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## Zebrafish Rohon-Beard Neuron Development: Cdk5 in the Midst

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### Abstract

Cyclin-dependent kinase 5 (cdk5) is a proline-directed serine/threonine kinase that is activated mostly by association with its activators, p35 and p39. Initially projected as a neuron-specific kinase, cdk5 is expressed ubiquitously and its kinase activity solely depends on the presence of its activators, which are also found in some non-neuronal tissues. As a multifunctional protein, cdk5 has been linked to axonogenesis, cell migration, exocytosis, neuronal differentiation and apoptosis. Cdk5 plays a critical role in functions other than normal physiology, especially in neurodegeneration. Its contribution to both normal physiological as well as pathological processes is mediated by its specific substrates. Cdk5-null mice are embryonically lethal, therefore making it difficult to study precisely what cdk5 does to the nervous system at early stages of development, be it neuron development or programmed cell death. Zebrafish model system bypasses the impediment, as it is amenable to reverse genetics studies. One of the functions that we have followed for the cdk5 ortholog in zebrafish in vivo is its effect on the Rohon-Beard (RB) neurons. RB neurons are the primary sensory spinal neurons that die during the first two days of zebrafish development eventually to be replaced by the dorsal root ganglia (DRG). Based on our studies and others', here we discuss possible mechanisms that may be involved in cdk5's role in RB neuron development and survival.

### Keywords

Rohon-Beard neuron; Neurogenesis; Protein kinases; Gene knockdown; Cell fate

## Introduction

Cyclin-dependent kinase 5 (cdk5) belongs to the family of serine/threonine cyclin-dependent kinases and was initially identified as a member of the cyclin-dependent kinase family [1]. Although cdk5 is found in mitotic cells [2], it is not involved in the cell cycle. Despite its high degree of homology to other cyclin-dependent kinases, cdk5 activity is stimulated not by an associated cyclin [3] but by neuron-specific activators, p35, and p39, and possibly p67 (Munc-18) [2, 4, 5]. The cdk5 activators p25 and p29 are cleavage products of p35 and p39, respectively, generated by calpain-mediated proteolysis. The major activator, p35, is expressed predominantly in post-mitotic neurons of the central nervous system, and correlates well with cdk5 kinase activity [6–8]. Although cdk5 is expressed in many tissues, its highest kinase activity is observed mainly in post-mitotic neurons of the CNS due to the expression of the activators p35 and p39 in these neurons. Cdk5 activity is controlled by regulatory phosphorylation, subcellular localization, and association with other proteins [9–14]. Although the various activators have been shown to differentially influence the kinetics and substrate specificity of the cdk5 [9, 15, 16], the significance of cdk5 activation by a range of activators is unknown. It is possible that differences in anatomical and subcellular localization of the activators may serve specific physiological functions.

## Cdk5 Function in the Nervous System

Cdk5 is a multifunctional protein kinase (Fig. 1). It is known to affect neurite outgrowth and axonogenesis in both mammalian and *Drosophila* development [17–22]. Cdk5 knockout mice exhibit defects in organization of the cortex and cerebellum and are embryonically lethal [19]. In addition, cdk5 plays an important role in a range of physiological and pathological processes that include involvement in nervous system development, dopaminergic function and neurodegeneration [2, 23–27]. Recent studies support potential roles of cdk5 in regulated exocytosis of growth factors [28, 29].

Furthermore, cdk5 has been implicated in the connectivity of developing neurons. Disturbed fasciculation of axonal tracts, such as the corpus callosum in p35 null mutant mice, together with effects of cdk5 on growth cone collapse and neurite actin dynamics, provide evidence for a role of cdk5 in axon guidance [2, 26, 27]. Recent investigations have focused on the role of cdk5 in synaptogenesis, another aspect of connectivity. Work in models of synapse formation has demonstrated that cdk5 promotes the formation of synaptic structures, possibly by inducing the expression and clustering of acetylcholine receptors in the post-synaptic membrane [30–32].

