Review Article

Notch and Cdk5 in Zebrafish *Mindbomb* Mutant: Co-regulation or Coincidence?

(neuron / delta / Ubiquitin ligase / *mindbomb* / Cdk5 / Notch)

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Abstract. Notch signalling is critical for the development of the nervous system. In the zebrafish *mindbomb* mutants, disruption of E3 ubiquitin ligase activity inhibits Notch signalling. In these mutant embryos, precocious development of primary neurons leading to depletion of neural progenitor cells results in a neurogenic phenotype characterized by defects in neural patterning and brain development. Cyclin-dependent kinase 5 (Cdk5), a predominant neuronal kinase, is involved in a variety of essential functions of the nervous system. Most recently, mammalian studies on Notch and Cdk5 regulating each other's function have been emerging. The status of Cdk5 in the *mindbomb* mutant embryos with excessive primary neurons is not known. *In situ* hybridization of the zebrafish *mindbomb* mutant embryos uncovered a robust upregulation in *Cdk5* expression but with a reduced Cdk5 activity. The implications of these findings in both the mammalian system and zebrafish are discussed in this mini-review to provide a glimpse into the relationship between Notch and Cdk5 that may explain certain neurodevelopmental defects associated with either mutations in ubiquitin ligase or altered expression of Cdk5.

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Abbreviations: APC – anaphase-promoting complex, Cdk5 – cyclin-dependent kinase 5, CHIP – Hsc70-interacting protein, CPM – counts per minute, DSL – Delta/Serrate/Lag-2, hpf – hours post-fertilization, Mib1 – Mindbomb 1, *Mib1* – *mindbomb* mutant, NICD – Notch intracellular domain, RING – really interesting new gene.

Introduction

Notch signalling is a highly conserved pathway that determines cell fate and is known to negatively affect neuronal cell fate *in vivo* (Weinmaster and Kintner, 2003). Several earlier *in vitro* studies using cultured cells also confirm this property of Notch signalling (Berezovska et al., 1999; Franklin et al., 1999; Sestan et al., 1999; Redmond et al., 2000). The Notch transmembrane receptors are activated by transmembrane ligands of the Jagged and Delta/Serrate/Lag-2 (DSL) family that includes Delta and Serrate/Jagged subfamilies (Kopan and Ilagan, 2009). Binding of Notch and DSL ligands elicits intracellular signal transduction when Notch and DSL originate from adjacent cells (*trans*-binding), and *cis*-binding occurs when Notch and DSL originate from the same cell inhibiting signalling (Miller et al., 2009; Sprinzak et al., 2010).

Notch/DSL *trans*-binding-induced cell signalling occurs only when DSL ligands are co-expressed with ubiquitin (Ub) E3 ligase (Le Borgne et al., 2005; Pitsouli and Delidakis, 2005). There are two families of really interesting new gene (RING) domain E3 ligases that can activate the DSL ligands: Neuralized (Neur) (Deblandre et al., 2001; Pavlopoulos et al., 2001; Yeh et al. 2001) and Mindbomb 1 (Mib1) (Itoh et al., 2003; Koo et al., 2005; Lai et al., 2005; Le Borgne et al., 2005; Pitsouli and Delidakis, 2005; Wang and Struhl, 2005). Since ubiquitination of plasma membrane proteins leads to their endocytosis (Acconcia et al., 2009; Clague and Urbe, 2010), Neur- and Mib1-mediated ubiquitination can potentially trigger DSL ligand endocytosis. Endocytosis of ubiquitinated Delta reportedly promotes the *trans*-activation of Notch (Weinmaster and Fischer, 2011; Meloty-Kapella et al., 2012).

Mib1 was first identified in genetic mutagenesis screens of zebrafish (Itoh et al., 2003). In zebrafish, Mib1 positively regulates the Notch pathway (Itoh et al., 2003) necessary for cell fate specification during development (Weinmaster and Kintner, 2003), since it facili-

tates the internalization of Notch ligands rather than their degradation (Itoh et al., 2003). Mib1-null mice are embryonically lethal (Koo et al., 2005). In zebrafish, its loss-of-function mutant (*mindbomb*) exhibits developmental defects that occur due to the loss of lateral inhibition induced by Notch signalling, wherein Mib1 enhances the ubiquitination followed by endocytosis of the Notch ligand Delta, thereby activating Notch signalling (Itoh et al., 2003). *Mib1* mutants (*mindbomb*) show a "neurogenic" phenotype characterized by increased supernumerary, early-born primary neurons. These embryos also exhibit compromised mesoderm development (Hwang et al., 2009).

