,4, 7, 14 and 23** of the revised manuscript for examples.

5) *The reviewer suggested that since "this review mostly concerns depression and not much is said about other types of pathology", we should change the title*

We agree with the reviewer's point of view and thus changed the expression "pathology" by "depression", in the title, as also suggested by the reviewer #1. The title of this revised manuscript is now: "Re-cycling paradigms: cell cycle regulation in adult hippocampal neurogenesis and implications for **depression**".

Re-cycling paradigms: Cell cycle regulation in adult hippocampal neurogenesis and implications for depression

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ABSTRACT

Since adult neurogenesis became a widely accepted phenomenon, much effort has been put in trying to understand the mechanisms involved in its regulation. In addition, the pathophysiology of several neuropsychiatric disorders, such as depression, has been associated with imbalances in adult hippocampal neurogenesis. These imbalances may ultimately reflect alterations at the cell cycle level, as a common mechanism through which intrinsic and extrinsic stimuli interact with the neurogenic niche properties. Thus, the comprehension of these regulatory mechanisms has become of major importance to disclose novel therapeutic targets. In this review, we first present a comprehensive view on the cell cycle components and mechanisms that were identified in the context of the homeostatic adult hippocampal neurogenic niche. Then, we focus on recent work regarding the cell cycle changes and signaling pathways that are responsible for the neurogenesis imbalances observed in neuropathological conditions, with a particular emphasis on depression.

KEYWORDS

Cell cycle, cell signaling, adult hippocampal neurogenesis, depression

Introduction

The view that no new neurons can be added to the adult brain was deconstructed over the past years. Neurogenesis in the adult mammalian brain is now a widely accepted neuroplastic event [1-3] that enables the brain to adapt to intrinsic and extrinsic stimuli. In fact, during the past few years, a large amount of data has provided evidence that the production, differentiation and survival of neurons in the adult brain have significant implications for several physiological processes, such as memory and learning [4-6]. Moreover, many studies have linked neurogenesis deregulation with the emergence of several pathological features in neuropsychiatric disorders. However, the role of neurogenesis in these disorders is yet to be completely established. Presently, much effort is devoted to the generation of behavioral and molecular data that establish a mechanistic link between neurogenesis and disease-state, so that ultimately directed therapeutic interventions can be designed.

Adult stem cells are responsible for tissue integrity, by adding new cells to the networks or by promoting the capacity to repopulate mature differentiated tissues as their constituting cells die due to damage or degeneration. The complex process of producing new cells throughout an organism's lifespan may rely on a common denominator - the cell cycle regulatory machinery (Fig. 1). However, the specificity of this transversal phenomenon in each tissue or cell type offers a wide spectrum of responses to a particular stem cell niche. In this review we aim to provide a comprehensive and integrated view of the cell cycle regulation in the adult hippocampal neurogenic niche both in basal conditions and in disease (namely in depression). With this, we offer a perspective on how the cell cycle machinery may constitute an interesting and still largely unrecognized link between alterations in postnatal hippocampal neurogenesis

and disease, highlighting its relevance for the discovery of new molecular targets for the treatment of neurobiological disorders.

Neurogenesis in the adult mammalian brain

Adult neurogenesis is the process by which neural progenitors divide mitotically to produce new neurons in the adult brain. This complex process involves several steps beyond cell division; namely, the commitment of the new cell to a neuronal phenotype, the migration and maturation of the cells, and the establishment of appropriate synaptic contacts that culminate with a full integration on the pre-existent network. Well described, adult neurogenesis is known to occur at least in two different regions of the mammalian brain: the subependymal zone (SEZ) of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) [7]. In both regions, astroglial cells act as the source of adult progenitor cells [8-9]. The neuroblasts born in the SEZ migrate along the rostral migratory stream (RMS) becoming mostly mature GABAergic granule and periglomerular interneurons in the olfactory bulb (OB). The cells born in the adult SGZ migrate into the granular cell layer (GCL) of the DG and differentiate into glutamatergic granule cells [7]. Additionally, although disputable [10-11], several reports describe the generation of new neurons in other regions of the adult brain, including the cortex [12-13], the amygdala [14-16], the hypothalamus [17-18], the striatum [19-20] and the substantia nigra [21-22]. However, in all these areas, neurogenesis appears to occur at very low levels or under non-physiological conditions [23].

Adult hippocampal neurogenesis

Neurogenesis in the adult DG occurs from a progenitor population residing in a narrow layer of about three nuclei wide, the SGZ. The first type of progenitor cells, defined as multipotent, are the neural stem cells (NSCs or Type-1 cells). These cells express nestin and glial fibrillary acidic protein (GFAP), among other markers, and can be divided in two subtypes based on their orientation in the SGZ: radial astrocytes/NSCs (rA) and horizontal astrocytes/NSCs (hA). Radial NSCs, morphologically characterized by having a single radial process, are slow dividing cells, whereas horizontal NSCs have a short horizontal process and divide faster than rA [24-25]. Either one or both of these cell subtypes will then divide asymmetrically into one daughter cell and one progenitor cell, both of which already committed to a neuronal lineage. These latter cells, designated by transit amplifying neural progenitors (tANPs, or Type-2 cells), are mitotically active and divide to give rise to neuroblasts (also known as Type-3 cells). This last stage corresponds to a transition from a slow proliferating neuroblast, which is exiting the cell cycle, to a postmitotic immature neuron, that will migrate a short distance into the GCL. Neuroblasts transiently express markers of the neuronal lineage, such as the calcium-binding protein calretinin, doublecortin (DCX) and polysialylated-neural cell adhesion molecule (PSA-NCAM) [10,26]. The newborn cells will then fully maturate into granule neurons, elongating their axons towards the hippocampal Cornus Ammonis 3 (CA3) area [23] and making the appropriate axonal connections [26]. These adult-born neurons become integrated in the pre-existing neuronal network 4 to 8 weeks after their birth [3,27-29] (Fig. 2a).