Cdk5 has been associated with neuronal differentiation. Loss of cdk5 during development leads to an inability of neurons to exit the cell cycle, coupled with their incomplete differentiation [33]. This is consistent with data from other cell types that suggest a general role for cdk5 in cellular differentiation [34].

The development of the CNS requires the programmed migration, differentiation and connection of neurons to form functional circuits capable of expressing synaptic plasticity. Phenotypically, cdk5 null mutant mice, as well as p35/p39 double null mutants, exhibit a

reversal of the normal cortical laminar architecture, as well as cytoarchitectural disturbances in the cerebellum, brainstem and hippocampus [19, 35], implicating cdk5 in neuronal migration. Cdk5 phosphorylates a large number of proteins, including the neurofilaments and tau [36–43]. Cdk5-mediated phosphorylation of a broad range of substrates including proteins involved in cytoskeletal dynamics and axonal transport suggests its role in neuronal migration (Fig. 1). Moreover, mice lacking p35 reveal cortical lamination defects [44]. Additional evidences suggest that p35 null mutant mice may carry confounding developmental defects, such as aberrant hippocampal circuitry forming excitatory feedback loops [45]. In *Xenopus*, cdk5 has two p35 homologs, and plays a role in eye, muscle and neuronal development [46–48].

Regulated and deregulated cdk5 kinase activity has been implicated in neuronal survival and death, respectively. Amyloid- $\beta$  peptide and ischemic insults [49, 50] induce the conversion of p35 to its truncated form, p25, which causes apoptosis in neurons. In contrast, the association of p35 and cdk5 is required for neuronal survival. Cdk5-mediated kinase crosstalk regulates neuronal survival [51–53]. Much less is known about the proteolytically cleaved activators p25 and p29, than their parent molecules, p35 and p39. The formation of p25 has so far been demonstrated only in neurotoxic and degenerative situations such as Alzheimer's disease, although there is still controversy on the latter issue [54]. Whether p25 and p29 serve any normal physiological function remain to be elucidated.

## Cdk5 and Neuronal Death

During vertebrate nervous system development, as many as half of the developing neurons die [55–57]. An anti-apoptotic role for cdk5 has been reported in mammalian systems [52, 58, 59], although its precise role in apoptosis is still uncertain. On the other hand, cdk5 has been implicated in the induction of apoptosis [58, 60–64], while others have reported a reduction in cdk5/p35 in apoptotic cells [65]. Furthermore, in the mammalian system, based on studies using siRNA and anti-sense RNA, it has been suggested that cdk5 activation is not the inducer of cell death, but is a result of cell death, thus placing cdk5 activation downstream of cell death [66].

Inhibition of de-regulated cdk5 activity has been shown to promote cell survival of neurons challenged by  $\beta$ -amyloid peptide [67–69]. However, cdk5-deficient cultured cortical neurons exhibit increased sensitivity to apoptotic stimuli [52]. How precisely cdk5 regulates cell survival or death seems cell-specific and probably requires a tight regulation that would conceivably depend on the coactivator level and the existing signaling molecules or circuitry to propagate its effect. Apparently, even if cdk5 activity were the same in various cells, its downstream or parallel signaling factors would be critical in determining the ultimate outcome.

## RB Neuron Development and Apoptosis

Death is an essential part of the developmental process. Several hypotheses have been proposed to explain the purpose of this death: reduction in the number of neurons to match target tissue size, elimination of neurons that make connectivity errors, and failure to obtain

adequate trophic support [70]. Irrespective of the mechanism, elimination of neurons eventually results in a nervous system comprising the right number of neurons having error-proof pre- and post-synaptic connections.

