Studies on the Notch Signaling in the Retinal Regeneration of Adult Newt, *Cynops pyrrhogaster*

A Dissertation Submitted to the Graduate School of
Life and Environmental Sciences
the University of Tsukuba
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Science
(Doctoral Program in Functional Biosciences)

Kenta NAKAMURA

Contents

Abbreviations

AT: acetylated tubulin

bHLH: basic helix loop helix

CGZ: circumferential germinal zone

CMZ: ciliary marginal zone

CNS: central nervous system

DAPT: N-[-N-(3,5-Diflurophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester

DDBJ: DNA Data Bank of Japan

DEPC: diethylpyrocarbonate

DIG: Digoxigenin

DSL: Delta/Serrate/Lag-1

FGF-2: fibroblast growth factor 2

GCL: ganglion cell layer

GFAP: glial fibrillary acidic protein

Hes: Hairy/Enhancer of split

IHC immunohistochemistry

INL: inner nuclear layer

IPL: inner plexiform layer

IR: immunoreactivity

ISH: in situ hybridization

Ngn: neurogenin

NICD: Notch intra cellular domain

NPR: non-pigmented region

ONL: outer nuclear layer

OPL: outer plexiform layer

ORF: open reading frame

PCM: pigmented ciliary margin

po: post-operative

RPE: retinal pigment epithelium

RT: room temperature

TEA: triethanolamine

1. **Abstract**

The retina is a part of the central nervous system (CNS) in the eye, and disease or traumatic injury of the retina is very serious against the normal life. In contrast to other vertebrates, newts can regenerate, even in adulthood, their entire retinas upon a surgical removal of the neural retina through a process of the transdifferentiation (cell-type switch) of the retinal pigment epithelium (RPE) cells. Cellular and molecular pathways underlying the retinal regeneration remain virtually unknown. Notch signaling is one of the most important systems for cell specification. In the present study, the involvement of Notch signaling in retinal regeneration by transdifferentiation of pigment epithelium cells was investigated in the adult newt *Cynops pyrrhogaster*.

The newt Notch receptor *Notch-1* was identified and its expression patterns were examined by *in situ* hybridization.

Notch-1 expressing cells appeared in solitary along the regenerating retina of one-two cells in thickness (stage E-3) and increased in number as the regenerating retina increases in thickness. *Notch-1* expression was decreased in the central retina in association with cell differentiation and became restricted in the peripheral immature retina. To attenuate Notch signaling, DAPT was injected into the intra-abdominal space or a small cotton ball containing DAPT was implanted into the anterior eye chamber. In both trials, a cluster of neurons appeared earlier than in the normal regeneration, along the regenerating retina of 1-3 cells in thickness (stage E-3 to I-1), suggesting that neuronal differentiation was hastened by DAPT administration. Immunoblot analysis revealed that DAPT could perturb the processing of Notch-1. Similar results were obtained in the newt embryonic retinal development. These results suggest that Notch-1 signaling system may be set again to regulate

neurogenesis during retinal regeneration. However, PCR analysis revealed that the adult newt RPE cells express *Hes-1*, *neurogenin1* and sometimes *Delta-1*, all of which are differently regulated in association with retinal regeneration, implying that Notch signaling might be involved also in early process of transdifferentiation.

2. **Introduction**

The retina is a part of the central nervous system (CNS) in the eye, and disease or traumatic injury of the retina is very serious against the normal life. Recent advances in stem-cell biology and regenerative medicine have revealed that pigment epithelium (PE) cells of adult eye, which transdifferentiate into retinal cells *in vitro*, are one of the most hopeful cell sources for future cell therapy of degenerated retina (Ahmad et al., 2000; Tropepe et al., 2000; Haruta et al., 2001; Amemiya et al., 2004; Coles et al., 2004). As yet, however, we do not have clinical strategies to regenerate the whole retina from PE cells in the eye of patient. In contrast, some urodele amphibians such as newts can regenerate the entire retina through transdifferentiation of the retinal pigment epithelium (RPE) cells even when the neural retina is completely lost (Mitashov, 1996; Raymond and Hitchcock, 1997, 2000; Grigoryan, 2001; Reh and Fischer, 2001; Del Rio-Tsonis and

Tsonis, 2003; Hitchcock et al., 2004; Tsonis and Del Rio-Tsonis, 2004; Chiba/Hoshino/Nakamura et al., 2006). Therefore, the urodelian retinal regeneration can be an *in vivo* transdifferentiation model of PE cells to gain critical information toward new clinical strategies for retinal regeneration. However, cellular mechanisms and molecular pathways underlying the retinal regeneration of adult urodeles remain virtually unknown because of technical limitations (Chiba et al., 2004; Ueda et al., 2005; Chiba/Hoshino/Nakamura et al., 2006).

Notch signaling is one of the most important systems for cell specification. In the present study, I addressed using *C. pyrrhogaster* experimental system, whether Notch signaling is involved in retinal regeneration of adult urodels.

2.1. Structure and function of the retina

Neural retina is a part of CNS in the eye, involved in early process of vision; it transduces light energy from environment into sensory neural responses. RPE gives many physiological supports to neural retina such as nutrient supply, phagosytosis of shed photoreceptor membrane-bound discs, and absorption of light. All known retinas of vertebrates contain at least five major types of retinal neuron (photoreceptor, horizontal, bipolar, amacrine, and ganglion cells) and at least one type of glia (Müllar cells)(Fig. 1). These cells are arranged in a penta-laminar array; three nuclear layers (outer nuclear layer, ONL; inner nuclear layer, INL; ganglion cell layer, GCL) and two synaptic layers (outer plexiform layer, OPL; inner nuclear layer, IPL).

2.2 Retinal development

The retina is considered as a part of the brain. Eyes of vertebrates derive from the neural tube during development, like other CNS organs. Diencephalon wall of embryo evaginates to form optic vesicle in early neural development. The optic vesicle invaginates to form two-layered optic cup. The inner layer increases its cell thickness and differentiates to multi-layered neural retina, while the outer layer differentiates into mono-layered RPE (Fig. 2).

2.3. Retinal repair and regeneration

2.3.1. Newts

A strong ability of eye regeneration in adult urodele amphibians such as newts has been studied for more than 100 years (for reviews, see Del Rio-Tsonis and Tsonis, 2003; Tsonis and Del Rio-Tsonis, 2004). Adult newt can regenerate the lens through transdifferentiation of the dorsal side of iris pigment epithelium (IPE) cells. This process is known as ‘Wolffian regeneration’, a classic example of how cells can regenerate an organ (for reviews, see Del Rio-Tsonis and Tsonis, 2003; Tsonis and Del Rio-Tsonis, 2004). Currently, studies on the molecular mechanisms of newt lens regeneration are progressing with modern biological techniques (Grogg et al., 2005). Retina regeneration in adult newts also has been studied as an example of CNS regeneration. Even though they lose their neural retinas, they can regenerate the entire retina through transdifferentiation of RPE cells (Mitashov, 1996; Raymond and Hitchcock, 1997,

2000; Grigoryan, 2001; Reh and Fischer, 2001; Del Rio-Tsonis and Tsonis, 2003; Hitchcock et al., 2004; Tsonis and Del Rio-Tsonis, 2004; Chiba/Hoshino/Nakamura et al., 2006). Upon removal of the neural retina, RPE cells undergo a loss of pigment granules (depigmentation) and proliferation, generating a retinal rudiment and the RPE itself (Wachs, 1920; Stone, 1950; Hasegawa, 1958; Keefe, 1973). In the retinal rudiment, cells continue to proliferate, producing retinal neurons and glia that eventually reform a new functional retina (Negishi et al., 1992; Saito et al., 1994; Chiba et al., 1997; Cheon et al., 1998; Chiba, 1998; Sakakibara et al., 2002, Oi et al., 2003a,b; Chiba et al., 2005). The process from RPE to neural retina is called 'transdifferentiation'. Recently, we re-described using modern biological techniques the process of retinal regeneration of the adult Japanese fire-bellied newt (*Cynops pyrrhogaster*) after a complete removal of neural retina (Chiba et al., 2006) and

succeeded in dividing this process into nine stages (Chiba/Hoshino/Nakamura et al., 2006; see Fig. 3).

In the adult newt eye, there are other cell sources for retinal regeneration for example, immature regions located in the anterior margin, which correspond to the inner non-pigmented epithelium (NPE) in the ciliary body and the ciliary marginal zone (CMZ) that contain the retinal stem cells and the retinal progenitor cells, respectively (Haynes and Del Rio-Tsonis, 2004; Chiba/Hoshino/Nakamura et al., 2006). Cells in these regions are capable of differentiating and forming all types of neural retina, contributing to the peripheral retinal regeneration.

2.3.2. Other vertebrates

As like newts, many vertebrates, from fishes to mammalians, have a capacity of regenerating their neural retinas following damage of their original retinas (for reviews, see Del Rio-Tsonis and Tsonis, 2003). However, such capacities of vertebrates are restricted to their immature stages. For example, in the embryonic chick, retinal regeneration after retinectomy is induced by fibroblast growth factor 2 (FGF-2) treatment (Park and Hollenberg, 1989, 1991, 1993), though the regenerated retina has the reverse polarity, its photoreceptor layer located in the innermost (closest to the lens) layer.

Fishes can repair their retinas even in adult, from several cell sources including neural precursors from the circumferential germinal zone (CGZ), rod precursors in the ONL, quiescent stem cells distributed throughout the retina, and Müller cells (for

reviews, see Reh and Levine, 1998; Del Rio-Tsonis and Tsonis, 2003; Haynes and Del Rio-Tsonis, 2004, Hitchcock et al., 2004).

Recently, it was reported that postnatal chick and mammalians (including human) also have capacities of retinal repair from Müller cells (Fischer and Reh, 2001), or pigmented ciliary margin (PCM), which has a proliferative potential *in vivo* (Ahmad et al., 2000). PCM cells can produce progenitors/stem cells *in vitro*, and even in mammalians, these cells can give rise to a variety of retinal cells (Ahmad et al., 2000; Tropepe et al., 2000). Although cells of vertebrates have abilities of transdifferentiation, no known vertebrates except urodeles, can regenerate their entire retinas.

2.4.Notch signaling

Notch signaling plays important roles in many biological events such as segmentation, angiogenesis, intestine cell renewal, lymphoid cell genesis, myogenesis, and neurogenesis as regulating stem cells (for reviews, see Aulehla and Herrmann, 2004; Leong and Karsan, 2006; Crosnier et al., 2006).