Importantly, not all cells expressing immature neuronal markers develop into fully mature neurons [30]. In fact, if not recruited to perform any function, the great majority of these newly-born cells are eliminated by apoptosis once they exit cell cycle [31-32]; a

mechanism that until recently was largely undescribed. However, a recent report by Lu *et al*. [33], has very elegantly shown that DCX-positive neuronal progenitors present a phagocytic activity in the DG as well as in the SEZ, with important implications for the neurogenic process [33].

The generation of new neurons in the hippocampal niche of the adult brain, depends on the harmonization of several processes and cellular activities, which include proliferation, cell cycle exit, activation of survival/death pathways, migration through the GCL and differentiation/maturation of the newborn neurons [34]. These processes are regulated by both intrinsic and extrinsic factors that are ultimately responsible for the modulation of the neurogenic phenomenon. While there are numerous focused studies on several of these steps, little is known about the cell cycle regulation in the context of adult hippocampal neurogenesis and its repercussions for disease states. Here, to provide an integrated view, we will consider the "expanded cell cycle" [35], taking into consideration some of the most well-described mitogenic signals and the interacting signaling pathways.

Cell cycle regulation in the adult hippocampal neurogenic niche

Providing important clues on the regulation of the adult neurogenic process, the expression of cell cycle proteins and their regulation have been extensively explored in the context of embryonic development [36-40]. On one hand, some of the mechanisms are common to both embryonic and adult brain; however, there are essential differences between them especially regarding niche properties. Whereas during development, the cellular environment is highly specialized to support proliferation, in the adult hippocampus the environmental context includes a population of fully mature and functionally active neurons [7,37], thus providing a different set of both intrinsic and

extrinsic signals. In fact, in the adult mammalian brain, the vast majority of neuronal cells are in a quiescent differentiated state (G0 phase of the cell cycle), which is probably promoted by an increase in the expression of region-specific Cdk inhibitors [34,41-42]. Nonetheless, the expression of cell cycle proteins in the postnatal brain and their definite role in this neurogenic niche are still being unveiled [36].

Contrary to the traditional concept that postmitotic mature neurons are stably maintained in a quiescent differentiated state, recent, albeit still controversial, evidence has demonstrated that in some disease conditions, such as Alzheimer's disease [43-44], traumatic brain injury [45] and cerebral hypoxia-ischemia [46], these cells are capable of responding to mitogenic signals and reenter the cell cycle [35,47]. However, apparently these neurons neither finish dividing nor revert to their G0 quiescent state, ultimately undergoing apoptotic cell death and suggesting that they lack the factors needed for cell cycle progression [35].

It is important to mention, at this point, that the expression of cell cycle proteins in neuronal populations is not always associated with cell proliferation or cell cycle reentry. Indeed, a small number of studies have demonstrated that the expression of key cell cycle components may be associated with other neuronal processes, such as neuronal migration, dendrite morphogenesis, synaptic maturation and plasticity [48-49]. Nonetheless, a few cell cycle molecules and signaling pathways have been implicated in the regulation of adult hippocampal neurogenesis. Moreover, their deregulation is often the cause for neurogenesis imbalances observed in several disorders, such as depression. As such, we will first provide a brief overview on these molecules and its functions in the context of adult hippocampal neurogenesis.

Cell cycle regulators

The cell cycle consists of a succession of events that lead to cell division. It comprises four distinct consecutive phases: the first gap (G1) phase, during which cells prepare for DNA replication in the synthetic (S) phase, followed by a second gap (G2) phase and mitosis (M). A highly coordinated network of molecules mediates progression through these four phases. There are two major classes of cell cycle regulators that cooperate in order to promote cell cycle progression: cyclins and cyclin-dependent kinases (Cdks). Cdks are serine/threonine kinases stably expressed during cell cycle progression that must bind to cyclins, their regulatory subunits, whose expression levels vary throughout the cell cycle phases, to form active catalytic heterodimers [50-51]. Each Cdk is able to associate with different cyclins, which will in turn determine the proteins to be phosphorylated by a specific Cdk-cyclin complex (Fig. 1).

