Mind Bomb1 and DeltaD are Localized into Autophagosome after Endocytosis in Zebrafish during Neurogenesis

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ABSTRACT : Endocytosis of the Notch ligand, DeltaD, by mind bomb1 is indispensable for activation of Notch in cell fate determination, proliferation, and differentiation during zebrafish neurogenesis. Loss of mind bomb1 activity as an E3 Ubiquitin ligase causes the accumulation of deltaD at the plasma membrane and results in the ectopic neurogenic phenotype by activation of Notch in early zebrafish embryogenesis. However, the regulatory mechanism of deltaD during neurogenesis is not identified yet. This study aims to analyze the pathway of mib1 and deltaD after endocytosis *in vivo* during zebrafish embryogenesis. Mind bomb1 and deltaD are co-localized into autophagosome and mutant form of mind bomb1 fails to cargo deltaD into autophagosomes. These findings suggest that mind bomb1 mediates deltaD regulation by autophagy in an ubiquitin-dependent manner during zebrafish embryogenesis.

Key words : Notch signaling, Mind bomb1, DeltaD, Autophagosome, Degradation, Neurogenesis, Zebrafish

INTRODUCTION

Notch signaling is essential for cell fate determination, proliferation, and differentiation during development (Lewis, 1998; Lai, 2004). Notch signaling is triggered by binding of delta or jagged ligands followed by Notch intracellular domain (NICD) cleavage by γ-secretase complex (Mumm and Kopan, 2000). NICD and ligands translocate to the nucleus and regulate transcriptional activity of target genes, like Hes/her or Hey genes (Iso et al., 2003).

In vertebrates, Notch activation can suppress the differentiation of neuronal progenitors and lead ligand-expressing cells to undergo neuronal differentiation in the neural epithelium (Simpson 1990; Muskavitch 1994). Moreover, control of the level of Notch expression is important to determine the potency of cell signaling as well as modulate the signaling pathway in disease pathogenesis (reviewed in MacGrogan et al., 2011). For example, overexpression of Notch3 is detectable in ovarian (Rose et al., 2010), lung (Westhoff et al., 2009), and breast cancers (Parr et al., 2004; Guo et al., 2011). Hair colors are progressively changed with each reduction in Notch1/2 alleles in knockout mice (Schouwey et al., 2007).

Recent studies have suggested degradation of NICD is regulated by the ubiquitin-proteosome system (UPS) (Jehn et al., 2002; Jia et al., 2009). Jehn et al. investigated that ubiquitination of N1-ICD by c-Cbl leads to degradation through lysosomal-dependent pathways. N1-ICD was significantly increased after lysosomal inhibitor treatment, but failed to change the NICD levels with proteosome inhibitors. Lysosomal degradation pathway is necessary to regulate Notch activity in cell-cell communication.

Autophagy is a crucial catabolic pathway which is required for the lysosomal degradation of cytoplasm, long-lived proteins, and cellular organelles (Klionsky & Nemchenko, 2011). It is highly active during differentiation and development. Classical studies about autophagy were focused on the degradation pathway of unfolded proteins and damaged cellular organelles etc. in cell death or a stress response mechanism. Recent studies revealed the novel function of autophagy in developmental regulatory system. Autophagy may rearrange

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and recycle cellular components and nutrients needed for signaling pathways in proliferation, death, and differentiation during embryogenesis (Cecconi & Levine, 2008). Moreover, novel role of autophagy has been suggested that it may be a significant process of cytosolic rearrangement in cell fate decisions (Mizushima & Levine, 2010). In Notch signaling pathways, recent studies prove that degradation of NICDs is mediated by lysosomes. Until now, no trial has been performed to determine the molecular fate of Notch ligands in the cell fate determination.

In zebrafish, Itoh et al. demonstrated that the E3 ubiquitin ligase, mind bomb1 (mib1), ubiquitinates deltaD, promotes its endocytosis (Itoh et al., 2003). The zebrafish mind bomb1 mutant is the well known for the severe ectopic neurogenic phenotype from reduction of Notch signaling. Itoh et al showed that interaction of deltaD with Notch can promote endocytosis of deltaD and regulate activation of Notch signaling during zebrafish neurogenesis. The turnover mechanisms of Notch ligands are still undefined, prompting to test whether autophagy is responsible for distribution and stability of deltaD. Using zebrafish *in vivo* system, cellular translocation of Notch ligands, deltaD, was carefully investigated to unveil the regulatory mechanism in zebrafish neurogenesis.