Notch signaling is crucial for the retinal development in vertebrates (Ishibashi et al., 1994; Austin et al., 1995; Ahmad et. al., 1995; Dorsky et al., 1995; Tomita et al., 1996; Ahmad et al., 1997; Dorsky et al., 1997; Bao and Cepko, 1997; Henrique et al., 1997; for reviews, see Beatus and Lendahl, 1998; Rapaport and Dorsky, 1998). Figure 4 is a schematic hypothetical diagram of Notch signaling in vertebrates. Notch is a single pass transmembrane receptor and activated by Delta/Serrate/Lag-1 (DSL) ligands presented by neighboring cells (for reviews, see Robey, 1997; Lewis, 1998; Artavanis-Tsakonas et. al., 1999;

Justice and Jan, 2002; Arias et. al., 2002). Upon activation, Notch is processed to release the intracellular domain (NICD) by γ -secretase and the NICD is transferred into the nucleus to form a complex with RBP-J, which is a transcriptional repressor. The NICD-RBP-J complex induces as transcription activator expression of down stream genes (for review, see Kageyama et al., 2005). Hairy/Enhancer of split (Hes), which is a basic helix-loop-helix (bHLH) transcription factor, is one of the intracellular effectors of Notch. Hes-1/Hes-5 represses the transcription of other bHLH genes such as neurogenin (Ngn), achaete-scute or atonal homologues (ASH; ATH, respectively) that positively regulate neurogenesis (Ishibashi et al., 1994; Tomita et al., 1996; Hojo et al., 2000; for review, Hatakeyama and Kageyama, 2004, Kageyama et al., 2005). Expression analysis have revealed that *Notch-1* and *Notch-3* are both expressed in the central retina, while *Notch-2* is expressed in the

peripheral retina and RPE (Lindsell et al., 1996; Bao and Cepko, 1997). Reduction of Notch-1 levels in early embryonic retina or embryonic retinal cells in culture enhance production of ganglion cells, which is the first-born retinal cell type (Austin et al., 1995, Silva et al., 2003). On the other hand, constitutively activated Notch-1 induces increasing of undifferentiated progenitor like cells (Dorsky et al., 1995) or cells having glial properties (Furukawa et al., 2000; Scheer et al., 2001), suggesting that Notch-1 contributes to maintenance of progenitor cells and negatively regulates the neuronal determination in retinal development.

In contrast to those developmental studies, there are few evidences for Notch signaling in retinal regeneration of adult urodeles (Kaneko et al., 2001).

2.5. Aims of the present study

To address whether Notch signaling is involved in retinal regeneration of adult urodeles by using *C. pyrrhogaster* experimental system, I isolated a Notch homologue Notch-1 of *C. pyrrhogaster* newt and analyzed its gene expression patterns and functions of Notch signaling during adult retinal regeneration in comparison with those during embryonic retinal development. In addition, I analyzed temporal gene expression patterns of homologues of Delta (a Notch ligand), Hes (a Notch intracellular effector) and neurogenin (a proneural gene) during retinal regeneration in comparison with those of the Notch-1 receptor and neural markers. The results provide the first evidence suggesting that Notch signaling is crucial for retinal regeneration by transdifferentiation of PE cells.

3. **Materials and Methods**

3.1. *Animals.*

Adult *C. pyrrhogaster* newts (total body-length: 11-12 cm) were purchased from local suppliers in Japan, and housed in polyethylene containers with water at room temperature (RT) under a normal day-night cycle of lighting. Fertilized eggs of newts were obtained as described previously (Cheon and Saito, 1999) and kept at 22°C. Developmental stages were determined according to the criteria of Ichikawa and Kajishima (1965). The research reported herein was performed under the guidelines established by the University of Tsukuba animal use and care committee.

3.2. *Retinectomy.*

Adult newts were anesthetized in tap water containing 0.1% FA100 (4-allyl-2-methoexxyphenol; Tanabe, Japan) in dark for 2

hr, and then placed under a binocular. Dorsal half of the left eye was cut open along the corneal-scleral junction and the neural retina together with the lens was carefully removed by a fine needle and forceps (Fig. 5). During this operation, the vitreous chamber was gently rinsed with a sterile newt saline solution (115 mM NaCl, 3.7 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 18 mM D-glucose, 5 mM HEPES, pH 7.5 adjusted with 0.3 N NaOH). After operation, the eye flap consisting of the iris and cornea was carefully placed back to its original position. The operated animals were maintained in moist containers and allowed to recover at 22°C (the day-night cycle was 12 hr: 12 hr). They were sacrificed on selected days post-operative (po) under anesthesia.

Figure 3 shows a schematic diagram of retinal regeneration after retinectomy.

3.3. *Molecular cloning.*

A small cDNA fragment (284 bp) of *Notch-1* was amplified by PCR from a cDNA library (6 x 10^6 pfu/ml) of newt embryos (neural plate to tailbud stages) constructed using ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene Cloning Systems, La Jolla, CA). The primer set:

sense, 5'-atgat(c/t)gc(a/c)tc(c/g)tg(c/t)ag(c/t)ggagg-3';

antisense, 5'-atctggaa(g/t)ac(a/g/t)cc(c/t)tg(a/g)gcatc-3'. On the basis of the nucleotide sequence information PCR-Digoxigenin (DIG) probes were synthesized (Roche Diagnostics, Mannheim, Germany) and a partial cDNA clone of *Notch-1* (3274 bp) was screened from the cDNA library. 5'-RACE PCR was performed according to the SMART RACE protocols (SMART RACE cDNA Amplification Kit, Clontech, Palo Alto, CA) and then a full-length clone (7885 bp) containing the open reading frame (ORF) was obtained by end-to-end PCR (Fig. 6). Cloning was

made more than three times independently and PCR-associated errors in the nucleotide sequence were corrected. The nucleotide sequence has been deposited to the DNA Data Bank of Japan (DDBJ) with Accession No. AB095016. Homology search for the cDNA clone was performed with FASTA algorithm on the DDBJ www server (http://www.ddbj.nig.ac.jp/E-mail/homology.html). Multiple alignment and phylogenetic tree analysis of homologous proteins were made with clustalW program on the same server. Sequence data compared in the present study were taken from DDBJ (Accession number is shown in parenthesis): *Drosophila melanogaster* (Dm) Notch (M11664), human (H) Notch-1 (AF308602), mouse (M) Notch-1 (AF508809), rat (R) Notch-1 (X57405), chick (C) Notch-1 (AF159231), *Xenopus laevis* (X) Notch-1 (M33874), zebrafish (Z) Notch-1 (U57973), H-Notch-2 (AF315356), M-Notch-2 (D32210), R-Notch-2 (M93661), H-Notch-3 (U97669), M-Notch-3 (X74760), R-Notch-3

(AF164486), Z-Notch-3 (AF152001), newt Notch-like-protein (AF296839). Molecular cloning of Delta-1 and Ngn-1 protocols were basically the same as that for Notch-1. At first, small cDNA fragments of *Delta-1* (317 bp) and *Ngn-1* (133 bp) were obtained by PCR with the following primer sets:

for *Delta-1*, [sense, 5’-aagtactccta(c/t)cg(a/t/c)tttgtgtg-3’;

antisense, 5’-tggcagttgca(c/t)tgccatggctg-3’];

for *Ngn-1*, [sense, 5’-gggagcggaa(c/t)cgcatgcacg-3’;

antisense, 5’-agcgccca(a/g)atgtagttctt-3’]. Then, using PCR-DIG probes, a partial cDNA clone of *Delta-1* (2574 bp) and a full-length clone of *Ngn-1* (1719 bp) were screened. For *Delta-1*, 5’-RACE PCR was performed, and a full-length cDNA clone (3646 bp) was obtained by end-to-end PCR. Cloning was made more than three times independently and PCR-associated errors in the nucleotide sequence were corrected. The nucleotide sequence

of each clone has been deposited to DDBJ with Accession No.; *Delta-1*, AB095017; *Ngn-1*, AB065284.

3.4. *In situ hybridization* (ISH).

Newt embryos (stage 26 to 60), juveniles (1-2 weeks after metamorphosis; 3-4cm in total body length) and adult eyeballs were fixed in 4% paraformaldehyde (PFA) in 80% PBT [80% PBS (pH 7.4, DEPC-treated)/0.1% Tween 20] for 72h at 4°C. Fixed preparations were rinsed with 80% PBT two times and transferred into 30% sucrose in 80% PBT. After the eyeballs had sunk in the sucrose solution (it took 2-3 hr), they were embedded into O.C.T. compound (Sakura Finetecnical Co.) and cryosectioned transversely at about 20 μm thickness to pre-cleaned MAS coated micro slide glass (76 x 26 mm, MATSUNAMI, Japan).

Full-length Notch-1 cDNA was amplified and inserted into the pCRII plasmid vector and cloned in both direction (pCRII-TOPO TA-cloning system, Invitrogen). Both clones were cut by restriction enzyme Not I. Digoxigenin (DIG)-labelled antisense/sense RNA probes for *Notch-1* ISH were produced from the *Notch-1* full-length cDNA clone by DIG RNA Labeling Kit (SP6/T7, Roche Diagnostics) according to the manufacturer's instruction and hydrolyzed into ~600 bp fragments by 40 mM $NaHCO_3$/60 mM Na_2CO_3 (Fig. 7). Probes for the voltage-gated Na^+-channel (*Cp-Na_V1*; Accession No. AF123593) and rhodopsin (Accession No. AB043890) were produced as described by Hirota et al. (1999) and Sakakibara et al. (2002), respectively. The probes were purified by removing unicorporated ribonucleotides and small RNA fragments using a quick spin column (G-50 Sephadex Columns for Radiolabeled RNA Purification, Roche Diagnostics).

The tissue sections were rinsed in 80% PBT for 10 min at room temperature (RT), those were treated with 10 μg/ml ProteinaseK (Roche Diagnostics) in 80% PBT for 5 min at 37°C, re-fixed in 4% paraformaldehyde for 30 min at RT, treated with 2 mg/ml glycine in 80% PBT for 10 min at RT, and rinsed twice in 80% PBT for 5 min each at RT. The tissue sections were further treated with 0.1 M triethanolamine (TEA) and then with 0.5% acetic anhydride in 0.1 M TEA for 10 min each at RT, and washed four times in 80% PBT for 5 min each at RT.