Several studies support the view that most cell cycle regulators are functionally redundant [52] and the need for a particular molecule is dependent on the cell type and on the niche [53]. This holds also true for the hippocampal neurogenic niche. As an example, studies on the role of Cdk4 and Cdk6 in the adult hippocampus unraveled a crucial role for Cdk6, but not Cdk4, in controlling the expansion of neuronal committed progenitors and thus the rate of neuronal production [54]. In fact, the absence of Cdk6 was shown to induce the lengthening of the G1 phase causing premature cell cycle exit and differentiation [54]. These findings lead to the "cell cycle length hypothesis" [55], which states that proliferative divisions exhibit a short G1 phase whereas neurogenic divisions are characterized by longer G1 phases. In a molecular perspective, it is proposed that the ability of a cell fate determinant to induce any cellular response is related with the time it has to act during G1 [55-56]. Additionally, overexpression of the Cdk4-cyclin D1 complex in the adult mouse hippocampal niche was shown to increase

the expansion of neural progenitor cells, in a specific and cell-autonomous manner, while inhibiting neurogenesis [57]. Indeed, stopping the overexpression of the Cdk4-cyclin D1 complex was accompanied by an overproduction of new neurons, corroborating its effect on the expansion of the neural progenitors at the expense of neuronal differentiation. Moreover, no effects were observed in the survival and maturation patterns of DCX+ immature neurons [57]. Altogether, results suggest that the Cdk4-Cyclin D1 complex is able to decrease the cell cycle length of cells that characteristically present longer cell cycles, whereas it is not able to change the short-length cycles [57]. In line, it is proposed [55,58] that beyond the cell cycle length itself, it is the length relative variation that may be underlying the changes in the fate of a given cell [57]; yet, more studies are needed to clarify this subject.

Cyclins, another important class of cell cycle regulators, are also implicated in the adult neurogenic phenomenon. Indeed, three types of cyclins D (D1, D2 and D3), which control Cdk4/6 activity, were already identified in mammals [59]. Although most cells express more than one cyclin D, several studies have demonstrated cell type-dependent roles for each of them [60-62]. One paradigmatic example of such specificity in the adult brain is the demonstration that cyclin D2, but not cyclin D1, knock-out (KO) mice, have a marked reduction of cell proliferation in the DG, measured by BrdU incorporation [59]. Other studies have corroborated this inability of cyclin D1 to promote neurogenesis in the hippocampus in the absence of cyclin D2 [63-64].

Cell cycle progression is also negatively regulated, at a post-translational level, by two families of cyclin-dependent kinase inhibitors: the Inhibitor of kinase 4/Alternative reading frame (Ink4/ARF) family and the CDK inhibitory protein/Kinase inhibitory protein (Cip/Kip) family. These intracellular proteins are responsible for slowing or arresting the progression through the cell cycle, by blocking imperative events. The

expression pattern and function of some of these Cdk inhibitors in the context of adult neurogenesis have also been characterized. P27kip1 (p27) is an important Cdk inhibitor, that induces cell cycle exit in proliferative cells [65]. In accordance, p27 expression decreases when cells are exposed to mitogenic signals, allowing their entrance in the S phase [66]. In the context of adult hippocampal neurogenesis, p27 protein is expressed in the SGZ of mice [67] and rats [64]; interestingly, many of the p27 cells co-express DCX in this region [67]. *In vitro* assays, using cultured neural progenitor cells (NPCs), showed rapid increases in p27 expression following differentiation by growth factor withdrawal, thus confirming its role in cell cycle arrest and NPCs differentiation [67]. Additionally, deletion of p27 promoted an increase in the proliferative pool of NPCs [67], in accordance with previously published results in the SEZ [68]. These data were corroborated by *in vivo* assays showing an increased number of BrdU positive cells in the SGZ and in the SEZ of p27 KO mice. These results, together with the increased levels of proliferating cell nuclear antigen (PCNA) expression in the KO animals, further suggest that the absence of p27 promotes NPCs proliferation in both adult neurogenic niches [67]. Adding to the p27 studies, Pechnick *et al.* carried out two separate studies exploring the function of p21cip1 (p21) in the mouse hippocampus [69-70]. Using a BrdU incorporation paradigm (1 injection every two hours in a total of three injections, and sacrifice 24h after the first injection), the authors showed, both *in vivo* and *in vitro,* that the absence of p21 lead to an increased proliferation of hippocampal neurons. Ultimately, the work suggests that p21 is responsible for blocking cell cycle progression in the adult hippocampal SGZ [69-70]. Interestingly, p21 expression is restricted to neuronal committed progenitors [70], unlike p27 that reveals no distinction on lineage preference [67]. Strikingly, the results are not in agreement with a previous report in which p21 deletion showed no impact on the proliferation of

neural progenitors in basal conditions [71]. However, it is worth mentioning that a different BrdU incorporation paradigm was used in this latter study. Indeed, the use of BrdU incorporation approaches has limitations as a direct measure of proliferation because does not always discriminates among effects that may underlie an increased S phase labeling; for example, G1/G2 phase shortening, increase in the growth fraction and lengthening of S phase [56]. The appropriate controls, the use of additional thymidine analogs and endogenous markers of proliferation should improve the analyses and may give a broader picture on the cell cycle regulatory mechanisms.