MATERIALS AND METHODS

1. Zebrafish Maintenance and Mutant Strains

Zebrafish wild-type AB*, mind bomb alleles (mib^{ta52b} and mib^{m178} (Kelsh et al., 1996; Kim & Kim, 2003) were maintained under standard animal laboratory conditions (Whitlock & Westerfield, 2000). Embryos were allowed to develop, staged using morphological criteria at 28.5℃ and fixed at hours or days after fertilization. To inhibit pigment formation, embryos were raised under 0.003% 1-phenyl-2-thiourea (PTU; Sigma, St Louis, MO).

2. Plasmids and Cloning

All plasmids for *in vivo* expression were made by subcloning zebrafish mib (wt, m178 : vector of full length of mib ORF was kindly provided by Yeo, S.Y. (Yeo & Chitnis, 2007)) and zebrafish DeltaD into a pCS2+ 3X FLAG (modified from pCS2+ FLAG) or pCS2+ vector. LC3-EGFP vector was kindly obtained from Lippincott-Schwartz's Lab, NIH, USA (Kim et al., 2008).

3. Whole-mount Immunohistochemistry

Embryos were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS). Whole-mount immunohistochemistry was performed as previously described with anti-rabbit LAMP-1 antibodies (abcam, 1:20) and anti-mouse monoclonal Zdd2 (abcam, 1:20) antibody. Secondary antibodies were used as follows : Alexa Fluor 488-conjugated goat anti-rabbit or mouse, Alexa Fluor 568-conjugated goat anti-rabbit or mouse (Jackson lab). After completion of immunohistochemistry, DAPI was used for nuclei staining.

4. Cell Culture, Transfection and Immunocytochemistry

COS-7 and HEK 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/㎖ penicillin and setreptomycin (Invitrogen) at 37℃ in 5% CO_2. Cells were transiently transfected with appropriate amount of plasmid DNAs using Fugene6 (Roche). After one day of transfection, cells were fixed in 4% paraformaldehyde/PBS and blocked with 10% goat serum in PBST. Immunocytochemistry was performed with anti-mouse monoclonal FLAG(Sigma) and Zdd2 (Abcam). Secondary antibodies were used as follows : anti-rabbit antibody conjugated with Alexa488 or 546. Nuclei were stained with DAPI.

5. Fluorescent Imaging

After immunohistochemistry, yolks were removed and embryos were mounted in 100% glycerol on glass slides and analyzed through a confocal laser scanning microscope (FV1000; Olympus). Cells on coverslips after cytochemistry were mounted in Prolong Gold antifade reagent (Invitrogen) and imaging was performed through a confocal laser scanning microscope (FV1000; Olympus).

RESULTS AND DISCUSSION

1. Distribution of DeltaD in the Developing Nervous System during Early Zebrafish Embryogenesis

DeltaD is known to be expressed in the neurogenic domain of the neural plate (Matsuda & Chitnis, 2009). DeltaD distribution was examined in zebrafish 3-somite (3ss), 10-somite (10ss), and 3 days post fertilization (3dpf) embryos. Throughout zebrafish neuronal development, deltaD distribution was restricted to the cytoplasmic puncta, not in the plasma membrane. DeltaD was expressed in the limited number of neuronal cells in the primary sensory neurons (Fig. 1A), primary motor neurons (Fig. 1B), and trigeminal ganglion cells (Fig. 1C) at 3-somite stages. DeltaD expression remained to be at the cytoplasmic puncta in the hindbrain at 10ss (Fig. 1E) and 30hpf (Fig. 1G).