The tissue sections were incubated in 2 x SSC/50% formamide/0.1% Tween 20 for 30 min at RT, and then with 100 ng/ml DIG RNA probe in hybridization mixture (1 mg/ml Torula tRNA, 20 mM Tris-HCl (pH 8), 2.5 mM EDTA (pH 8), 1 x Denhardt's solution, 300 mM NaCl, 10% Dextran Sulfate, 50% formamide) for 30 min at 70°C and sequentially overnight at 50°C. The tissue sections washed in 2 x SSC/50%

formamid/0.1% Tween 20 for 30 min at 45°C, twice in 10 mM Tris-HCl (pH 8)/500 mM NaCl for 5 min each at RT, and treated with 20 µg/ml RNase A in 10mM Tris-HCl (pH 8)/500 mM NaCl for 30 min at 37°C. Those were washed in 2 x SSC/50% formamid/0.1% Tween 20 for 30 min at 45°C, in 1 x SSC/50% formamid/0.1% Tween 20 for 30 min at 45°C, in 1 x SSC/50% formamid/0.1% Tween 20 for 30 min at RT, and finally six times in 100mM Tris-HCl (pH 7.5)/150 mM NaCl/0.1% Tween 20 for 15 min each at RT.

The tissue sections were incubated with 1% (w/v) Blocking Reagent (Roche Diagnostics) in 100 mM maleic acid/150 mM NaCl for 30 min at RT, and then with sheep anti-DIG alkaline phosphatase antibody (Roche Diagnostics, 1:2000 (0.375 U/ml) in 100 mM maleic acid/150 mM NaCl) overnight at 4°C. those were washed six times in 100 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.1% Tween 20 for 15 min each at RT, treated twice with 2

mM levamisole in 100 mM Tris-HCl (pH 9.5)/100 mM NaCl/50 mM $MgCl_2$ for 5 min each at RT, and then mounted with NBT/BCIP (Roche diagnostics, 1:50 in 100 mM Tris-HCl (pH 9.5)/100 mM NaCl/25 mM $MgCl_2$/2 mM levamisole/0.1% Tween20/10% polyvinyl alcohol) and incubated in the dark at RT (Fig. 8). The reaction was terminated as the signal to background noise ratio reached the maximal. The tissue sections were washed twice in PBS for 30min each at RT. The tissue sections were mounted with 50% glycerol/5 mM levamisole in PBS and covered.

3.5. *Immunohistochemistry* (IHC).

Tissues for IHC were fixed in 4% PFA in PBS for 15 hr at 4°C. Fixed preparations were washed thoroughly in 80% PBT or PBS,

and both were cryosectioned transversely at about 20 μm in thickness.

The primary antibodies for IHC were as follows: for retinal neurons, rabbit polyclonal anti-Na^+ channel (III-IV loop) antibody (1:200; Cat. #06-811, Upstate Biotechnology, Lake Placid, NY; Cheon et al., 1998) and mouse monoclonal anti-acetylated tubulin (AT) antibody (1:2000; Clone 6-11B-1, Cat. #T6793, Sigma, Saint Louis, MO; Kaneko et al., 1999); for retinal progenitor cells and Müller glia cells, Cy3 conjugated mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:2000; Clone G-A-5, Cat.#C9205, Sigma; Umino and Saito, 2002). The secondary antibodies were biotinylated goat anti-rabbit IgG antibody (1:500; Cat. #BA-1000, Vector, Burlingme, CA) and FITC conjugated goat anti-mouse IgG antibody (1:150; Cat. #115-095-003, Jackson ImmunoResearch, West Grove, PA). Cell

nuclei were sometimes counter-stained with DAPI (1:50000; Molecular Probes, Eugene, OR).

For immunofluorescence labeling, tissue sections were rinsed in PBS, 0.2% Tritonx-100 in PBS, and PBS for 15 min each, and incubated in a blocking solution (2% serum of host animal of secondary antibody/0.2% TritonX-100 in PBS) for 2 hr. Double labeling of tissue sections was performed conventionally. In experiments where tissue sections were double-labeled with the Cy3-GFAP and acetylated tubulin mouse antibodies, labeling was performed separately (acetylated tubulin was first, and then Cy3-GFAP). In brief, sections were incubated in the AT antibody diluted with the blocking solution overnight at 4°C, washed thoroughly in PBS, incubated in the FITC-conjugated secondary antibody diluted with PBS containing 0.2% TritonX-100 for 4 hr at RT. After the sections washed in PBS, incubated in 10% normal mouse serum (Cat. #015-000-120, Jackson

ImmunoReserch) diluted with PBS for 2 hr at RT, washed again in PBS, and incubated in non-labeled goat anti-mouse IgG antibody (Fab fragment) (Cat. #115-007-003, Jackson ImmunoResearch; 1:100) diluted with PBS overnight at 4°C, washed again in PBS, and then, incubated in the Cy3-GFAP antibody diluted in PBS (Fig. 9). Following immunofluorescence labeling, cell nuclei were sometimes counter-stained with DAPI (Molecular Probes; 1:50000).

3.6. *Administration of DAPT.*

To attenuate Notch signaling, we used a γ-secretase inhibitor DAPT (N-[-N-(3,5-Diflurophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester, Cat.#565770, Calbiochem, San Diego, CA) which is known to perturb Notch signaling through inhibiting release of a Notch intracellular domain (NICD), which translocate to the

nucleus to regulate transcription of effector genes (Geling et al., 2002; James et al., 2004; Schweisguth, 2004; Bernardos et al., 2005). DAPT was dissolved in DMSO (Cat. #D2650, Sigma) at a concentration of 10 mM and stored in the dark at -20°C. In experiments to examine effects of DAPT on retinal development, newt embryos of stage 26 were cultured in 1.7% agarose-coated 35-mm dishes (Falcon 3001; Becton Dickinson, Franklin Lakes, NJ) filled with ~3 ml of 0.1 x MMR (in mM: 10 NaCl, 0.2 KCl, 0.1 $MgSO_4$, 0.01 EDTA, 0.5 HEPES, pH7.6) containing 5-100 μM DAPT for 5 days in the dark at 25°C. The number of embryos in a dish was controlled less than 6. Immunohistochemical data shown here were obtained from embryos cultured in the presence of 100 μM DAPT because under this condition every embryo showed a morphological abnormality consistently (see Fig. 14B).

In experiments to examine effects of DAPT on retinal regeneration of adult newt, intra-abdominal injection of DAPT

and intraocular implantation of a cotton ball containing DAPT were carried out. In the intra-abdominal injection, 50 μl of a physiological saline solution (in mM: 115 NaCl, 3.7 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 18 D-glucose, 5 HEPES, pH 7.5 adjusted with 0.3 N NaOH) containing 200 μM-10 mM DAPT was injected into the abdominal space of animal (body-weight: ~5 g) manually through a needle (27G, TERUMO, Japan) attached to a 1ml syringe between 17 and 23 days po. The number of injections and their intervals were varied (see Results). In the intraocular implantation, a knot (~0.5 mm in diameter) of a cotton thread was soaked in 200 μM-10 mM DAPT solution and carefully implanted into the anterior eye chamber through a slit made on the cornea (see Fig. 6L). For the mock control, the same experiments were carried out at least three times with solutions containing the solvent DMSO only. Apoptotic cell death was assayed with NeuroTACS Kit (Cat.

4820-30-K, Trevingen, Inc., Gaithersburg, MD) according to the manufacturer's instruction.

In this study, I tried intraocular injection of DAPT as well. Since one-time injection of DAPT seemed not to affect retinal regeneration, I tried every day or every other day injection from 19 days po (2 to 5 times in total). Under these conditions, morphological abnormalities such as a local enlargement or a 'rosette' formation were sometimes observed in regenerating retinas. However, these abnormalities included artifacts by injection because similar abnormalities were observed even by the injection of normal saline solution only. Therefore, I gave this method up and do not show the results here (for injection conditions we tried, see supplemental materials).

3.7. *Immunoblot analysis.*

Posterior half of eyeballs (eye-cup) of 19 days po, which contain E-3 to I-1 stage regenerating retina (see Fig. 3), were prepared under sterilized conditions. Anesthetized animals were beheaded, and their heads was rinsed in 70% ethanol. The eyeballs were enucleated and collected containing PBS. The eyeballs were put on nitrocellulose filter membranes (0.42 μm pore size, Millipore) cornea side up, and cut along its equator, and then the anterior half of them were carefully removed to make eye-cups. The eye-cups were placed in 35-mm culture dishes (one eye-cup per dish) filled with 80% L-15 medium (Invitrogen, Carlsbad, CA) containing either 100 μM DAPT/2% DMSO or 2% DMSO, and incubated for 20 hr in the dark at 25°C. Three eye-cups of each condition were transferred into 60 μl of 2 x sample buffer [100 mM Tris-HCl, pH 7.5, 4% (w/v) SDS, 10% 2-mercaptoethanol (Sigma), 20% glycerol, 0.01% (w/v)

bromophenol blue (Wako, Japan)] in a 1.5 ml tube on ice, frozen in liquid nitrogen, thawed on ice, and sonicated in chilled water for 10 min. The samples were immediately boiled for 5 min at 95°C and centrifuged (12,000 rpm) for 10 min at 4°C. Supernatants (10 μl each) were placed on wells of 7.5% gel (Ready gel J, Bio-Rad, Hercules, CA), and proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Immun-Blot PVDF membrane, 0.2 μm pore size, Bio-Rad) electrophoretically (Mini transblot cell, Bio-Rad; 100 V, 1 hr). The blotting membrane was rinsed in TBS [100 mM Tris-HCl (pH 7.4), 150 mM NaCl] for 10 min, and incubated in a blocking solution (2% goat normal serum/2% non-fat skim milk in TBS) containing Avidin D (1:50; Avidin/Biotin Blocking Kit, Vector) for 1hr. After the membrane was washed 3 times in TBS (10 min each), it was incubated in rabbit anti-Notch-1 antibody (1:500; Cat. #N5375-01, United States Biological, Swampscott, MA)

diluted with the blocking solution containing Biotin (1:50; Avidin/Biotin Blocking Kit, Vector Labs) overnight at 4°C. This antibody recognizes a peptide corresponding to the residues 2459-2474 within the intracellular domain of newt Notch-1. The membrane was washed three times again in TBS and incubated in the biotinylated goat anti-rabbit IgG antibody (1:1000; Vector) diluted with the blocking solution for 4 hr at RT. After a complete wash of the antibody in TBS, the membrane was incubated in a mixture of Avidin and Biotin Complex (Vectastain ABC Elite kit, Vector), and reactions were visualized with a DAB substrate kit (Vector).