A final word to mention E2F1, an element that is part of a broad family of transcription factors involved in the regulation of cell cycle progression [42], but also with important roles in the adult neurogenic process. Contrary to what is described for most members of this family, E2F1 has been reported to induce cell death, by forcing postmitotic cells to re-entry cell cycle [42,72]. In the context of adult neurogenesis, E2F1 was shown to be important for cell proliferation and differentiation. Using a single BrdU injection paradigm, two hours before sacrifice, Cooper-Kuhn *et al.* showed that E2F1-deficient mice have decreased cell proliferation and diminished neurogenesis, both in the hippocampal DG and the SVZ [72]. These authors also described a decrease of about 60 to 70% in apoptotic cells, in the hippocampal neurogenic niche of E2F1-deficient mice compared to wild-type (WT), further corroborating the role of this gene in regulating cell death in the context of adult neurogenesis [72]. Fig. 1 depicts the cell cycle regulators described in the context of adult hippocampal neurogenesis.

Signaling pathways

Cell cycle entry promotion and initial progression through G1 phase is induced by mitogens or growth factors present in the extracellular environment [64,73-74]. The

interplay between cell cycle regulation and cell fate determination is also a topic of great relevance [56]. In particular, the G1 phase length is crucial for the switch from proliferation to differentiation and is modulated by cell cycle regulators and cell fate determinants [56]. Thus, signaling from the niche is suggested to be responsible for key processes in the regulation of adult neurogenesis homeostasis, including: the balance between quiescence versus proliferation, the mode of cell division, and the prevention of stem cell depletion [75-76]. In this section we will briefly describe some of the signaling pathways activated in the adult hippocampal neurogenic niche.

The role of Notch signaling in NPCs in the adult hippocampus was investigated *in vivo* through inducible gain- and loss-of-function experiments. Activated Notch1 overexpression induced proliferation of endogenous progenitors, whereas inhibition or ablation of Notch1 signaling promoted cell cycle exit, inducing the transition from neural stem or progenitor cells to transit-amplifying cells or neurons [77]. On the other hand, in maturing neurons, Notch1 proved to be relevant for survival and structural plasticity modulation [77].

Bone morphogenetic proteins (BMPs) are other key regulatory components of the adult hippocampal neurogenic niche, restricting the proliferation of the stem cell pool, through BMP receptor-IA (BMPR-IA) activation, and thus maintaining the equilibrium between stem cell proliferation and quiescence [75]. Downregulation of endogenous BMP signaling promoted an increased proliferation of SOX2+ cells by recruiting quiescent radial cells into the cell cycle. Moreover, the canonical BMP signaling pathway is reactivated shortly after neuronal fate commitment, possibly to promote cell cycle exit of newly born neurons [75].

Sonic hedgehog (Shh) is an evolutionarily conserved secreted protein that plays an important role in many aspects of developmental control [78], as well as in adult

hippocampal neurogenesis [79]. Shh signaling pathway was shown to induce a dose-dependent proliferative response in progenitors *in vitro*, whereas inhibition of Shh signaling reduced proliferation *in vivo*. These studies confirmed Shh signaling pathway as an important regulator of adult hippocampal neural progenitors [79], suggesting also its involvement in cell cycle regulation.

Like Shh, Wnt proteins are also well-known key regulators of neural stem cell behavior during embryonic development [80]. Wnt signaling has been reported as a regulator of adult hippocampal neurogenesis [81], through the activation of the proneural gene NeuroD1 [82]. Activation of NeuroD1 is important for the generation of granule cells in the hippocampus and cerebellum [83], possibly by promoting cell cycle exit.

Altogether, these data highlight the complex orchestration of the cell cycle process in the context of adult hippocampal neurogenesis, as well as the interplay between cell cycle regulators and upstream molecular signaling pathways. Interestingly, there is a certain level of functional redundancy among cell cycle regulatory components, possibly as an evolutionary mechanism to prevent severe damage upon deficiency of one of these molecules. On the other hand, most of these studies point to tissue and cell specificity as a hallmark of these systems, proving that these regulators may operate at different levels of the cell cycle and implying the need for their fine tuning in the homeostatic control of adult hippocampal neurogenesis.

Cell cycle regulation in neuropathological scenarios

The molecular mechanisms and pathways regulating adult hippocampal neurogenesis in response to deleterious stimuli, and the contrasting actions of pro-neurogenic drugs, are still largely undisclosed. It is legitimate to consider that these alterations in adult

neurogenesis may be attributable to direct or indirect changes in cell cycle regulatory mechanisms. As such, the cell cycle machinery is possibly a convergent pathway through which intrinsic and extrinsic factors, such as stress and toxins, manifest their effects. Indeed, cell cycle deregulation in the context of adult neurogenesis has been associated with the pathogenesis of neurodegenerative disorders, such as Alzheimer's [84] and Parkinson's disease [85], neuropsychiatric diseases, as is the case of schizophrenia [86] and major depression [87], and injury, namely stroke [88-89]. These changes in cell cycle dynamics, as observed in several disease states [90-92], further reinforce the need for additional studies examining the role of core cell cycle players as targets for disruption. Next, we will briefly explore the case of depression as a paradigmatic example of how cell cycle deregulation can lead to the development of pathological traits.