Therefore, death serves to eliminate an obsolete or unnecessary cell type [66]. One such population is RB spinal sensory neurons. RB cells have been described in amphibians [55, 71–73]. Zebrafish RB neurons share the same attributes as amphibian RB neurons: large cell bodies with huge nuclei and granular cytoplasm [74]. RB neurons are derived from the same neural plate domain that generates neural crest cells [75], and manipulation of Delta/Notch signaling, as in the *mind bomb* mutants, reveals that RB neurons are the preferred fate, and neural crest cells the nonpreferred fate within this domain [75]. Why RB neurons die during development is unknown. In amphibians, the RB neurons die gradually and their death coincides with dorsal root ganglion (DRG) development [71, 73, 76].

Although our studies have shown that *cdk5* is essential to maintain the RB neurons, as analyzed in 24 h embryos [77, 78], it's likely that *cdk5* also plays a role during differentiation of RB neurons from neuronal precursors. During neuronal differentiation, proneural genes, such as *neurogenin1* (*ngn1*), induce downstream bHLH genes, such as *neuroD*, to elicit the transition from proliferative neural precursor cells to postmitotic neurons that express neuron-specific markers, N tubulin, and *HuC* [79–82]. In *Xenopus*, however, *cdk5* activation has been shown to be downstream from the expression of inducers of terminal neural differentiation, *ngn1* and *neuroD* [48], suggesting that *cdk5* activity is not required for neuronal differentiation. However, in zebrafish, when we analyzed the expression of *HuC* by in situ hybridization at 11.5 h of development, there was a significant difference in the number of *HuC*-expressing cells between control siRNA-injected and *cdk5* siRNA-injected embryos (Fig. 2a–d). The trigeminal ganglion primordia (T) were smaller in the *cdk5* knockdown embryos compared to those in the control embryos. Primary neurons include neurons in the trigeminal ganglia (T), RB neurons in the lateral neural plate, and primary interneurons (I) and motoneurons (M) in the intermediate and medial neural plates, respectively [83]. In our studies, a similar reduction in the number of early-born RB neurons (R), were also observed in the *cdk5* knockdown embryos. Later at 24 h of development, immunostaining of embryos with an antibody against islet-1, a marker of primary neurons, revealed a significant reduction in RB neurons in the *cdk5* siRNA-injected embryos compared to the control siRNA-injected embryos (Fig. 2e, f). Quantitative analyzes of islet-1 positive neurons showed a significant (~50%) reduction in the number of RB neurons in the *cdk5* siRNA-injected embryos (Fig. 2g). In this context, the question arises, whether *cdk5* is involved in RB neuron differentiation.

Most recently, phosphorylation and stabilization of p27<sup>Kip1</sup> by *cdk5* has been reported in the mammalian system [[84], and our unpublished data]. It's homolog, the *cdk* inhibitor p27<sup>Xic1</sup> in *Xenopus*, stabilizes *neurogenin1* and promotes early neuron development [85], whereas Notch suppresses p27<sup>Xic1</sup> expression in both the myotome and the lateral stripe of primary neurons [86]. Mammals express three members of the Cip/Kip family members, p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup> [87]. However, redundancy and inaccessibility has made it impossible to precisely determine the function of these Cip/Kip family members in nervous system development of null mouse models [88–90]. Whether *cdk5* regulates early neurogenesis in

zebrafish by modulating p27<sup>Kip</sup> function warrants further investigation. Here, we present a possible scenario of how cdk5 might be involved in zebrafish RB neuron differentiation from neuronal precursors along with the well-studied pathway of Notch inhibition resulting in RB neuron differentiation (Fig. 3). It is known that Notch inhibition induces *Ngn1* gene expression, which, in turn, can translate Ngn1 protein that has been shown in *Xenopus* to be stabilized by p27<sup>Kip</sup>. Ngn1 is an inducer of *NeuroD* and both are key players in RB neuron differentiation.