3.8. *PCR analyses.*

To exclude a possibility of contamination of intrinsic neural stem/progenitor cells in the peripheral margin of neural retina, the

total RNA was isolated from the eye-cup. Muscles and connective tissues attached to the sclea were removed as much as possible. Six eye-cups were collected at selected days po in a 1.5 ml RNase-free siliconized tube (Ambion) filled with PBS (DEPC-treated) on ice. Six eye-cups without neural retinas prepared from isolated normal eyeballs were used for the day-0 sample (i.e., immediately after retinectomy). Six neural retinas without peripheral margin were also collected for comparison. Total RNA of each-day sample was isolated with SV Total RNA Isolation System (Promega) according to the manufacturer's instruction. This system incorporates a DNase treatment step that substantially reduces genomic DNA contamination. The eluted RNA solution (100 μl) of each day sample was vacuum-dried and re-suspended in 13 μl nuclease-free water. Then, using SMART cDNA Library Construction Kit (Clontech), the first-strand cDNA was synthesized and nonselectively amplified by a long distance

PCR of 32 cycles according to the manufacturer's instruction. The amplified SMART cDNA was used as the template for subsequent PCR gene expression analysis. The templates were used at a concentration of 1 μl per 50 μl PCR mixture.

To investigate gene expression of adult RPE cells, single-cell RT-PCR analysis was carried out using solitary RPE cells isolated from the normal eyeballs as follows. RPE-choroid tissues were collected from retina-less eye-cups by separating them from the sclera with a fine needle. The tissues were treated with 1 mg/ml elastase (Cat. #11027905001, Roche Diagnostics) in EGTA solution (115 mM NaCl, 3.7 mM KCl, 10 mM EGTA, 18 mM glucose, 10 mM HEPES and 0.001% Phenol red, pH7.5) for 2 hr at 28°C. After the tissues were rinsed several times in PBS, RPE cells were isolated by a gentle pipetting and collected in a plastic dish. Template cDNAs for PCR were constructed using Cells-to-cDNA^{TM} II Kit (Ambion, Austin, TX) according to the

manufacturer's instruction. One solitary RPE cell was picked up using a micropipetter (20 μl pipetman set at 0.5-1 μl) under a binocular and transferred into a tube (1.5 ml RNase-free tube, Ambion) containing 25 μl Cell Lysis Buffer. Following a treatment with DNaseI, the solution was divided into two tubes; one was allowed the reverse transcription to obtain the template cDNA and the other was used for the negative control. In the present study, we used template cDNAs with which the intense product band of RPE marker *RPE65* (Accession No. AB095018; Chiba/Hoshino/Nakamura et al., 2006) was detected by the first 45 cycle PCR. The template cDNAs and PCR products for nested PCR were used at a concentration of 0.5 μl per 50 μl PCR mixture.

See Table 1) and 2) for each PCR conditions and primers,

Notch-1, *Delta-1*, *Hes-1*, *Ngn-1* and neural markers [*N-CAM* (Accession No. D85084), *Cp-Na$_V$1*, Opsin (*Cp-L;* Accession No.

AB043891)] were examined for their expression patterns as well as those of *RPE65*, *Pax-6* (Accession No. D88741) and *Ef1-*α (Accession No. AB005588). PCR primers, product sizes and cycle numbers are listed in Supplemental Table 1 and 2. KOD Dash (Toyobo, Japan) was used for PCR enzyme. To analyze quantitative changes of gene expression in association with retinal regeneration, the number of PCR cycle was tested in the range between 20 and 45, and selected so that differences in intensity of product band reflect the quantitative difference of mRNA.

3.9. *Data analysis.*

Bright-light and fluorescence images of tissue sections were acquired using a personal computer and a color CCD camera (C4742-95 ORCA-ER system, Hamamatsu Photonics, Japan). Figures were prepared using PhotoShop 5.0 graphics software

(Adobe, San Jose, CA). Image, brightness, contrast, and sharpness were adjusted. Statistical data in the text were presented as the mean ± S.E.M.

4. **Results**

4.1. Molecular cloning of newt genes

A cDNA clone (*Cp-Notch-1*; 7885 bp) from neural plate to tailbud stage embryos of the newt *C. pyrrhogaster* was obtained (Fig. 10). This clone encoded a protein (2528 aa, Fig. 11) with characteristics of Notch receptors (Fig. 12A): 36 EGF repeat region, LIN/Notch repeat domain, transmembrane (TM) domain, RAM domain, CDC10/ankyrin repeat domain, OPA repeat region, and PEST region (Coffman et al., 1990; Bierkamp and Campos-Ortega, 1993; Tamura et al., 1995). The identity of amino acid sequence was 75-78% in total and higher than 91% (91.5-94.4%) in CDC10/ankyrin repeat domain as compared with other vertebrate Notch-1 (Fig. 12B). Phylogenetic tree analysis revealed that this clone belongs to the Notch-1 family (Fig. 12C). Therefore, I concluded that this clone encodes a Notch-1 receptor of the newt.

Candidate genes for involving to Notch signaling were also isolated from the cDNA library. The cDNA clone of *Delta-1*, one of Notch ligands, containing ORF was obtained. Its deduced amino acid sequence (726 aa) contained characteristics of DSL ligands: the 8 EGF repeat region (225-519, 295 aa) and the DSL domain (180-224, 45 aa). Compared with DSL domain of other vertebrate DSL ligands, this clone had the highest homology (80-93.3%) to other vertebrates Delta-1 (Fig. 13). For another candidate gene, I tried to obtain bHLH transcription factors, which positively regulate neurogenesis. One of the bHLH transcription factor cp-*Neurogenin-1 (cp-NGN-1)* cDNA clone (1719 bp) was obtained. Deduced amino acid of Cp-NGN-1 (220 aa) contained bHLH domain, which had the identity of 51-58% in total as compared with other vertebrate Ngn-1, and that of the b-HLH domain was higher than 83% (83.6-87.3%) (Fig. 14).

4.2. *Notch-1* is re-expressed during retinal regeneration of adult newt with a similar pattern to that during embryonic retinal development

I examined expression patterns of *Notch-1* during embryonic retinal development of the newt by *in situ* hybridaization (Fig. 15). *Notch-1* expression became obvious in the central region of the inner cell layer of the optic cup as the optic vesicle invaginates to form the optic cup (Fig. 15A, B). As the development proceeds, the *Notch-1* positive [*Notch-1*(+)] region extended toward the periphery, while *Notch-1* expression started decreasing from the centre of developing retina where the cell differentiation is more advanced (Fig. 15C,D). As the retina is stratified with apparent two synaptic layers and grows anteriorly, *Notch-1* expression was diminished in the central retina and became restricted in the peripheral immature retina (Fig. 15E, F). In juveniles, the *Notch-1*(+) region was still observed in the peripheral retina but

the signal was very low (Fig. 15G). In adult, *Notch-1* expression was not detected even in the non-pigmented region (NPR) of *pars ciliaris retinae* or *ora serrata* (Fig. 15H), i.e., the homologous regions of the non-pigmented ciliary epithelium (NPE) and the ciliary marginal zone (CMZ), respectively (Haynes and Del Rio-Tsonis, 2004; Chiba/Hoshino/Nakamura et al., 2006). Interestingly the non-pigmented epithelium adjacent to the growing retina was consistently *Notch-1* negative [*Notch-1*(-)] throughout the development (green asterisks in Fig. 15B-G), and this could be the origin of the adult NPR (green asterisk in Fig. 15H).

Using the same techniques, the expression of *Notch-1* during retinal regeneration of the adult newt was also examined (Fig. 16). *Notch-1* expression was first detected in a small number of cells in regenerating retinas of stage E-3 (Fig. 16A,B). *Notch-1*(+) cells increased in number as the regenerating retina increases in

thickness, and in regenerating retinas of stage I-1, almost all the cells expressed *Notch-1* intensely (Fig. 16C,D). *Notch-1* expression started decreasing in association with retinal cell differentiation (Fig. 16E-J). In regenerating retinas of stage I-2, premature ganglion cells became *Notch-1* (-) (Fig. 16E) and complementarily expressed a type of voltage-gated Na^+ channels *Cp-*Na_V*1* (Fig. 16F). Immediately before segregation of synaptic layers, i.e., stage I-3, not only the presumptive ganglion cell layer but also the presumptive photoreceptor cell layer became *Notch-1*(-) (Fig. 16H). A small number of photoreceptor cells started expression of *rhodopsin* (Fig. 16I). As the regenerating retina segregates into synaptic layers, *Notch-1*(+) cells in the inner nuclear layer decreased in number and eventually disappeared (Fig. 16K,L). In regenerating retinas with immature synaptic layers, i.e., stage L-1, a small number of cells in the inner nuclear layer remained *Notch-1*(+) (Fig. 16K). The *Notch-1*(+) cells

sometimes looked like Müller glia cells. In more advanced regenerating retinas, i.e., transitional stage between L-1 and L-2 on 45-days po, *Notch-1* expression in the central retina had declined to undetectable levels (Fig. 16L). In the peripheral region of retina, the *Notch-1*(+) region became restricted with progress of regeneration (Fig. 17A-C). Cells in the NPR and CMZ participate in regeneration of the peripheral retina (Chiba/Hoshino/Nakamura et al., 2006). Interestingly, the *Notch-1*(-) non-pigmented epithelium adjacent to the peripheral regenerating retina was preserved throughout regeneration (green asterisks in Fig. 17A-C). Thus, except at stage E-3, the spatial expression patterns of *Notch-1* during adult retinal regeneration were very similar to those during embryonic retinal development.

4.3. Administration of a Notch signaling inhibitor DAPT to newt embryos results in overproduction of early born retinal neurons at the expense of retinal progenitor cells

DAPT is one of the γ-secretase inhibitor, which is known to perturb Notch signaling through inhibiting release of a NICD (Geling et al., 2002; James et al., 2004; Schweisguth, 2004; Bernardos et al., 2005). To examine effects of DAPT on the newt retinal development, newt embryos of stage 26 were cultured in the presence of 100 μM DAPT for 5 days. In this dose, all of treated animals showed an abnormal ‘dorsal flexion’ phenotype consistently (Fig. 18). In eyes of ‘dorsal flexion’ embryos, the excess of neurons was observed in the central retina (Fig. 19). Those neurons expressed a type of voltage-gated Na^+ channels (Fig. 19A), which is a marker of retinal ganglion and some amacrine cells (Cheon et al., 1998). The territory of them obviously enlarged to the most distal margin of retina as

compared to the mock control (Fig. 19D) where the Na^+ channel expression is restricted to the presumptive ganglion cell layer. On the other hand, GFAP-labelled cells, possibly retinal progenitor cells, drastically decreased in number in the same region (Fig. 19B). It was compared that the number of cells within the central retina between the DAPT and mock treatment. The total cell number was not changed by DAPT (Fig. 19G), but the ratio of Na^+ channel expressing neurons increased significantly while that of GFAP-labelled cells decreased (Fig. 19H). Apoptotic cells were not observed in either group. These results suggest that in the presence of DAPT early born retinal neurons such as ganglion cells are overproduced at the expense of retinal progenitor cells.