Cyclin-dependent kinase 5 (Cdk5) is a member of the family of serine/threonine (S/T) cyclin-dependent kinases (Meyerson et al., 1992). Unlike other Cdk family members, Cdk5 is highly expressed with a robust kinase activity in the neurons (Cheung et al., 2006; Dhavan and Tsai, 2001). Cdk5 is a multi-functional protein kinase involved in a wide range of neuronal functions including neuronal migration and cell survival (Cruz and Tsai, 2004). Although Cdk5 is found in mammalian mitotic cells, its activity is higher in the neurons since neurons express the Cdk5 activators, p35 and p39 (Dhavan and Tsai, 2001; Ko et al., 2001). Cdk5 is activated upon direct interaction with its neural-specific activator p35 in normal conditions, but gets hyperactivated in various pathological conditions when conversion of p35 to p25 occurs via calpain-mediated cleavage (Patrick et al., 1999). Cdk5 plays a crucial role in neuronal differentiation (Cicero and Herrup, 2005) and is essential for the regulation of various post-mitotic cellular processes in the nervous system including neuronal migration, protein trafficking, cell survival, neurite and synapse development, dopamine response, learning, and memory (Dhavan and Tsai, 2001; Cheung et al., 2006). Deregulation of Cdk5 activity causes a wide range of pathological processes of the nervous system development including neurodegeneration (Dhavan and Tsai, 2001; Shelton and Johnson, 2004). Axon guidance defects were observed in p35 (Cdk5 activator) knock-out mice (Kwon et al., 1999) and in *Drosophila* with abnormal Cdk5 activity (Connell-Crowley et al., 2000). Deregulation of Cdk5 activity has been implicated in an array of neurodegenerative diseases (Patrick et al., 1999; Cheung and Ip, 2012).

In cultured cortical neurons, Cdk5 activity suppression compromised neurite outgrowth, while ectopic expression of exogenous p35 and Cdk5 led to the development of longer neurites (Nikolic et al., 1996). A major function of Cdk5 in cell survival has been shown in studies where Cdk5 protected cultured neurons from cell death by direct interaction and activation of the anti-apoptotic protein Bcl-2 (Cheung et al. 2008; Wang et al. 2006). Cdk5-null mice exhibit defects in organization of the cortex and cerebellum and are embryonically lethal (Ohshima et al., 1996). Very little is known regarding the question on whether Cdk5 and Notch regulate each other's activity. In this mini-review, we attempt to put into context our current findings on the zebrafish *mindbomb* embryos and all that is known about Cdk5 and Notch co-regulation in the mammalian cells, including our own studies on the rat cortical neurons (Kanungo et al., 2008).

Potential link between Notch and Cdk5

It has been shown that Mib1 is a substrate of p35/Cdk5 *in vitro*, and in rat forebrain neurons, Cdk5 activity induced degradation of Mib1, possibly by its phosphorylation, since Mib1 degradation was suggested to rely on the interaction with Cdk5/p35 (Choe et al., 2007). The activity of many ubiquitin ligases can be modulated by phosphorylation, as exemplified by E3s, such as anaphase-promoting complex (APC) (Tang et al., 2004), Itch (Gao et al., 2004) and the C-terminus of Hsc70-interacting protein (CHIP) (Kim et al., 2016). It is possible that Mib1 is similarly regulated by Cdk5-mediated phosphorylation.

Mib1 has been shown to regulate neurite morphogenesis by physically and functionally interacting with p35/Cdk5 (Choe et al., 2007). This interaction between Mib1 and Cdk5/p35 links Notch to Cdk5, thus integrating these two different signalling pathways. Even in non-neuronal cells, Cdk5 and Notch inter-regulation has been reported (Merk et al., 2016). Endothelial Cdk5 plays a role in vascular development and tumour angiogenesis. In the endothelial-specific Cdk5 knockout mice, Notch function was disrupted in the endothelial cells with further biochemical studies revealing that Cdk5 inhibition disrupted Notch function by reducing generation of the active Notch intracellular domain (NICD) (Merk et al., 2016). It was also confirmed that Cdk5 inhibition only affected Delta (Dll4)-Notch signalling, whereas Jagged-Notch signalling remained intact (Merk et al., 2016). Cdk5 inhibition, both chemically and genetically, inhibited Notch signalling in zebrafish and potentiated pancreatic β-cell differentiation, although increased β-cell population occurred in the Cdk5 mutant zebrafish independent of Notch signalling inhibition (Liu et al., 2018). The Cdk5 and Notch pathways may possibly have similar interaction in neurons. In our previous study, we have shown that suppression of Cdk5 activity generated supernumerary cranial and motor neurons *in vivo* in zebrafish (Kanungo et al., 2009). What happens to Cdk5 expression and activity, should Notch signalling be disrupted, can elucidate the interplay of Cdk5 and Notch. In this context, *mindbomb* zebrafish is an ideal model to explore this connection.