Major depression

Major depression is a chronic and debilitating disease, and one of the most common psychiatric disorders in modern society. It is estimated that about 16% of the population will be affected by this disease once or several times during lifetime [93]. Like other psychiatric disorders, depression is a complex and heterogeneous clinical entity [94], dependent on the interaction between genetic susceptibility [95-96] and environmental factors [97]. Depressive patients present symptoms of depressed-mood, learned helplessness, anhedonia and impaired cognition, and present a high co-morbidity with anxiety disorders [94].

Strikingly, depression is characterized by several pathophysiological alterations in the brain such as differences in size of specific brain regions, changes in neuronal morphology, neurochemical and signaling alterations, and changes in genetic and

epigenetic regulation [98-99]. Knowledge of the etiopathogenesis of depression has progressed substantially in the last years [100], in part due to studies employing animal models. Animal models of depression use known etiological factors (etiological validity) to induce behavioral and neurobiological symptoms in animals similar to those of the human disease (face validity). Moreover, a valid animal model for the formulation of hypotheses and for the development of novel therapeutic strategies should respond to clinically effective treatments (predictive validity) [94,101]. There are several animal models of depression: chronic mild stress (CMS), social stress, early life stress, fear conditioning and olfactory bulbectomy [97]. Although none of them can fully recapitulate the complexity and heterogeneity of the human disease, they are considered robust approaches to study the depression in humans. For example, the CMS animal model presents alterations in the three behavioral domains known to be affected in depressive patients, i.e. mood, anxiety and cognition [94]. Despite this large contribution of data from studies in animal models of depression, and from post-mortem studies of human brains, the neurobiological basis of this disorder is still poorly defined. Importantly, the fact that approximately half of the patients presenting clinical depression show incomplete remission or relapse after treatment with the currently available antidepressants [97] further reinforces the need for finding new molecular targets and more efficient treatments.

There are currently several leading hypotheses that attempt to elucidate the neural and molecular mechanisms of depression. The monoamine hypothesis of depression [102] has been the most prevalent. The main support of this hypothesis is the fact that most classic antidepressants induce an increase of the serotonin and noradrenaline levels [103]. More recently, additional studies have shown that other mechanisms are implicated in the neurobiology of this disorder; this is mostly based on the observation

that other factors are altered in depressed individuals [87,104-105] and on the efficacy of new antidepressants, in which the mechanisms of action do not rely on the monoamine transmission systems [106-109]. Thus, several other hypotheses on the etiology of depression have been put forward, including: the neurotrophin hypothesis, the cytokine hypothesis, the hypothalamic pituitary adrenal (HPA) axis modulation hypothesis and the neurogenic hypothesis. Although none of these are mutually exclusive, herein we will focus mostly on the role of adult hippocampal neurogenesis and on the molecular processes that can regulate it at the cell cycle level.

The neurogenic hypothesis of depression and cell cycle (de)regulation

Studies showing reduced hippocampal neurogenesis in several animal models of depression [110-112] constitute the basis for the neurogenic hypothesis of depression. Importantly, all major classes of antidepressants [87,113], and most of the environmental factors that confer antidepressant-like behavioral effects, such as environmental enrichment [114-115], physical activity [115] and learning [7], are also known to promote hippocampal neurogenesis. These facts have lead to the proposal that neurogenesis may have a role in the etiopathogenesis of depression; however, the currently available data strongly reinforces the need for restructuring this possibly oversimplified view. Indeed, the functional implications of decreased neurogenesis for the precipitation and maintenance of the depressive state are yet to be completely established, as the experimental approaches and time frames of analysis diverge. Some studies have implicated neurogenesis in the emergence of behavioral deficits observed in animal models of depression and in the actions of antidepressants [116-118]. While, other studies showed that at least the short-term mood-improving actions of antidepressants depend on neuronal remodeling in the hippocampus and prefrontal

cortex (PFC), rather than on neurogenesis [110]. Moreover, recently published data from our lab showed that the appropriate incorporation of new cells in the adult rat hippocampus is a key factor for the long-term spontaneous recovery from depressive-like behavior as well as for the action of antidepressants [119]. Using a longer experimental time frame, to allow the full differentiation and integration of newborn cells in the pre-existing neuro-glial circuitry, it was possible to fate-map the new cells generated during antidepressants treatment and understand their impact in distinct behavioral dimensions [119]. These findings further reinforce the need for an integrated time-dependent overview of the neurogenic phenomenon with great emphasis on the functional role of newly generated cells in the adult hippocampus. Importantly, most of the stimuli affecting adult neurogenesis, are also responsible for inducing changes at the cell cycle level in the progenitor cells of the hippocampal niche. Some of the most relevant reports on cell cycle regulation in the context of adult hippocampal neurogenesis and stress-related disorders have disclosed a major role for Cdk inhibitors [64,69-70]. Heine *et al.* evaluated the role of p27 in the regulation of the cell cycle in the DG of rats following exposure to stress [64]. After three weeks of chronic exposure to unpredictable stress, rats presented significantly decreased numbers of proliferating cells, measured by ki67 immunostaining, and increased numbers of p27 positive cells in the SGZ. Notably, this effect was not observed upon acute stress exposure. Moreover, the proliferation levels returned to normal after a 3–week recovery period from chronic stress, suggesting a transient p27-dependent G1 arrest in the SGZ cells of chronically stressed animals [64]. Somehow unexpectedly, neither Cyclin-E nor Cyclin-D1 protein levels were significantly altered in these animals when compared to controls [64].