Recently, we have shown that a Notch inhibitor, DAPT, downregulates cdk5 activity in rat primary neurons [91]. However, it's not known whether DAPT inhibits cdk5 activity in neuronal precursors and what would the fate of the neuronal precursors be, if such were the case. It is likely that in the neuronal precursors, Notch inhibition, during the process of inducing neuronal differentiation, might not suppress cdk5 activity. This assumption is based on the fact that cdk5 is essential for complete differentiation of neurons from the neuronal precursors in mammals [33]. During neuronal differentiation, it is possible that the impact of the proneural gene *Ngn1* expression, induced by Notch inhibition, becomes more pronounced by stabilization of Ngn1 protein by p27<sup>Kip1</sup>, the latter being stabilized by cdk5 phosphorylation. Thus, a synergistic effect of Notch inhibition that regulates the expression of the pro-neural gene, *Ngn1*, and cdk5 activity that could stabilize the Ngn1 protein, during RB neuron differentiation seems plausible (Fig. 3).

### Cdk5 and RB Neuron Death

As in amphibians, zebrafish RB neurons die over a protracted period of time [92, 93]. A number of factors may be responsible for the death of the RB neurons. It has been shown recently that zebrafish RB neuron death is correlated with reduced expression of neurotrophin-3 (NT-3) receptor, trkC1, suggesting that RB neuron survival involves the neurotrophin NT-3 [92, 93]. RB neuron death also appears delayed by inhibiting caspase activity, suggesting that RB death is caspase-dependent [92, 93]. A decrease in sodium current-mediated electrical activity has been shown to cause RB neuron death [94]. Recently, our data suggest that a reduction in cdk5 levels results in RB neuron death as seen in 24 h zebrafish embryos [77] and also causes a reduction in the trigeminal ganglia primary sensory neurons [78]. Although zebrafish RB neurons begin showing signs of programmed cell death, such as DNA fragmentation, as early as the first day of development, the cells remain present for considerably longer and degenerate over a protracted period of time and unlike in amphibians, independent of any developmental link to DRG neurons [93].

The DNA of RB neurons is fragmented before neuronal degeneration and this can be detected using the TUNEL assay [95]. It is also of interest to note that a drastic reduction in TUNEL-positive RB neurons in the cdk5 siRNA and cdk5 mRNA-co-injected embryos occurs compared to the cdk5 siRNA-injected embryos, coincidentally with an up-regulation in cdk5 catalytic activity [77]. These results support the notion that cdk5 may have a role in RB neuron survival. A tight regulation of cdk5 expression during RB neuron development, and their (RB neurons') disappearance in two days, must occur for the normal development of zebrafish embryos. In this context, other signaling cascades, along with cdk5, could have a major role in the proper development of the spinal sensory nervous system.

Previously, we cloned the zebrafish ortholog of human cdk5 [78]. The zebrafish cdk5 is strikingly homologous (97% identity) to all vertebrate cdk5s, conservation consistent with the view that this kinase plays a major role in development and function of the vertebrate nervous systems. Compared with other vertebrates, particularly the closely related *Xenopus* [96], zebrafish cdk5 protein is expressed very early during cleavage, before the mid-blastula transition. Moreover, cdk5 kinase activity, although low, was measurable during the first 12 h of development. Cdk5 is present maternally in the early embryos much before muscle or neuronal differentiation occurs [78]. In contrast, cdk5 in *Xenopus*, first appears after the mid blastula transition [46]. Using siRNA-mediated cdk5 knockdown and cdk5 mRNA misexpression studies, we have shown that cdk5 influences RB neuron survival although the exact mechanism remains to be understood [77]. Immunostaining with the islet-1 antibody revealed the reduction of RB neurons in the cdk5 siRNA-injected embryos that can be rescued when cdk5 mRNA is coinjected. These studies were further supported by our observation that the primary sensory neurons of the trigeminal ganglia of the peripheral nervous system were also depleted in the cdk5 siRNA-injected embryos [78].