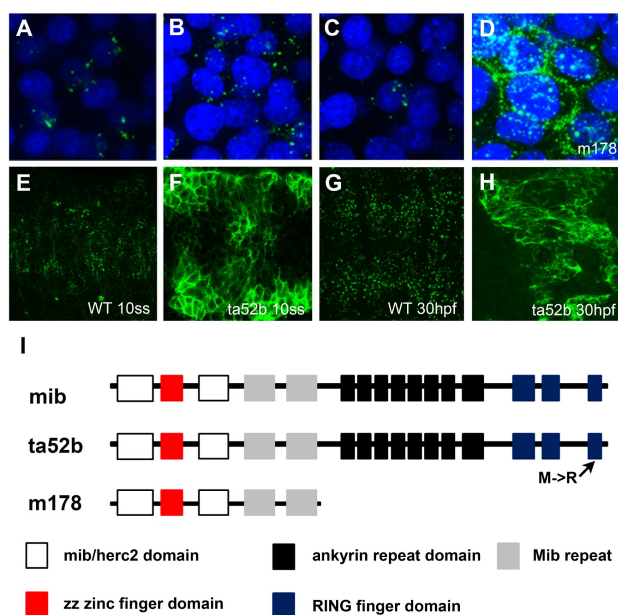

Fig. 1. Comparison of deltaD localization in the developing neuronal domain of zebrafish embryos. DeltaD is localized in the cytoplasmic puncta in wild type and accumulated in the plasma membrane in mib mutant zebrafish embryo. (A-D) DeltaD expression in wild-type (WT) and *mib*^{m178} 3-somite stage (3ss) zebrafish embryos. A. DeltaD in the primary sensory neurons. B. DeltaD in the primary motor neurons. C. DeltaD in the trigeminal ganglion cells. D. DeltaD in the neuronal domain of mib mutant (m178). (E,F) DeltaD in WT(E) and *mib*^{ta52b}(F) 10-somite stage (10ss) zebrafish embryos. (G,H) DeltaD in WT(G) and *mib*^{ta52b}(H) 30 dpf zebrafish embryos. In mib mutants, expression of deltaD is accumulated in the plasma membrane of the neurogenic domain. I. Scheme of mind bomb domain in wild type and mutant alleles.

DeltaD protein was highly detected in the plasma membrane in the mib mutants. The mib mutant (m178) did not utilize the ubiquitination of deltaD by lack of RING domain (Fig. 1I). DeltaD was accumulated in the cell surface of neuronal domain at the 3ss embryos (Fig. 1D). The mib mutant (ta52b) which has a point mutation of the third RING domain had the strongest neurogenic phenotype compared to m178 (Itoh et al., 2003). Even though level of neurogenic phenotype showed the different level of neurogenesis, the distribution pattern of deltaD was not changed in the mib mutant (ta52b) embryos (Fig. 1F and 1H).

In mib mutants, Matsuda and Chitnis confirmed that accumulation of deltaD in a plasma membrane is not due to higher production of deltaD (Matsuda & Chitnis 2009). These results suggest that mib1-mediated ubiquitination of deltaD may lead to endocytosis and degradation pathway to regulate Notch-Delta signaling in the neural tissue.

2. DeltaD Promotes Internalization by Mind Bomb1 in COS-7 Cells

To investigate the effect of E3 Ub ligase activity on the distribution of deltaD, COS-7 cells were transfected with mib-FLAG and zebrafish deltaD. When mib1 was transfected alone, it was broadly expressed and also localized in the cytoplasmic puncta on COS-7 cells (Fig. 2A). These pattern of mib localization was dependent on the expression levels of transfected mib1. In contrast, the mutant form of mib (m178) was not localized into the cytoplasmic puncta (Fig. 2B). It was also distributed into the plasma membrane. Zebrafish deltaD was mainly expressed in the surface and membrane of transfected cells (Fig. 2C).

When mib1 and deltaD were co-transfected, membrane-bound deltaD was endocytosed and internalized with mib1