Other animal studies have focused their attention on the role of Cdk inhibitors regarding the pro-neurogenic action of antidepressants [69-70]. Pechnick *et al.* showed that naïve

mice chronically treated with imipramine, a tricyclic antidepressant, not only show increased neurogenesis in the DG but also decreased the expression of p21 Cdk inhibitor in the SGZ, when comparing to saline-treated controls [69]. In a more recent study, the same group analyzed the effects of chronic administration of other classes of antidepressants on SGZ p21 expression and neurogenesis; all antidepressants tested (fluoxetine, imipramine and desipramine) were able to specifically inhibit p21 expression in the mice DG and this effect was linked to increased neurogenesis [70]. Unexpectedly, no change was noted in p27 expression following antidepressants administration [70], possibly suggesting specific roles for each of these Cdk inhibitors following different stimuli. It is worth mentioning that Pechnick *et al.* did not include an animal model of depression in their studies, possibly accounting for these results. Together, these findings support the involvement of cell cycle molecules in the mechanistic association between stress and the action of antidepressants, in the context of neurogenesis regulation.

A recent study has implicated the cyclin dependent kinase 5 (Cdk5)/p35 complex in the development of depressive-like behavior and in the action of antidepressants [120]. Cdk5 still has no recognizable function in the progression of the cell cycle [37,47], although structurally similar to other Cdks. In fact, Cdk5 expression and activity occur almost exclusively in postmitotic neurons, both in the developing and in the adult brain [37]. This kinase works as a cell cycle inhibitor in postmitotic neurons, repressing aberrant cell cycle reentry, a phenomenon linked to the development of several neurodegenerative disorders [37,121]. Cdk5 regulation requires activators that are specifically expressed in postmitotic neurons. One of these activators is p35, a regulatory subunit that translocates from the cytosol to the membrane to induce Cdk5 activity [120-121]. In a recent study, it was reported an increased Cdk5 kinase activity

together with the translocation of p35 to the cell membrane, in the DG of rats exposed to CMS. They also observed that inhibition of Cdk5 specifically in the DG, but not in the CA1 or CA3 of the hippocampus, prevented the CMS-induced behavioral impairments, further suggesting the involvement of the Cdk5/p35 complex in the etiology of depressive-like behavior. Remarkably, p35 overexpression blocked the antidepressant behavioral effects of venlafaxine, a selective serotonin reuptake inhibitor (SSRI) antidepressant [120]. These data suggest an association between Cdk5 activity and the development of stress-related disorders [120], similarly to what has been previously described for some neurodegenerative disorders [121]. Moreover, the studies may also suggest that the effect of Cdk5 activation is attributable to the impairments typically observed in hippocampal neurogenesis induced by CMS exposure [120]. Complementary studies with analyses of cell proliferation and neurogenesis would help to better define Cdk5 function in the adult hippocampus. Figures 2b and 2c are schematic representations of the adult hippocampal neurogenesis changes observed in animal models of depression and after antidepressant treatment, and the corresponding cell cycle alterations.

Changes in the signaling pathways known to be involved in the modulation of adult hippocampal neurogenesis have also been indirectly associated with the development of depressive-like behavior in animal models. Indeed, Wnt knockdown-mediated neurogenesis ablation was shown to impair several hippocampal-dependent cognitive functions, such as long-term retention of spatial memory and object recognition memory [122]. Importantly, these cognitive behavioral deficits were linked with depression onset and maintenance [94,123]. Wnt signaling was further implicated in the actions of fluoxetine, a SSRI antidepressant; chronic treatment with this antidepressant was able to stimulate the expression of Wnt3a protein in the hippocampal DG.

However, Wnt activity appears to be preferentially implicated in fluoxetine's reported induction of neural plasticity and not in its pro-neurogenic actions [124].

Notch and BMP signaling have also been shown to be mediators of the pro-neurogenic actions of physical exercise. Physical exercise is a stimulus with recognized antidepressant effects [125-126]. Moreover, it has been consistently reported to robustly induce adult hippocampal neurogenesis, by promoting the proliferation of progenitors and the survival and maturation of newborn neurons [127-130]. More recently, some studies investigated the molecular signaling correlates of these cellular events [131-133]. Using thymidine analogs incorporation paradigms, Brandt *et al* showed that voluntary exercise (i.e. mice that had access to a running wheel) preferentially promotes the proliferation of DCX+ type-3 precursor cells and Notch1-dependent cell cycle exit. Since Notch1 is known to induce proliferation and inhibit differentiation in earlier neural progenitor cells (type-1 and 2a cells) [77], it is interesting to notice these contrasting pro-neurogenic functions in more committed progenitors [132]. Altogether the findings support the use of experimental designs that specifically address the role of molecular determinants in each hippocampal cell type. Table 1 summarizes the most relevant studies regarding the cell cycle and signaling alterations implicated in adult hippocampal neurogenesis imbalances in the context of depressive-like behavior.