How cdk5 regulates RB neuron death is not known. Whether during RB neuron death, cdk5 level decreases prior to the onset of apoptosis remains to be elucidated. This issue is complicated since *in situ* hybridization analyzes do not reveal a detectable expression of cdk5 in the RB neurons of zebrafish at 24 h of development. In this context, whether cdk5 functions cell autonomously or non-autonomously needs to be examined. Another possibility is that the effect of cdk5 could be indirect based on cdk5's role in exocytosis of growth factors [28, 97–99]. P39-mediated cdk5 activation seems to positively regulate glucose-dependent insulin secretion, but p35-mediated cdk5 activation seems to negatively regulate glucose-dependent insulin secretion [99–101]. Cdk5/p39 stimulates Ca<sup>2+</sup>-dependent exocytosis in primary  $\beta$ -cells via the phosphorylation of Munc18-1 [99]. It has been reported that antibodies, which deplete NT-3, induce RB cell death while exogenous application of NT-3 reduces death and RB neurons express the neurotrophin receptor trkC1 [93]. Thus, Neurotrophin-3-triggered RB neuron survival may be mediated via cdk5. For example, if the neurotrophin secretion is compromised in the neighboring cells in cdk5 knockdown embryos, the RB neuron survival may be in jeopardy, suggesting a cell non-autonomous mechanism triggered by cdk5 in RB neuron survival. Yet another possibility is the upregulation of the anti-apoptotic molecule, Bcl-2, by cdk5. Inhibition of cdk5 has been shown to attenuate Bcl-2 expression in mammalian cells [53]. The possibility of cdk5-induced Bcl-2 expression in RB neurons thus leading to their survival in a cell-autonomous manner remains to be tested. Based on the available studies on the programmed cell death of RB neurons, a summary of possible scenarios of potential pathways operating to bring about this important developmental process is presented in Fig. 4, where we show a potential involvement of cdk5 in RB neuron survival dependent on the regulation of neurotrophin secretion from adjacent non-neuronal cells.

## Conclusions

Cdk5's resume as a multifunctional kinase that includes a range of its participation in cell death (pro-apoptotic), cell survival (anti-apoptotic), neuron migration, and nervous system development, is topped by its general regulation of endocytosis/exocytosis in both neuronal



and non-neuronal cells. The latter, which could regulate neuronal growth factor secretion, may also have a major role in neuronal survival. In zebrafish, although we have reported that in 24 h of development, RB neurons are scanty in the siRNA-mediated cdk5 knockdown embryos, suggesting a role of cdk5 in RB neuron survival, the exact mechanism(s) of this process remains to be determined. In 11.5 h of development, when early neurons begin to develop, we observed that the primary neurons were significantly less in the trigeminal ganglion placode of the cdk5 knockdown embryos. RB primary neurons were also reduced in number in these embryos, suggesting a possible role of cdk5 during early primary neuron differentiation. We speculate that cdk5 may impart dual functions on RB neuron development; an early role during their differentiation from the neuronal precursors, and a later role in their survival as fully differentiated neurons.