Cdk5 mRNA, but not activity, is upregulated in the zebrafish *mindbomb* mutant

With excessive development of primary neurons in the *mindbomb* mutants, it is not known whether Cdk5

expression is also altered. *In situ* hybridization of *mindbomb* embryos at 11.5 hours post-fertilization (hpf) showed excessive expression of Cdk5 mRNA compared to the wild-type embryos (Fig. 1A, B). In the 24 hpf *mindbomb* embryos, Cdk5 mRNA expression expanded beyond the brain and the central nervous system, suggesting that an up-regulation of Cdk5 transcription occurred in neuronal as well as non-neuronal cells (Fig. 1C, D). It is likely that Cdk5 mRNA expression was induced either by transcriptional or post-transcriptional mechanisms through Notch signalling disruption. Surprisingly, Cdk5 activity was significantly reduced in the Mib$^{-/-}$ embryos (Fig. 1E). In zebrafish, Cdk5 mRNA over-expression alone without its partner p35 adversely affected motor neuron development *in vivo* (Kanungo et al., 2009). Likewise, it has been reported that in the Cdk5 transgenics, Cdk5 activity was surprisingly reduced while the mice were normal (Tanaka et al., 2001). However, Cdk5 activity in the brain extracts of these mice was increased upon addition of p35 protein in kinase assays, suggesting that the Cdk5 transgene was functional, but over-expressed Cdk5 either auto-inhibited its binding to p35 or p35 protein levels were limiting for Cdk5 activity (Tanaka et al., 2001). In the zebrafish *mindbomb* embryos, should sustained over-expression of Cdk5 reduce its catalytic activity or remain inactive because of limiting levels of p35, neuronal survival would be adversely affected and maturation of neurons would be compromised. In such a scenario, activating the excess Cdk5 by co-expressing its activator, p35, may inhibit neuronal death and possibly help retain the cellular integrity of non-neuronal cells.

It is not known whether in the *mindbomb* embryos *Cdk5* over-expression drives certain non-committed progenitors of the non-neuronal lineage to commit to a neuronal fate (Fig. 1), or neuronal commitment occurs prior to *Cdk5* expression in these cells. Cdk5 knockdown has been shown to induce neurogenesis with the formation of supernumerary motor neurons (Kanungo et al., 2009), which suggests that *Cdk5* over-expression in the *mindbomb* embryos possibly occurs after the neuronal commitment of cells. Chemical inhibition of Notch signalling by N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT) suppressed Cdk5 activity in rat cortical neurons despite inducing Cdk5 mRNA transcription *in vitro* (Kanungo et al., 2008), suggesting that Cdk5 activity succeeds Notch activation (Fig. 2). Altered distribution of Tau and neurofilament proteins was observed in these neurons (Kanungo et al., 2008), which could have been a consequence of reduced Cdk5 activity.

Based on our preliminary observation on the *mindbomb* embryos, another hypothesis arises with the assumption that in the neurons once generated, Cdk5 mRNA expression occurs because of neuronal intrinsic signalling, but sustained Notch inhibition predisposes these neurons to apoptosis. Most likely, first, *mindbomb* embryos produce supernumerary neurons at the expense of non-neuronal progenitors, thus disrupting body pat-