4.4. Administration of DAPT to adult newts results in hasty production of neurons in regenerating retina

To examine effects of DAPT on retinal regeneration of the adult newt, I tried two tactics: intra-abdominal injection of DAPT and intraocular implantation of a cotton ball containing DAPT (Fig. 20). As the marker of retinal neurons, here I used an acetylated tubulin (AT) antibody as well as the Na^+ channel antibody. The AT antibody labels cell bodies of most retinal neurons except photoreceptors, as well as processes along the outer and inner plexiform layers and the optic fiber layer in mature retina of the newt (Kaneko et al., 1999). During normal retinal regeneration, AT-IR (AT-immunoreactivity) first appears in short processes, probably the axon of premature ganglion cell, at stage I-1 (Fig. 20A), and then in cell bodies of premature ganglion cells at stage I-2 (Fig. 20B). I must note that NaCh-IR (Na^+ channel immunoreactivity) is first detected, later than AT-IR,

in cell bodies of premature ganglion cells at stage I-2 and then in the optic fibers (Cheon et al., 1998).

In the intra-abdominal injection, I injected higher concentrations (200 μM-10 mM) of DAPT into the abdominal space of newt from 17 to 23 days po at various intervals, and analyzed regenerating retinas of 4 days after the last injection by immunohistochemistry. Morphological abnormalities of regenerating retina were not recognized under most injection conditions, except a delay of regeneration. The delay of regeneration is probably due to a side effect of DMSO because it was also observed in mock experiments. On the other hand, under a condition where 10 mM DAPT was injected on 17 and 19-23 days po (once a day) (Fig. 20C), I observed, in 5 of 15 eyes (33%), hasty production of neurons in regenerating retinas of 4 days after the last injection, i.e., 27 days po (Fig. 20D-G). The regenerating retina was typically 4-5 cells in thickness (stage I-1 to I-2) as

observed in the mock experiments (Fig. 20H-K), but occasionally contained thinner regions. In three eyes, along the regenerating retina of one-two cells in thickness, I observed a cluster of neurons of which cell bodies and processes were intensely labelled with the AT antibody (Fig. 20E). Such an intense immunorecativity was not observed in cell bodies in other 4-5 cell regions of the same regenerating retina but only in the optic fibers, as observed in the mock experiments (Fig. 20I). In the other two eyes, I observed a cluster of neurons around a crack of regenerating retinas of 4-5 cells in thickness (Fig. 21). In those cases, a large number of neurons expressing voltage-gated Na^+ channels were generated throughout the thickness of regenerating retina.

Similar results were obtained by intraocular implantation of a cotton ball containing DAPT (Fig. 20L-N). In all cases (n=3) where a cotton ball containing 10 mM DAPT was implanted into

the anterior chamber of the eyeball of 19 days po, It was observed in the eye of 4 days after implantation a cluster of AT-labelled neurons along the regenerating retina of 1-3 cells in thickness. The cluster of neurons tended to appear along the ventral side of regenerating retina (3/3). Interestingly, under this condition, cell bodies of neurons were present along the border between the regenerating retina and RPE layer, and extended processes along the most distal margin of regenerating retina (Fig. 20N). In the mock control, such abnormally AT-IR was not observed in regenerating retina (3/3; Fig. 22).

To ascertain whether DAPT could inhibit Notch signaling in regenerating retinas, I carried out immunoblot analysis with the Notch-1 antibody, which recognizes the intracellular domain of Cp-Notch-1 (Fig. 20O). In eye-cups of 19 days po cultured in the presence of 100 μM DAPT for 20 hr, the protein band (~87kD) corresponding to the Cp-Notch-1 intracellular domain (Cp-NICD)

was too pale to detect while that (~97kD) of membrane-tethered intracellular domain (NTM) was evident. On the other hand, in the mock control Cp-NICD was detected as well as NTM. These results indicate that DAPT blocks processing of Notch-1, suggesting that DAPT could inhibit at least signaling through Notch-1 receptor in regenerating retinas.

4.5. RPE cells express *Delta-1*, *Hes-1* and *Ngn-1* which are regulated differently in association with retinal regeneration

To examine whether genes involved in the Notch signaling are also expressed during retinal regeneration, cDNA clones encoded Cp-homologues of a Notch ligand Delta-1 and a bHLH proneural factor Ngn-1 was isolated (Fig. 13 and 14), and investigated their gene expression patterns as well as that of a Notch intracellular effector Hes-1 during retinal regeneration in comparison with

those of Notch-1 and neural markers (N-CAM, voltage-gated Na^+ channel $CpNa_V1$ and opsin Cp-L) by PCR techniques (Fig. 23). At first I used a series of template cDNAs (0, 10, 14, 19, 23 and 45 days po) constructed from six eye-cups each and that from six normal neural retinas only (*N*) for comparison (Fig. 23A). *Notch-1* expression was not detected in the retina-less eye-cups of day 0 po (stage E-0). *Notch-1* was abruptly expressed at the maximal level at 19 days po (stage E-3 to I-1), and then the expression was slightly decreased at 23 days po (stage I-2 to I-3) and eventually down-regulated to the minimal level (see 45 days po; stage L-1). These results agree well with those obtained by *in situ* hybridization technique. On the other hand, the product band of *Notch-1* was detected in the sample of the neural retina only (*N*), although it was very faint at this cycle number, suggesting that *Notch-1* may be expressed in the mature retina at a lower level than the sensitivity of *in situ* hybridization.

Interestingly, *Delta-1*, *Hes-1* and *Ngn-1* were all expressed in the sample of day 0 po and apparently up-regulated before the onset of *Notch-1* expression. The level of *Delta-1* expression apparently rose at 14 days po (stage E-2), gradually increased at least until 23 days po (stage I-2 to I-3) and then decreased gradually. *Hes-1* and *Ngn-1* started increasing their expression levels at least from 10 days po (stage E-1). *Hes-1* expression reached the maximal level at 19 days po (stage E-3 to I-1) when *Notch-1* expression re-starts, while *Ngn-1* expression increased at least until 23 days po (stage I-2 to I-3) similar to *Delta-1* expression. The levels of *Hes-1* and *Ngn-1* expression decreased to near the normal levels as the neural retina had segregated into synaptic layers (see 45 days po; stage L-1). It must be noted that *Delta-1*, *Hes-1* and *Ngn-1* as well as *Notch-1* were all expressed in the mature neural retina (*N*) at low levels (the product band of *Delta-1* is obscure at this cycle number).

A neural marker N-CAM has two RNA spliced variants, *N-CAM-140* and *N-CAM-180*. The expression level of *N-CAM-140* in the normal retina was higher than that of *N-CAM-180*. During retinal regeneration, *N-CAM-140* was first expressed at 19 days po (stage E-3 to I-1) and then increased in its expression level gradually. On the other hand, *N-CAM-180* was not expressed even in regenerating retinas at 45 days po (stage L-1) but in regenerated retinas (stage L-2) later than 65 days po (data not shown).

Voltage-gated Na^+ channel $CpNa_V1$ and o*psin Cp-L* could be the functional differentiation markers of ganglion cells/some amacrine cells and a type of cone photoreceptor cells, respectively (Cheon et al., 1998; Sakakibara et al., 2002). These genes were first expressed, later than *N-CAM-140*, at 23 days po (stage I-2 to I-3), and then their expression levels increased to reach the normal levels at 45 days po (stage L-2). I obtained the same result

with *rhodopsin* (data not shown). The onset time of expression (23 days po) of these marker genes corresponds to the time when the expression levels of *Notch-1* and *Hes-1* decline slightly while those of *Delta-1* and *Ngn-1* continue to elevate.

To examine whether the PCR bands of *Delta-1*, *Hes-1* and *Ngn-1* at day 0 po reflect the gene expression of intact RPE cells, I carried out single-cell RT-PCR analysis using RPE cells acutely isolated from adult eyeballs (Fig. 23B). I carefully picked up one solitary RPE cell that preserved both a non-pigmented basal region containing the nucleus and a region with pigmented microvili (photomicrograph in Fig. 23B) from cell suspension to construct the single strand cDNA. I examined gene expression only with samples with which the intense product band of the RPE marker *RPE65* (Chiba/Hoshino/Nakamura et al., 2006) was detected by the first series of PCR. In one of five RPE cells examined, the product bands of *Hes-1* and *Ngn-1* were detected in

the first series of nested PCR, and that of *Delta-1* was detected in the second series of nested PCR while those of *Notch-1* and *Pax-6* were not. This result is consistent with that from PCR with eye-cups of day 0 po (Fig. 23A). However, in the remaining four cells, the expression of *Delta-1* was not detected. These results demonstrate that RPE cells express both *Hes-1* and *Ngn-1*, and imply that RPE cells may have heterogeneity.

5. **Discussion**

Embryos and larvae of many vertebrates have the ability to regenerate their retinas through the process of transdifferentiation of RPE cells. However, this capacity is lost in most animals during maturation. In contrast, certain urodele amphibians such as newts retain this potential throughout their lifetime (for a recent review, see Haynes and Del Rio-Tsonis, 2004). Recent advances in stem cell biology and regenerative medicine reveal that, even in adult mammals including human, RPE cells and cells in the pigmented ciliary margin (PCM) or the iris pigment epithelium (IPE) have a capacity to transdifferentiate into retinal cells in culture or after transplantation into eyes (Ahmad et al., 2000; Tropepe et al., 2000; Haruta et al., 2001; Amemiya et al., 2004; Coles et al., 2004). Therefore, comparative studies between the retinal regeneration of adult urodeles (i.e., *in vivo* transdifferentiation model of PE cells) and *in vitro*

transdifferentiation of mammalian PE cells must provide critical information not only for biology but also for medicine aiming at retinal repair/regeneration from PE cells of patient eyes. In the present study, using the adult *C. pyrrhogaster* newt retinal regeneration system, I addressed a question whether Notch signaling is required for retinal regeneration by transdifferentiation of PE cells.

A Notch-1 receptor of the newt was identified and its expression pattern during adult retinal regeneration was examined by *in situ* hybridization in comparison with that during embryonic retinal development. During retinal development, *Notch-1* expression started from the central region of the inner cell layer of the optic cup, decreased in association with cell differentiation and became restricted in the peripheral retina. This expression pattern is very similar to those reported in other animals (zebrafish: Bierkamp and Campos-Ortega, 1993, *Xenopus*:

Coffman et al., 1990; Dorsky et al., 1995, 1997, chick: Austin et al., 1995; Ahmad et al., 1997; Henrique et al., 1997, mouse: Guillemot and Joyner, 1993, rat: Ahmad et al., 1995; Bao and Cepko, 1997).