Conclusions and perspectives

Sixty years after the first report of ongoing neurogenesis in the adult brain, we are now at the point of evaluating the physiological relevance of the incorporation of new

neurons in pre-existing neuronal networks. The integrated studies on adult neurogenesis in its various stages – progenitor cells proliferation, cell cycle exit, migration and differentiation – have brought new players into the complex network of factors and molecular mediators that directly or indirectly participate in the process. Nonetheless, we were not yet able to establish the precise molecular cascades that regulate the homeostasis in adult neurogenic niches. Therefore, the future of this field of research needs to build up an integrated view of the molecular processes, by specifically targeting candidate molecules using conditional approaches to overcome the limitations of full-KO models. This approach will allow the exclusion of possible compensatory mechanisms promoted during embryonic development, a strategy that seems to be of particular importance in the case of cell cycle regulators. Additionally, most of the literature on the regulation of adult neurogenesis relies on the use of thymidine analogs incorporation, such as BrdU. The use of these strategies to study cell cycle regulation in the context of adult hippocampal neurogenesis requires careful interpretation of the data. In this way, the appropriate controls and additional strategies should be considered to ensure that the results definitely reflect the generation of new neural cells. Moreover, caution is needed when comparing different studies, as distinct experimental paradigms may draw contrasting conclusions.

More than a physiological phenomenon, adult hippocampal neurogenesis is a process by which the etiology of many neurodegenerative and neuropsychiatric disorders may be unraveled. More importantly, the neurogenic process is a substrate from which new molecular targets for treating these disorders may arise. The diverse ways of approaching the topic provide unique perspectives on how neurogenesis may be implicated in homeostatic responses and in the development of pathological states. The data reviewed here strongly supports that both direct and indirect cell cycle regulatory

events may constitute relevant pieces to elucidate the complex mechanisms underlying the response to anti- and pro-neurogenic stimuli, in both basal conditions and in disease. These reports further emphasize the pertinence of modulating cell cycle regulators as targets for the development of new therapeutic approaches for disorders associated with neuroplastic imbalances.

Particularly in the case of major depression, new theories beyond monoamines have created a broader picture on how etiological factors are translated into disease and the action of antidepressants into the alleviation of the most common symptoms. In this context, much of the mechanisms are now being explored, including those interfering with adult hippocampal neurogenesis. However, the study of depression still represents a challenge for research since it involves the interplay between an individual's genetic predisposition and molecular responses to environment. Certainly, the discovery of new molecular mediators will give us important clues on susceptibility or predisposition targets, promoting the establishment of novel disease models, in a feedback loop that would nourish the field with new perspectives.

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FIGURE CAPTIONS

Fig. 1 Cell cycle regulation in the adult hippocampal neurogenic niche. Some niche-specific cell cycle regulators in the adult hippocampus have been identified. Cdk6-cyclin D2 and Cdk4-cyclin D1 complexes promote the expansion of the neural progenitor pool. P21 and p27 Cdk inhibitors have a role in proliferation arrest, both at the G1 and G2 phase. E2F1 has an important role in the neurogenic process by inducing the expression of genes involved in cell proliferation and differentiation. Cdk5 activity is associated with cell cycle reentry inhibition in postmitotic neurons. Signaling pathways, such as Notch, BMP, Shh and Wnt, are also involved in proliferation regulation, and in the balance between proliferation induction and stem cell quiescence maintenance. Nevertheless, several key molecules remain to be identified in this process in the context of adult hippocampal neurogenic niche (represented in the figure by a question mark). Rb – retinoblastoma protein.

Fig. 2 (a) Neurogenesis in the hippocampus comprises several steps, including proliferation of neural stem cells and transit amplifying neural progenitors in the subgranular zone (SGZ), cell cycle exit, neuroblasts migration throughout the granule cell layer (GCL), and maturation of the newborn neurons. (**b,c**) Cell cycle regulators implicated in neurogenesis imbalances observed in animal models of depression and in the pro-neurogenic effects of antidepressant drugs and other stimuli. (**b**) Neurogenesis imbalances have been observed in animal models of depression. These imbalances are attributed to an increased expression of p27 Cdk inhibitor (green arrow) in the DGs of animal models of depression. P27 inhibits neural progenitor cells proliferation in this

neurogenic niche. Cdk5 is involved in the development of depressive-like signs in an animal model of depression. The increased activity of Cdk5 (green arrow), together with the translocation of p35 activator to the membrane, was observed in chronic mild stress (CMS) exposed animals. (**c**) The pro-neurogenic actions of antidepressant drugs and stimuli, such as physical exercise, have also been correlated with alterations in the "expanded cell cycle". Antidepressants are able to specifically inhibit p21 expression (red arrow) in the DG, while increasing neurogenesis. Additionally, signaling pathways with recognized effects over the cell cycle regulation, such as Wnt, Notch and BMP, were implicated in the modulation of adult hippocampal neurogenesis in the context of depression and antidepressant stimuli.