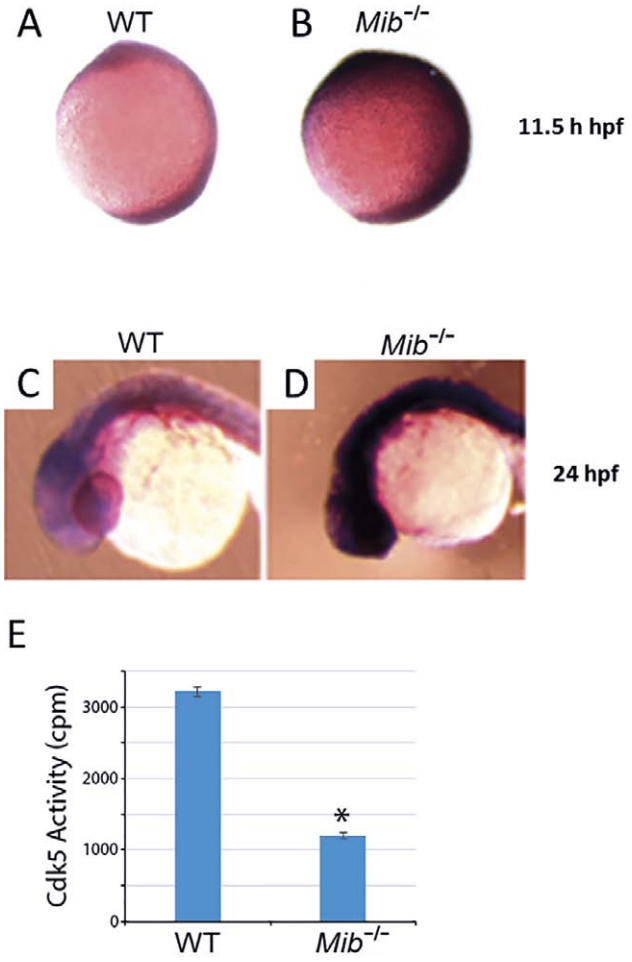

Fig. 1. Cdk5 mRNA expression and Cdk5 activity in the WT and *Mib*$^{-/-}$ embryos. Whole-mount *in situ* hybridization shows Cdk5 mRNA expression at the neurula stage at 11.5 hpf (**A**-**B**); 24 h embryos, lateral views (**C**-**D**). Whole-mount *in situ* hybridization of the zebrafish embryos was carried out following methods we previously described (Kanungo et al., 2007). Cdk5 activity in the WT zebrafish embryos was greater than that of the *Mib*$^{-/-}$ embryos (**E**). Cdk5 activity was measured in the 24 hpf WT and *Mib*$^{-/-}$ embryos following procedures described in our previous studies (Veeranna et al., 1998; Kanungo et al., 2009). Briefly, homogenates containing 200 µg total protein each from the WT and *Mib*$^{-/-}$ embryos were immunoprecipitated on Protein A-Sepharose beads using the anti-Cdk5 antibody. Kinase assay was performed in a final volume of 50 µl using histone H1 as the substrate. Acetic acid was added to a final concentration of 30 % to stop the reaction. The reaction products (25 µl each) were spotted onto P81 phosphocellulose discs (Whatman, Merck, Darmstadt, Germany). The discs were washed extensively, first with 30% acetic acid following three washes with 15% acetic acid. The discs were finally washed with acetone, air-dried, and counted in a scintillation counter. The CPM (counts per minute) values as averages of three reactions each are shown with standard deviation, and statistical significance (*) was set at $P < 0.05$ (**E**).

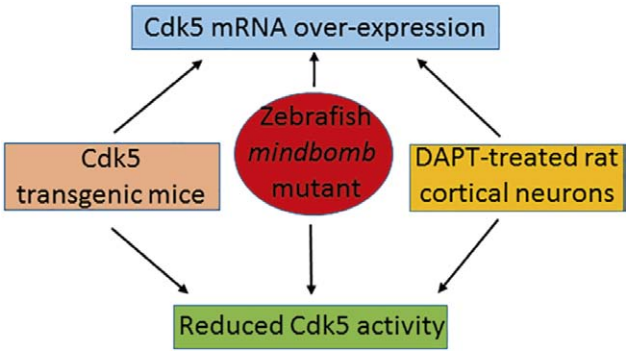

Fig. 2. Schematic presentation showing *Cdk5* over-expression both in transgenic mice through transgene expression (as reported in Tanaka et al., 2001) and rat cortical neurons treated with the Notch inhibitor DAPT (as reported in Kanungo et al., 2008) indicates that Cdk5 over-expression resulting in reduced Cdk5 activity in the Cdk5 transgenic mice and DAPT-treated neurons, as shown in those two studies, may have resulted from auto-inhibition of Cdk5 activity at the protein level.