In the adult newt eye, *Notch-1*(+) signal was not detected even in the NPR of *pars ciliaris retinae* or *ora serrata*. These regions correspond to the NPE of the chick and the CMZ of the frog, which contain the retinal stem cells and the retinal progenitor cells, respectively (Haynes and Del Rio-Tsonis, 2004; Chiba/Hoshino/Nakamura et al., 2006). In the adult *Xenopus*, *Notch-1*(+) cells are present within the CMZ (Dorsky et al., 1995). In the adult newt, *Notch-1*(+) region may be narrower and/or the expression level of *Notch-1* may be lower than the sensitivity of *in situ* hybridization.

During retinal regeneration, *Notch-1*(+) signal was first detected in the small number of cells in solitary along the

regenerating retina of one-two cells in thickness (stage E-3). Such an expression pattern was never observed in developing retina because the inner cell layer of the optic cup where *Notch-1* was first expressed had already increased to several cells in thickness (compare Fig. 15B and Fig. 16A). During further retinal regeneration, the spatial expression pattern of *Notch-1* was very similar to that during retinal development. The *Notch-1*(-) non-pigmented epithelium was observed in the most peripheries of growing and regenerating retinas (green asterisks in Fig. 15B-H and Fig. 17A-C), suggesting that the retinal stem cells are maintained in the periphery throughout the development and regeneration.

In the newt, the development of the techniques to manipulate molecular functions is on the way (Chiba et al., 2004; Ueda et al., 2005; Chiba/Hoshino/Nakamura et al., 2006). Therefore, in the present study, I tried to attenuate Notch signaling by

pharmacological techniques. I cultured newt embryos of stage 26 immediately before Notch-1 expression in the presence of 100μM DAPT, and found in every embryo overproduction of neurons at the expense of retinal progenitor cells. These neurons expressed a type of voltage-gated Na^+ channels, a phenotype of the early born neurons such as retinal ganglion cells. In the retinal development of other animals, Notch signaling has been implicated in several different processes including maintenance of a proliferating pool of retinal progenitor cells, lateral inhibitory interactions that prevent differentiation of retinal neurons, and promotion of Müller glial differentiation (Perron and Harris, 2000; Livesey and Cepko, 2001; Ahmad et al., 2004; Hatakeyama and Kageyama, 2004; Mu and Klein, 2004; Pujic and Malicki, 2004). Blocking Notch activity early in retinal development with antisense oligonucleotides or γ-secretase inhibitors forces progenitor cells to differentiate prematurely and adopt early neuronal fates, such

as retinal ganglion cells (Austin et al., 1995; Silva et al., 2003; Bernardos et al., 2005; Kubo et al., 2005). Our results with DAPT are consistent well with these findings.

To attenuate Notch signaling in regenerating retina, I tried intra-abdominal injection of DAPT and intraocular implantation of a cotton ball containing DAPT. In both trials, I observed a cluster of neurons, possibly ganglion cells, along the regenerating retina of 1-3 cells in thickness that looked like stage E-3 to I-1. Their cell bodies and processes were intensely labelled with the AT antibody. Normally, AT-IR is not recognized in cell bodies at stage E-3 to I-1. A possibility that neurons migrated from more advanced tissues can be excluded because neurons with the AT-labelled cell body were not observed in either the other regions of the same regenerating retina or the mock control. Consequently, these observations suggest that neuronal differentiation was hastened by DAPT administration. The pattern

of neurons was apparently different from that in the retina of embryos cultured in the presence of DAPT (compare Fig. 20E and N to Fig. 19A). This may reflect the difference in the expression pattern of *Notch-1* between the regeneration and development (compare Fig. 16A to Fig. 15B). The immunoblot analysis suggests that DAPT could perturb the processing of Notch-1 and intracellular signaling in regenerating retina.

Such an abnormality was observed infrequently and required high concentrations of DAPT. In the intra-abdominal injection, we occasionally observed a morphological abnormality in the regenerating retina of 4-5 cells in thickness. In those cases, a large number of neurons were generated throughout the thickness of regenerating retina, being responsible for a crack of tissue. In the intraocular implantation of a cotton ball, the cluster of neurons tended to appear along the ventral side of regenerating retina. Such fluctuations of result probably reflect difficulties in

controlling the concentrations of drugs in the vitreous chamber of living animal. In the intraocular implantation of a cotton ball, neurons extended their processes along the distal border of regenerating retina. This may be due to a perturbation of the gradient of diffusible factors or the polarity in the eye chamber by the implant, but further examinations are necessary.

Taken together, in the retinal regeneration of adult newt, Notch signaling at least through Notch-1 receptors may be involved in maintenance of retinal progenitor cells and inhibition of differentiation of retinal neurons. However, to discuss contributions of Notch-1, we need more direct evidences. In the regenerating retina of adult newt, the expression of a Notch-like protein has been reported (Kaneko et al., 2001). In vertebrate developing retinas, Notch-3 homologues are also known to be expressed (Lindsell et al., 1996; Sullivan et al., 1997).

PCR analysis with eye-cups revealed that *Delta-1*, *Hes-1* and *Ngn-1* as well as *Notch-1* are regulated in association with retinal regeneration. *Hes-1* expression reached the maximum at 19 days po when *Notch-1* expression re-starts with the maximal level, and both *Hes-1* and *Notch-1* expression declined slightly at 23 days po, while *Delta-1* and *Ngn-1* expression continue to elevate at least until 23 days po when neural marker genes started expression. These results support our hypothesis. However, since *Hes-1*, *Ngn-1* and sometimes *Delta-1* are expressed in RPE cells (see below), their changes in expression level may partially reflect those in RPE cells. This idea should be tested further by *in situ* hybridization and immunohitochemistry in the future.

Single-cell RT-PCR analysis demonstrated for the first time that RPE cells of the adult newt express *Hes-1*, *Ngn-1* and sometimes *Delta-1*. PCR analysis with eye-cups suggests that both *Hes-1* and *Ngn-1* are up-regulated by 10 days po (stage E-1)

and *Delta-1* is by 14 days po (stage E-2). These results suggest a possibility that Notch signaling may be involved also in early process of transdifferentiation of RPE cells. It has been reported in the embryonic rat that RPE cells express *Notch-2* (Lindsell et al., 1996; Bao and Cepko, 1997). Regulations without receptors may be also possible (Kubo et al., 2005). Isolated RPE cells of the adult newt can express neuronal phenotypes such as voltage-gated Na^+ channels and acetylated tubulin very early in culture (Sakai and Saito, 1994, 1997; Susaki and Chiba, 2005) similar to those of neonatal and adult rats (Botchkin and Matthews, 1994; Engelhardt et al., 2005) and adult human (Wen et al., 1994). Ngn-1 and Hes-1 are known to be a positive and negative regulator of neurogenesis. It have already started to investigate involvement of Notch signaling and these transcription factors in *in vitro* transdifferentiation of adult newt RPE cells in comparison with that of mammalian PE cells.

In conclusion, I propose that Notch signaling is involved in the transdifferentiation of the RPE cells into neural retina (Fig.24): at first, it is involved in the retinal regeneration as the signal of classically lateral inhibition like developing retina, second, it may be involved in the early step of transdifferentiation or the RPE cell state itself.

Regeneration is a dream of we human for a long time, because except for some rare cases, we have a restricted capacity of regeneration. As one of the rare case, I investigated adult newt retinal regeneration in this study. Adult newt retinal regeneration itself has been known for centuries, and believed that it is well known phenomenon, but the molecular mechanisms underlying the retinal regeneration of adult newts are virtually unknown.

I believe my work will help to find the breakthrough of this field and answer as how the mature cell produces the tissue progenitors.

6. **References**

Ahmad I, Zaqouras P, Artavanis-Tsakonas S (1995) Involvement of Notch-1 in mammalian retinal neurogenesis: association of Notch-1 activity with both immature and terminally differentiated cells. Mech Dev 53:73-85.

Ahmad I, Dooley CM, Polk DL (1997) Delta-1 is a regulator of neurogenesis in the vertebrate retina. Dev Biol 185:92-103.

Ahmad I, Tang L, Pham H (2000) Identification of neural progenitors in the adult mammalian eye. Biochem Biophys Res Commun 270:517-521.

Ahmad I, Das AV, James J, Bhattacharya S, Zhao X (2004) Neural stem cells in the mammalian eye: types and regulation. Semin Cell Dev Biol 15:53-62.

Amemiya K, Haruta M, Takahashi M, Kosaka M, Eguchi G (2004) Adult human retinal pigment epithelial cells capable of differentiating into neurons. Biochem Biophys Res Commun 316:1-5.

Arias AM, Zecchini V, Brennan K (2002) CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? Curr Opin Cenet Dev 12:524-533.

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

Aulehla A, Herrmann BG (2004) Segmentation in vertebrates: clock and gradient finally joined. Genes Dev 18:2060-2067.

Austin CP, Feldman DE, Ida JAJ, Cepko CL (1995) Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development 121:3637-3650.

Bao ZZ, Cepko CL (1997) The expression and function of Notch pathway genes in the developing rat eye. J Neurosci 17:1425-1434.

Beatus P, Lendahl U (1998) Notch and Neurogenesis. J Neurosci Res 54:125-136.

Bernardos RL, Lentz SI, Wolfe MS, Raymond PA (2005) Notch-Delta signaling is required for spatial patterning and Müller glia differentiation in the zebrafish retina. Dev Biol 278:381-395.

Bierkamp C, Campos-Ortega JA (1993) A zebrafish homologue of the *Drosophila* neurogenic gene *Notch* and its pattern of transcription during early embryogenesis. Mech Dev 43:87-100.

Botchikin LM, Matthews G (1994) Voltage-dependent sodium channels develop rat retinal pigment epithelium cells in culture. Proc Natl Acad Sci USA 91:4564-4568.

Cheon EW, Kaneko Y, Saito T (1998) Regeneration of the newt retina: Order of appearance of photoreceptors and ganglion cells. J Comp Neurol 396:267-274.

Cheon EW, Saito T (1999) Choline acetyltransferase and acetylcholinesterase in the normal, developing and regenerating newt retinas. Dev Brain Res 116:97-109.

Chiba C, Matsushima O, Muneoka Y, Saito T (1997) Time course of appearance of GABA and GABA receptors during retinal regeneration in the adult newt. Dev Brain Res 98:204-210.

Chiba C (1998) Appearance of glutamate-like immunoreactivity during retinal regeneration in the adult newt. Brain Res 785:171-177.