TABLE CAPTION

Table 1 Summary of the cell cycle and signaling alterations implicated in neurogenesis imbalances observed in animal models of depression and mediating the pro-neurogenic effects of antidepressant drugs and stimuli. SGZ – Subgranular zone; DG- dentate gyrus; CUS – chronic unpredictable stress; CMS – chronic mild stress; AD – antidepressants; DCX – doublecortin [64,69-70,120,124,132]

Table
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TABLE CAPTION

Table 1 Summary of the cell cycle and signaling alterations implicated in neurogenesis imbalances observed in animal models of depression and mediating the pro-neurogenic effects of antidepressant drugs and stimuli. SGZ – Subgranular zone; DG- dentate gyrus; CUS – chronic unpredictable stress; CMS – chronic mild stress; AD – antidepressants; DCX – doublecortin [64,69-70,120,124,132]

TABLE 1

Experimental model	Proliferation/neurogenesis in the hippocampal DG	Molecular changes	Reference
Cell cycle regulators			
CUS exposed mice	↓	↑ p27kip1 + cells in the SGZ of the DG	[64]
Naïve mice chronically treated with fluoxetine, imipramine and desipramine	↑	↓ p21cip expression in the SGZ of the DG	[69-70]
CMS exposed rats treated with venlafaxine, mirtazapine, and aripiprazole	(Not assessed)	↑ Cdk5 activity and translocation of p35 activator to the membrane	[120]
Signaling pathways			
Naïve animals chronically treated with fluoxetine	↑	↑ Wnt3a expression	[124]
Voluntary exercise in mice (antidepressant stimulus)	↑	↑ Notch1 activity in DCX+ cells (cell cycle exit promotion)	[132]

Figure 1

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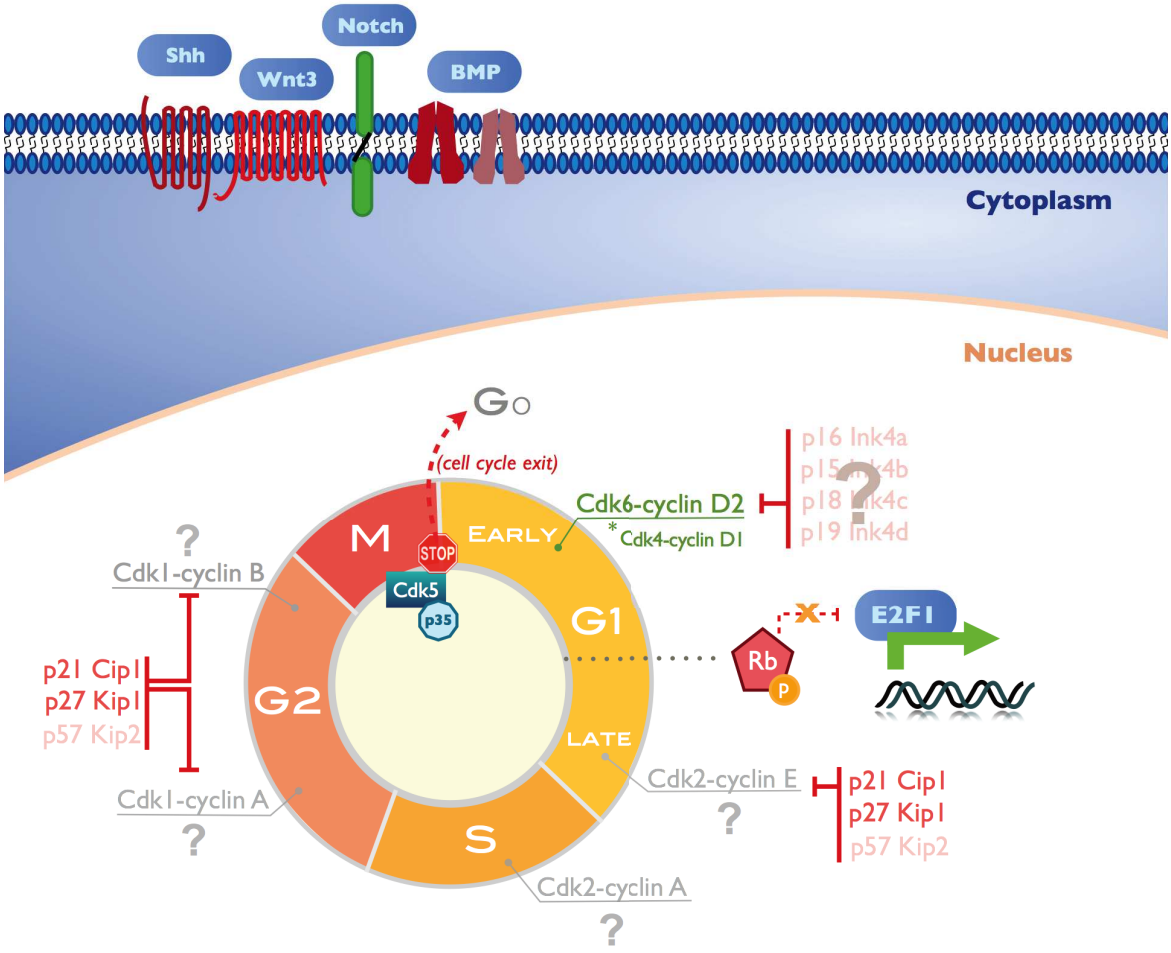

Figure 2
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