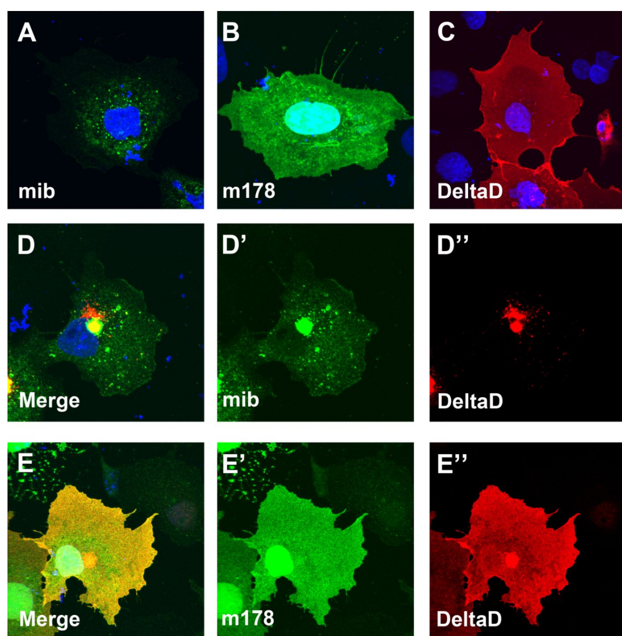

Fig. 2. Internalization of deltaD is dependent on mib activity in COS-7 cells. (A-C) Expression of mib - FLAG (A), mib mutant (m178) - FLAG (B), deltaD detected by zdd2 antibody (C) in COS-7 cells. (D-D'') Co-transfection of mib-FLAG (D': green), DeltaD (D'': red) and merged image of D'and D''(D). DeltaD is internalized with mind bomb expression in Cos7 cells. (E-E'') Co-transfection of mib mutant (m178) FLAG (E': green), DeltaD (E'': red) and merged image of E'and E''(E). DeltaD is mainly localized in the cell surface with mib mutant (m178) expression. Each transfection has been done for three times.

together (Fig. 2D-D''). Double positive vesicles were strongly increased in deltaD and mib1 co-transfection. However, no change of deltaD distribution occurred in m178 and deltaD co-transfected cells (Fig. 2E-E''). By ubiquitination of deltaD, mib1 can promote endocytosis of deltaD, sort it into the vesicular structures and internalize deltaD. From Itoh et al.'s results, m178 still bound to deltaD, but failed to ubiquitinate deltaD (Itoh et al., 2003). Therefore, ubiquitination of deltaD is an essential step to be removed from cell surface. Non-ubiquitinated Delta may accumulate in the plasma membrane, interfere with activation of Notch signaling and finally induce ectopic neuronal differentiation during zebrafish embryogenesis.

3. Mib1 is localized in the Autophagomal Vesicles in HEK 293T Cells

To determine the characterization of the vesicles in which mib1 was located, several markers were tested. Some vesicles of mib1 and Xdelta1 are known to be colocalized with GFP-tagged Rab9, a GTPase, seen in late endosomes and lysosomes (Itoh et al., 2003). Interestingly, the well known autophagosomal marker, LC3-EGFP, was co-localized with mib1-positive vesicles (Fig. 3A-C). In comparison to COS-7 cells, mib1 was more broadly distributed and few vesicles were LC3-GFP and mib1 double-positive in HEK 293T cells. However, mib1 was localized in the cytoplasmic puncta of both COS-7 cells and HEK 293T cells. Since mutant form of mib (m178) did not develop any vesicular structure, it was not surprising not to localize in the autophagosome (Fig. 3D-F). This colocalization study raises the possibility that mib1 might utilize the cytoplasm to vacuole targeting pathway mediated autophagy to regulate Notch signaling pathway.

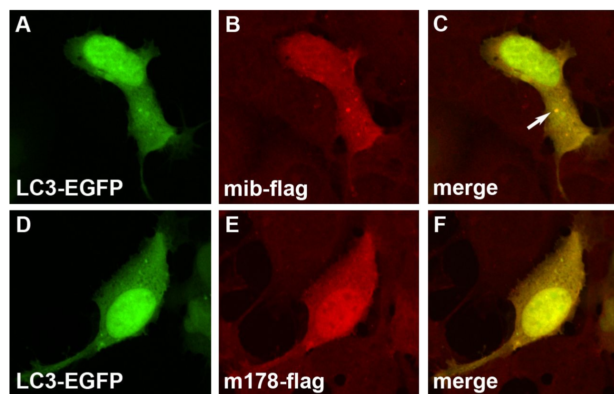

Fig. 3. The cytoplasmic puncta of mind bomb is colocalized with autophagosome in HEK 293T cells. (A-C) Co-transfection of LC3-EGFP (A) and mib-flag (B). Merged image of A and B (C). White arrow indicates the autophagosomal vesicles are colocalized with mind bomb. (D-F). Co- transfection of LC3-EGFP (D) and mib mutant (m178)- flag (E). Merged image of D and E (F). LC3-positive vesicles are not colocalized with mib mutant (m178). Each transfection has been done for three times.