Chiba C, Nakamura K, Unno S, Saito T (2004) Intraocular implantation of DNA-transfected retinal pigment epithelium cells: a new approach for analyzing molecular functions in the newt retinal regeneration. Neurosci Lett 368:171-175.

Chiba C, Oi H, Saito T (2005) Changes in somatic sodium currents of ganglion cells during retinal regeneration in the adult newt. Brain Res Dev Brain Res 154:25-34.

Chiba C, Hoshino A, Nakamura K, Susaki K, Yamano Y, Kaneko Y, Kuwata O, Maruo F, Saito T (2006) Visual cycle protein RPE65 persists in new retinal cells during retinal regeneration of adult newt. J Comp Neurol 495:391-407.

Coffman C, Harris W, Kintner C (1990) *Xotch*, the *Xenopus* homolog of *Drosophila notch*. Science 249:1438-1441.

Coles BLK, Angénieux B, Inoue T, Del Rio-Tsonis K, Spence JR, McInnes RR, Arsenijevic Y, van der Kooy, D (2004) Facile isolation and the characterization of human retinal stem cells. Proc Natl Acad Sci USA101:15772-15777.

Crosnier C, Stamataki D, Lewis J (2006) Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nature Rev Genet 349-359.

Del Rio-Tsonis K, Tsonis PA (2003) Eye regeneration at the molecular age. Dev Dyn 226:211-224.

Dorsky RI, Rapaport DH, Harris WA (1995) *Xotch* inhibits cell differentiation in the Xenopus retina. Neuron 14:487-496.

Dorsky RI, Chang WS, Rapaport DH, Harris WA (1997) Regulation of neuronal diversity in the *Xenopus* retina by Delta signalling. Nature 385:67-70.

Engelhardt M, Bogdahn U, Aigner L (2005) Adult retinal pigment epithelium cells express neural progenitor properties and the neuronal precursor protein doublecortin. Brain Res 1040:98-111.

Fischer AJ, Reh TA (2001) Transdifferentiation of pigmented epithelial cells: A source of retinal stem cells? Dev Neurosci 23:268-276.

Furukawa T, Mukherjee S, Bao ZZ, Morrow EM, Cepko CL (2000) rax, Hes1, and notch1 promote the formation of Müller glia by postnatal retinal progenitor cells. Neuron 26:383-394.

Geling A, Steiner H, Willem M, Bally-Cuif L, Haass C (2002) A γ-secretase inhibitor blocks Notch signaling *in vivo* and causes a severe neurogenic phenotype in zebrafish. EMBO Rep 3:688-694.

Grigoryan EN (2001) Differentiation markers of retinal cell types in studies on vertebrate eye development and regeneration. Ontogenez 32:85-105.

Grogg MW, Call MK, Okamoto M, Vergara MN, Del Rio-Tsonis K, Tsonis PA (2005) BMP inhibition-driven regulation of six-3 underlies induction of newt lens regeneration. Nature 438:858-862.

Guillemot F, Joyner AL (1993) Dynamic expression of the murine *Achaete-Scute* homologue *Mash-1* in the developing nervous system. Mech Dev 42:171-185.

Haruta, M; Kosaka, M; Kanegae, Y; Saito, I; Inoue, T; Kageyama, R; Nishida, A; Honda, Y; Takahashi, M (2001) Induction of photoreceptor-specific phenotypes in adult mammalian iris tissue. Nat Neurosci 4: 1163-1164.

Hasegawa M (1958) Restitution of the eye after removal of the retina and lens in the newt, *Triturus pyrrhogaster*. Embryologia 4:1-32.

Hatakeyama J, Kageyama R (2004) Retinal cell fate determination and bHLH factors. Semin Cell Dev Biol 15:83-89.

Haynes T, Del Rio-Tsonis K (2004) Retina repair, stem cells and beyond. Curr Neurovascul Res 1:231-239.

Henrique D, Hirsinger E, Adam J, Le Roux I, Pourquié O, Ish-Horowicz D, Lewis J (1997) Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Curr Biol 7:661-670.

Hirota K, Kaneko Y, Matsumoto G, Hanyu Y (1999) Cloning and distribution of a putative tetrodotoxin-resistant Na^+ channel in newt retina. Zool Sci 16:587-594.

Hitchcock P, Ochocinska M, Sieh A, Otteson D (2004) Persistent and injury-induced neurogenesis in the vertebrate retina. Prog Retin Eye Res 23, 183-194.

Hojo M, Ohtsuka T, Hashimoto N, Gradwohl G, Guillemot F, Kageyama R (2000) Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. Development 127:2515-2522.

Ichikawa M, Kajishima T (1965) Development of newt. In: Embriology in Vertebrate (Kume M, ed), pp242-245. Tokyo: Baifukan (in Japanese).

Ishibashi M, Moriyoshi K, Sasai Y, Shiota K, Nakanishi S, Kageyama R (1994) Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J 13:1799-1805.

James J, Das AV, Rahnenführer J, Ahmad I (2004) Cellular and molecular characterization of early and late retinal stem cells/progenitors: differential regulation of proliferation and context dependent role of Notch signaling. J Neurobiol 61:359-376.

Justice NJ, Jan YN (2002) Variations on the Notch pathway in neural development. Curr Opin Neurobiol 12:64-70.

Kageyama R, Ohtsuka T, Hatakeyama J, Ohsawa R (2005) Roles of bHLH genes in neural stem cell differentiation. Exp Cell Res 306:343-348.

Kaneko Y, Matsumoto G, Hanyu Y (1999) *Pax-6* expression during retinal regeneration in the adult newt. Deve Growth Differ 41:723-729.

Kaneko Y, Hirota K, Matsumoto G, Hanyu Y (2001) Expression pattern of a newt *Notch* homologue in regenerating newt retina. Dev Brain Res 128:53-62.

Keefe JR (1973) An analysis of urodelian retinal regeneration: I. Studies of cellular source of retinal regeneration in *Notophthalmus viridescens* utilizing ^{3}H-thymidine and colchicine. J Exp Zool 184:185-206.

Kubo F, Takeichi M, Nakagawa S (2005) Wnt2b inhibits differentiation of retinal progenitor cells in the absence of Notch activity by downregulating the expression of proneural genes. Development 132:2759-2770.

Leong KG, Karsan A (2006) Recent insights into the role of Notch signaling in tumorigenesis. Blood 107:2223-2233.

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

Lindsell CE, Boulter J, diSibio G, Gossler A, Weinmaster G (1996) Expression patterns of *Jagged*, *Delta1*, *Notch1*, *Notch2*, and *Notch3* genes identify ligand-receptor pairs that may function in neural development. Mol Cell Neurosci 8:14-27.

Livesey FJ, Cepko CL (2001) Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci 2:109-118.

Mitashov VI (1996) Mechanisms of retinal regeneration in urodeles. Int J Dev Biol 40:33-844.

Mu X, Klein WH (2004) A gene regulatory hierarchy for retinal ganglion cell specification and differentiation. Semin Cell Dev Biol 15:115-123.

Negishi K, Shinagawa S, Ushijima M, Kaneko Y, Saito T (1992) An immunohistochemical study of regenerating newt retinas. Dev Brain Res 68:255-264.

Oi H, Chiba C, Saito T (2003a) The appearance and maturation of excitatory and inhibitory neurotransmitter sensitivity during retinal regeneration of the adult newt. Neurosci Res 47:117-129.

Oi H, Chiba C, Saito T (2003b) A decay of gap junctions associated with ganglion cell differentiation during retinal regeneration of the adult newt. Vis Res 43:2847-2859.

Park CM, Hollenberg MJ (1989) Basic fibroblast growth factor induces retinal regeneration in vivo. Dev Biol 134:201-205.

Park CM, Hollenberg MJ (1991) Induction of retinal regeneration in vivo by growth factors. Dev Biol 148: 322-333.

Park CM, Hollenberg MJ (1993) Growth factor-induced retinal regeneration in vivo. International Rev Cytol 146:49-74.

Perron M, Harris WA (2000) Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors. Cell Mol Life Sci 57: 215-223.

Pujic Z, Malicki J (2004) Retinal pattern and the genetic basis of its formation in zebrafish. Semin Cell Dev Biol:105-114.

Rapaport DH, Dorsky R (1998) Inductive competence, its significance in retinal cell fate determination and a role for Delta-Notch signaling. Semin Cell Dev Biol 9:241-247.

Raymond PA, Hitchcock PF (1997) Retinal regeneration: common principles but a diversity of mechanisms. In: Advances in Neurology, vol 72 (Seil FJ, ed), pp171-184. Philadelphia: Lippincott-Raven.

Raymond PA, Hitchcock PF (2000) How the neural retina regenerates. Results Probl Cell Differ 31:197-218.

Reh TA, Levine EM (1998) Multipotential stem cells and progenitors in the vertebrate retina. J Neurobiol 36:206-220

Reh TA, Fischer AJ (2001) Stem cells in the vertebrate retina. Brain Behav Evol 58:296-305.

Robey E (1997) Notch in vertebrates. Curr Opin Genet Dev 7:551-557.

Saito T, Kaneko Y, Maruo F, Niino M, Sakaki Y (1994) Study of the regenerating newt retina by electrophysiology and immunohistochemistry (bipolar- and cone-specific antigen localization). J Exp Zool 270:491-500.

Sakai H, Saito T (1994) Development of voltage-dependent inward currents in dissociated newt retinal pigment epithelial cells in culture. Neuroreport 5:933-936.

Sakai H, Saito T (1997) Na^+ and Ca^+ channel expression in cultured newt retinal pigment epithelial cells: comparison with neuronal types of ion channels. J Neurobiol 32:377-390.

Sakakibara S, Hiramatsu H, Takahashi Y, Hisatomi O, Kobayashi Y, Sakami S, Saito T, Tokunaga F (2002) Opsin expression in

adult, developing, and regenerating newt retinas. Mol Brain Res 103:28-35.

Scheer N, Groth A, Hans S, Campos-Ortega JA (2001) An instructive function for Notch in promoting gliogenesis in the zebrafish retina. Development 128:1099-1107.

Schweisguth F (2004) Regulation of Notch signaling activity. Curr Biol 14:R129-R138.

Silva AO, Ercole CE, McLoon SC (2003) Regulation of ganglion cell production by Notch signaling during retinal development. J Neurobiol 54:511-524.

Stone LS (1950) The role of retinal pigment cells in regenerating neural retinae of adult salamander eyes. J Exp Zool 113:9-32.

Sullivan SA, Barthel LK, Largent BL, Raymond PA (1997) A goldfish *Notch-3* homologue is expressed in neurogenic regions of embryonic, adult, and regenerating brain and retina. Dev Genet 20:208-223.