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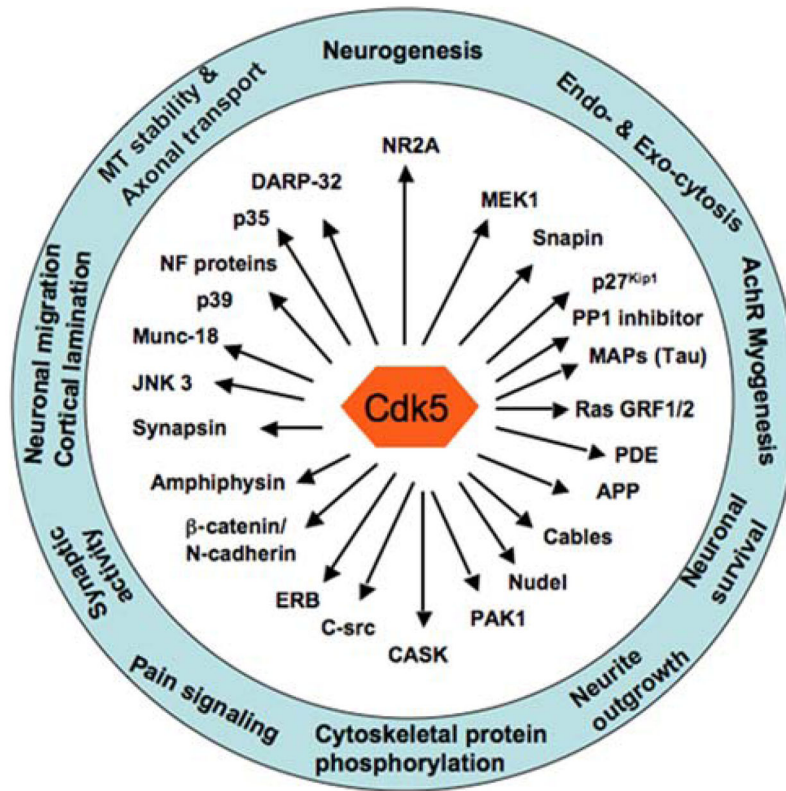


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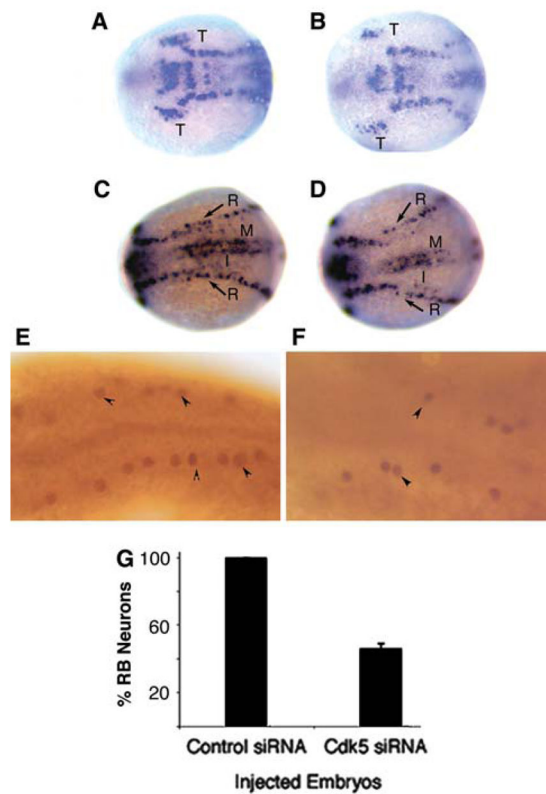
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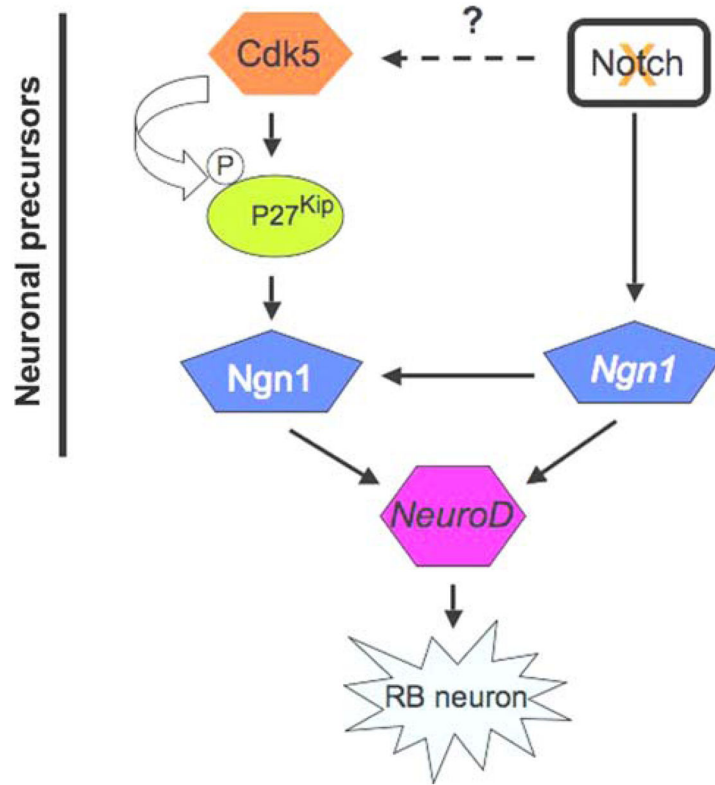