4. Co-localization of DeltaD in the Autophagosomal Membrane in Zebrafish Embryos

Even though mib1 itself was localized in autophagosome, transfection study may reflect on the exaggeration of mib expression *in vitro*. To determine the nature of vesicles of deltaD in zebrafish neurogenic domain, LAMP-1 was used to detect autophagosome in zebrafish 30dpf embryos. LAMP-1 is the lysosomal-associated membrane protein which requires for both the completion of autophagy and the fusion of autophagosomes with lysosomes. DeltaD was expressed as a cytoplasmic puncta at the hindbrain. In the hindbrain, deltaD expression was matched with LAMP-1 staining (Fig. 4A-C). However, not all the vesicular structures were double-positive of deltaD and LAMP-1. Since deltaD is known to be degraded by ubiquitin-proteosomal pathway (Itoh et al., 2003), deltaD may be internalized and degraded by both ubiquitin-proteosomal and autophagosomal systems.

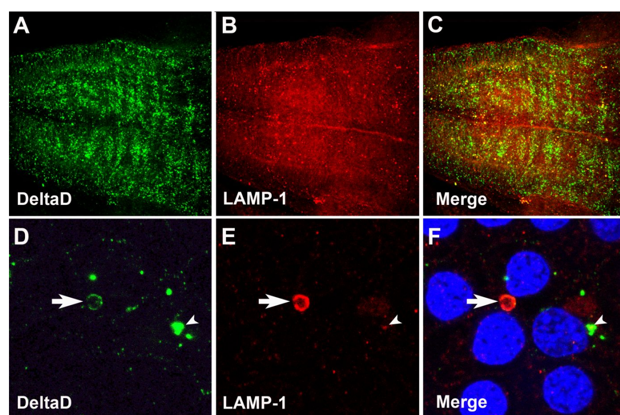

Fig. 4. Autophagy is contributed to deltaD degradation in the zebrafish embryos. DeltaD is co-localized on the autophagosome in the developing nervous system of zebrafish embryo. (A-C) DeltaD is expressed in the proneural domain of hindbrain as cytoplasmic puncta at 30hpf (A). LAMP-3 is expressed as a vesicular form in the zebrafish (B). Merged image of A and B. Yellow vesicles indicate the colocalization of deltaD and autophagosome. (D-F) LAMP-1 is showing the typical vesicular structure of autophagosome (E) and colocalized with deltaD (D). Merged image of D and E (F). White arrow is the typical processing form of autophagosome. White arrow and arrowhead indicate the co-localization of deltaD and LAMP-1.

At the high resolution imaging, typical processing autophagosomal structure was revealed by LAMP-1 staining (Fig. 4D-F). Surprisingly, deltaD was not located in the autophagosomal vacuole. It was localized in the autophagosomal membrane together. When cytosolic compounds are destined to be degraded through lysosome-dependent pathway, they are located in the vacuole engulfed by autophagosome, not in the autophagosomal membrane. Since the biogenesis of autophagosomal membrane is not clearly identified yet, the co-localization of deltaD in the autophagosomal membrane was unexpected. These data implies that unlike other cytosolic compounds, deltaD in the membrane is ubiquitinated by mib1, endocytosed and targeted into autophagosomal membrane. This may provide the new evidence that the plasma membrane is the one source of autophagosomal membrane. DeltaD, which may not be ubiquitinated by mib mutant, might not be internalized from cell surface, and sustain deltaD activity in the plasma membrane. So ubiquitination of deltaD is the essential step for endocytosis and regulate the balance of deltaD and Notch in neuronal plate.

In conclusion, these studies suggest that autophagy may be a novel pathway to regulate the Notch and deltaD protein level on the cell surface to determine lateral inhibition mechanism during embryogenesis. In addition, deltaD, a membrane-bound ligand, might be a good candidate to provide the source into autophagosomal membrane in cellular processing pathways. Future studies will be focused on the investigation of the candidates mediated by mib1 to regulate the activation/inactivation of Notch and its ligands for selection of cell fate from progenitors in autophagosomal pathway during embryogenesis.

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REFERENCES

Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284(5415):770-776.