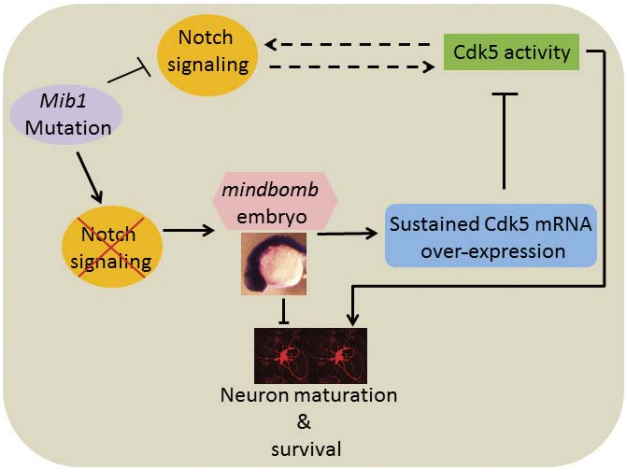

Fig. 3. Schematic presentation of potential link(s) between Cdk5 and Notch in zebrafish as deduced from the *mindbomb* zebrafish embryo phenotype, our previous report on the rat cortical neurons (Kanungo et al., 2008) and mice (Tanaka et al., 2001; Hellstrom et al., 2007). Mib1 mutation inhibits Notch signalling, resulting in enhanced primary neurogenesis. Whether Notch signalling inhibits Cdk5 mRNA expression is not known, but reduced Cdk5 activity due to Cdk5 mRNA over-expression may prove detrimental to the maturation and survival of the overproduced primary neurons. Notch inhibition resulting in reduced Cdk5 activity, both in the zebrafish and mammals, would potentially point to a direct relationship between Notch and Cdk5 (dotted lines).

terning, and second, the generated primary neurons lack the Notch survival signal (Ables et al., 2011) along with reduced Cdk5 activity that are necessary for proper neuronal development and maturation (Cho et al., 2014).

Conclusions

Functional studies on the potential link between Notch and Cdk5, although rare, are emerging (Table 1). In vivo, zebrafish *mindbomb* embryos appear to be a good model system to study the cross-talk between Notch and Cdk5. Both Notch regulating Cdk5 activity and *vice versa* have been reported (Kanungo et al., 2008; Merk et al., 2016). However, an organism with Notch signalling turned off as in the *mindbomb* embryos that abundantly over-express Cdk5 mRNA uniquely shows that Notch could negatively regulate Cdk5 transcription. On the other hand, sustained Cdk5 mRNA over-expression *in vivo,* as in the *mindbomb* embryos, may be the reason for the down-regulation of Cdk5 activity, as reported in Cdk5 transgenic mice (Tanaka et al., 2001) (Fig. 2). *Cdk5* knock-out mice resemble mice with disrupted Notch signalling (Hellstrom et al., 2007), and chemical and genetic inhibition of Cdk5 in zebrafish also results in Notch inhibition (Liu et al., 2018). Therefore, it is possible that both Notch signalling and Cdk5 activity (not mRNA) positively regulate each other, since Notch inhibition results in sustained Cdk5 mRNA over-expression (Figs. 1A-D and 2) potentially leading to auto-inhibition of Cdk5 activity (Figs. 1E, and 2-3), as has been shown in Cdk5 transgenic mice (Tanaka et al., 2001). Subsequently, a direct correlation between Notch and Cdk5 signalling becomes conceivable in the context that elevated Cdk5 mRNA expression is a direct consequence of Notch inhibition and loss of Cdk5 activity thereafter is a secondary effect (Fig. 3). Thus, suppressed Cdk5 activity along with Notch disruption would functionally result in the patterning defects of the nervous system as observed in the *mindbomb* embryos.

Table 1. Functional link between Notch and Cdk5 in zebrafish, mice, and rat cortical neurons in culture.

Functional evidence of potential link between Notch and Cdk5	**Reference**
Cdk5 mutant zebrafish display an increased number of pancreatic β-cells, an effect also seen upon Notch inhibition.	Liu et al., 2018
In endothelial-specific Cdk5 knockout mice, Notch function is disrupted in the endothelial cells.	Merk et al., 2016
Chemical inhibition of Notch signaling down-regulated Cdk5 activity in rat cortical neurons in culture resulting in altered distribution of cytoskeletal proteins.	Kanungo et al., 2008

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.

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