**Fig. 1.** Cdk5 is a multi-functional kinase. Cdk5 is involved in a wide range of neuronal functions. These functions are mediated by proteins that are phosphorylated by cdk5. Some of the cdk5 substrates are indicated by *arrows*

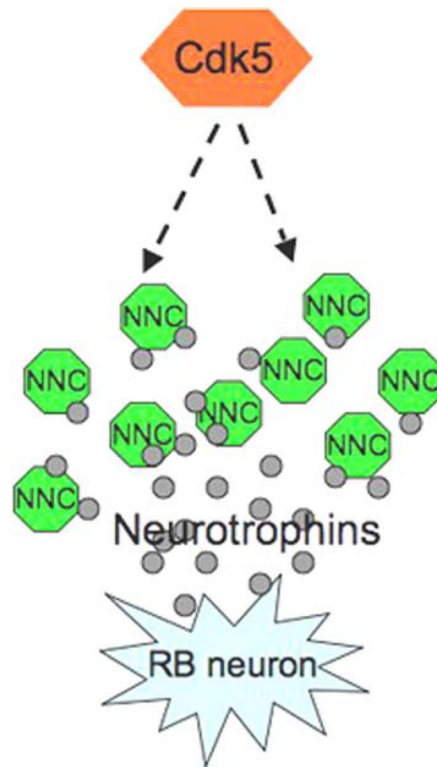


**Fig. 2.** Cdk5 is required for primary sensory neurogenesis. (a–d) Whole-mount in situ hybridization was carried out as described [102] by using digoxigenin-11-UTP-labeled probes (Roche). The antisense probe for *HuC* (marker for primary neurons) was obtained from the *HuC* gene with 3'UTR cloned in pGEMT vector. The anitsense DIG-labeled RNA was synthesized by using SP6 Polymerase after linearization with *NcoI*. The results show *HuC* mRNA expression at 11.5 h embryos (dorsal views) injected with control siRNA (a, c) or cdk5 siRNA (b, d). T indicates *trigeminal ganglion placode*. Arrows (R) indicate Rohon-Beard (RB) neurons. R, RB neurons; M, motor neurons and I, interneurons. (e–g) Immunostaining of 24 hpf embryos with an antibody against islet-1 marks (lateral views of the anterior regions of the embryos) RB neurons in control siRNA-injected (e), cdk5 siRNA-injected (f) embryos. Arrowheads indicate some of the RB neurons. Quantitative analyzes of islet-1 positive neurons are presented (g)





**Fig. 3.** Proposed model shows a possible role of cdk5 in Rohon-Beard neuron differentiation. Cdk5 stabilizes p27<sup>Kip</sup> by phosphorylating it in mammalian cells. On the other hand, in *Xenopus*, p27<sup>Kip</sup> has been shown to stabilize Ngn1 protein leading to neuronal differentiation. *Ngn1* is known to induce *NeuroD*. This model is presented with an established pathway, in which inhibition of Notch signaling is known to promote RB neuron generation via upregulation of *Ngn1* and subsequently, *neuroD*



**Fig. 4.** Model shows a potential role of cdk5 in Rohon-Beard (RB) neuron survival in a cell non-autonomous pathway. Neurotrophin (especially NT-3) secretion by non-neuronal neighboring cells (NNC), such as muscle cells, may be regulated by cdk5, thus providing trophic support to the embedded RB neurons