Cecconi F, Levine B (2008) The role of autophagy in mammalian development: cell makeover rather than cell death. Dev Cell 15:344-357.

Ehebauer M, Hayward P, Arias AM (2006) Notch, a universal arbiter of cell fate decisions. Science 314(5804): 1414-1415.

Guo S, Liu M, Gonzalez-Perez RR (2011) Role of Notch and its oncogenic signaling crosstalk in breast cancer. Biochim Biophys Acta 1815(2):197-213.

Itoh M, Kim CH, Palardy G, Oda T, Jiang YJ, Maust D, Yeo SY, Lorick K, Wright GJ, Ariza-McNaughton L, Weissman AM, Lewis J, Chandrasekharappa SC, Chitnis AB (2003) Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Dev Cell 4(1): 67-82.

Jehn BM, Dittert I et al (2002) c-Cbl binding and ubiquitin-dependent lysosomal degradation of membrane-associated Notch1. J Biol Chem 277(10):8033-8040.

Jia L, Yu G, Beyer S, von der Mark K, Bielke W. (2009) Lysosome-dependent degradation of Notch3. Int J Biochem Cell Biol 41(12):2594-2598.

Kelsh RN, Brand M, Jiang YJ, Heisenberg CP, Lin S, Haffter P, Odenthal J, Mullins MC, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Kane DA, Warga RM, Beuchle D, Vogelsang L, Nüsslein-Volhard C. (1996) Zebrafish pigmentation mutations and the processes of neural crest development. Development 123:369-389.

Kim H, Kim C (2003) Zebrafish as a tool for functionnal genomics. Development and Reproduction 7(2):69-80

Kim PK, Hailey DW, Mullen RT, Lippincott-Schwartz J. (2008) Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. Proc Natl Acad Sci USA 105(52):20567-20574.

Klionsky DJ, Nemchenko A (2011) There is more to autophagy than induction: regulating the roller coaster. Autophagy 7:801-802.

Lai EC (2004) Notch signaling: control of cell communication and cell fate. Development 131(5):965-973.

Lewis J (1998) Notch signalling and the control of cell fate choices in vertebrates. Semin Cell Dev Biol 9(6): 583-589.

MacGrogan D, Luna-Zurita L, de la Pompa JL (2011) Notch signaling in cardiac valve development and disease. Birth Defects Res A Clin Mol Teratol 91(6):449-459.

Matsuda M, Chitnis AB (2009). Interaction with Notch determines endocytosis of specific Delta ligands in zebrafish neural tissue. Development 136(2):197-206.

Mizushima N, Levine B (2010) Autophagy in mammalian development and differentiation. Nat Cell Biol 12:823-830.

Mumm JS, Kopan R (2000). Notch signaling: from the outside in. Dev Biol 228(2):151-165.

Muskavitch MA (1994) Delta-notch signaling and Drosophila cell fate choice. Dev Biol 166(2):415-430.

Parr C, Watkins G, Jiang WG (2004) The possible correlation of Notch-1 and Notch-2 with clinical outcome and tumour clinicopathological parameters in human breast cancer. Int J Mol Med 14(5):779-786.

Rose SL, Kunnimalaiyaan M, Drenzek J, Seiler N. (2010) Notch 1 signaling is active in ovarian cancer. Gynecol Oncol 117(1):130-133.

Schouwey K, Delmas V, Larue L, Zimber-Strobl U, Strobl LJ, Radtke F, Beermann F (2007) Notch1 and Notch2 receptors influence progressive hair graying in a dose-dependent manner. Dev Dyn 236(1):282-289.

Simpson P (1990) Notch and the choice of cell fate in *Drosophila neuroepithelium.* Trends Genet 6(11):343-345.

Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, Pelosi G, Spaggiari L, Mazzarol G, Viale G, Pece S, Di Fiore PP. (2009) Alterations of the Notch pathway in lung cancer. Proc Natl Acad Sci USA

106(52):22293-22298.

Whitlock KE, Westerfield M (2000) The olfactory placodes of the zebrafish form by convergence of cellular fields at the edge of the neural plate. Development 127(17): 3645-3653.

Yeo SY, Chitnis AB (2007) Jagged-mediated Notch signaling maintains proliferating neural progenitors and regulates cell diversity in the ventral spinal cord. Proc Natl Acad Sci USA 104:5913-5918.

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