Susaki K, Chiba C (2005) Isolated retinal pigment epithelium cells of adult newt transdifferentiate into neurons and glia under serum-free culture conditions. Soc Neurosci Abstr (Program No. 717.9).

Tamura K, Taniguchi Y, Minoguchi S, Sakai T, Tun T, Furukawa T, Honjo T (1995) Physical interaction between novel domain of the receptor Notch and the transcription factor RBP-jκ/Su(H). Curr Biol 5:1416-1423.

Tomita K, Ishibashi M, Nakahara K, Ang SL, Nakanishi S, Guillemot F, Kageyama R (1996) Mammalian hairy and enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron 16:723-734.

Tropepe V, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, McInnes RR, van der Kooy D (2000) Retinal stem cells in the adult mammalian eye. Science 287:2032-2036.

Tsonis PA, Del Rio-Tsonis K (2004) Lens and retina regeneration: transdifferentiation, stem cells and clinical applications. Exp Eye Res 78:161-172.

Ueda Y, Kondoh H, Mizuno N (2005) Generation of transgenic newt *Cynops pyrrhogaster* for regeneration study. Genesis 41:87-98.

Umino Y, Saito T (2002) Spatial and temporal patterns of distribution of the gap junctional protein connexin43 during retinal regeneration of adult newt. J Comp Neurol 454:255-262.

Wachs H (1920) Restitution des auges nach extirpation von retina und linse bei tritonen [Neue Versuche zur Wolffschen Linsenregeneration; II. Teil]. Wilhelm Roux'Arch. Entwickel-Mech Org 46:328-390.

Wen R, Lui GM, Steinberg RH (1994) Expression of a TTX-sensitive Na^+ current in cultured human retinal pigment epithelial cells. J Physiol 476:187-196.

7.Figures and legends

Figure 1. Newt retina.

Left: Normal newt retina. *Right*: Schematic diagram showing cells of newt retina and organization of retinal layers.

R, rod photoreceptor cell; C, cone photoreceptor cell; H, horizontal cell; B, bipolar cell; A, amacrine cell; G, ganglion cell; M, Müllar cell; PCL, pigment cell layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

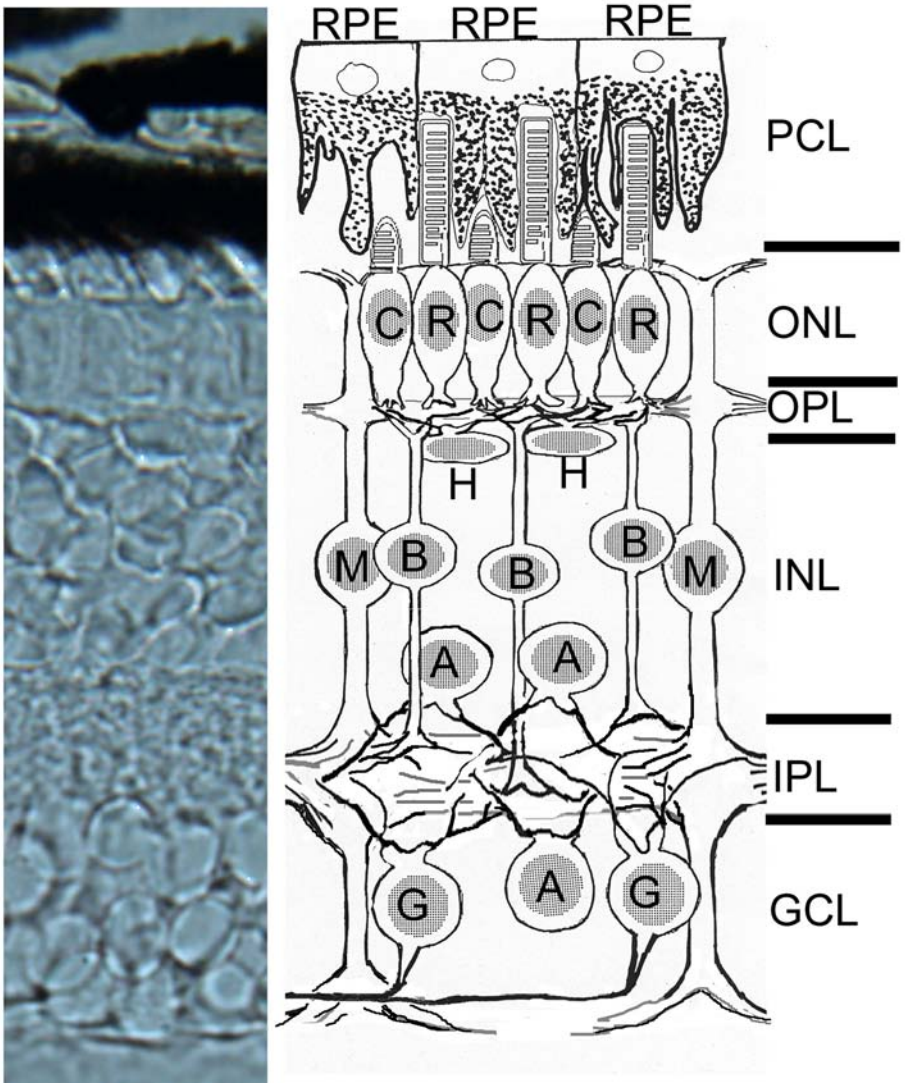

RPE
RPE
RPE
PCL
C
R
C
R
C
R
ONL
OPL
H
H
M
B
B
B
M
INL
A
A
IPL
G
A
G
GCL

Figure 2. Diagram of vertebrate eye development. ***A,*** five brain vesicles and a spinal code. Optic vesicles evaginate from diencephalon. ***B,*** eye development. The optic vesicle invaginates to form a layered optic cup. Outer layer differentiate into retinal pigment epitherium (RPE), inner layers differentiate into neural retina. The overlying ectoderm produces the lens placode. Note: Both neural retina and RPE cells have common progenitor.

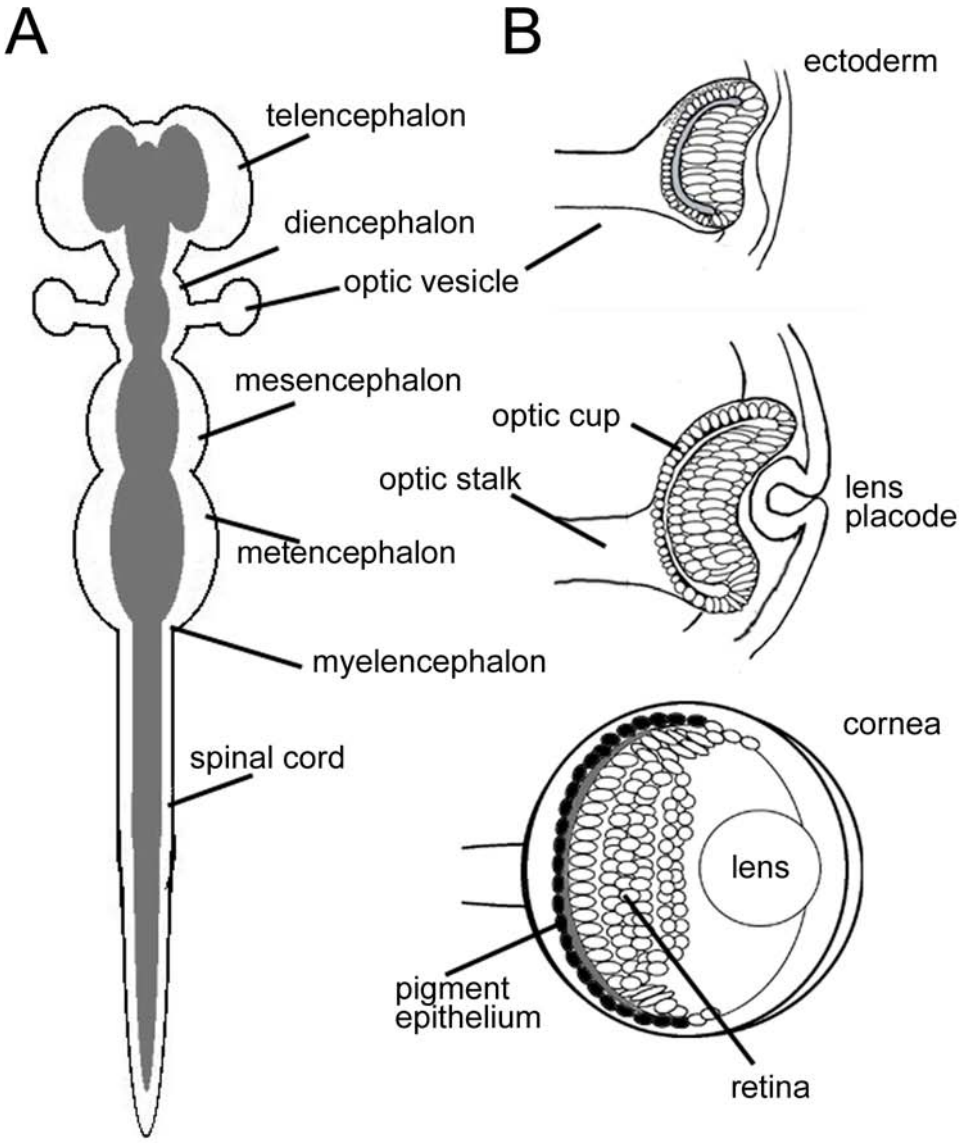

A
B
telencephalon
diencephalon
optic vesicle
mesencephalon
metencephalon
myelencephalon
spinal cord
ectoderm
optic cup
optic stalk
lens
placode
cornea
lens
pigment
epithelium
retina

Figure 3. Schematic diagram of adult newt retinal regeneration. The process of retinal regeneration after retinectomy was divided into nine stages. Stage E-0: RPE cells immediately after retinectomy. E-1: most RPE cells are oval/spindle shape and slightly de-pigment, and enter the cell cycle. E-2: cells of which the de-pigmentation proceeded considerably start generating the new retinal rudiment and the RPE cell-layer itself. E-3: the retinal rudiment/regenerating retina de-pigments almost completely and is 1-2 cells in thickness. I-1: the regenerating retina is 3-4 cells in thickness. I-2: round cell bodies of premature ganglion cells appear in the most proximal region. I-3 stage: the regenerating retina has more increased thickness and is just before formation of the plexiform layers. L-1: the outer and inner plexiform layers have appeared but are still immature. L-2: the regenerating retina is indistinguishable from the normal retina. The number in parenthesis indicates corresponding day post-operative (po) under the present experimental conditions. RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

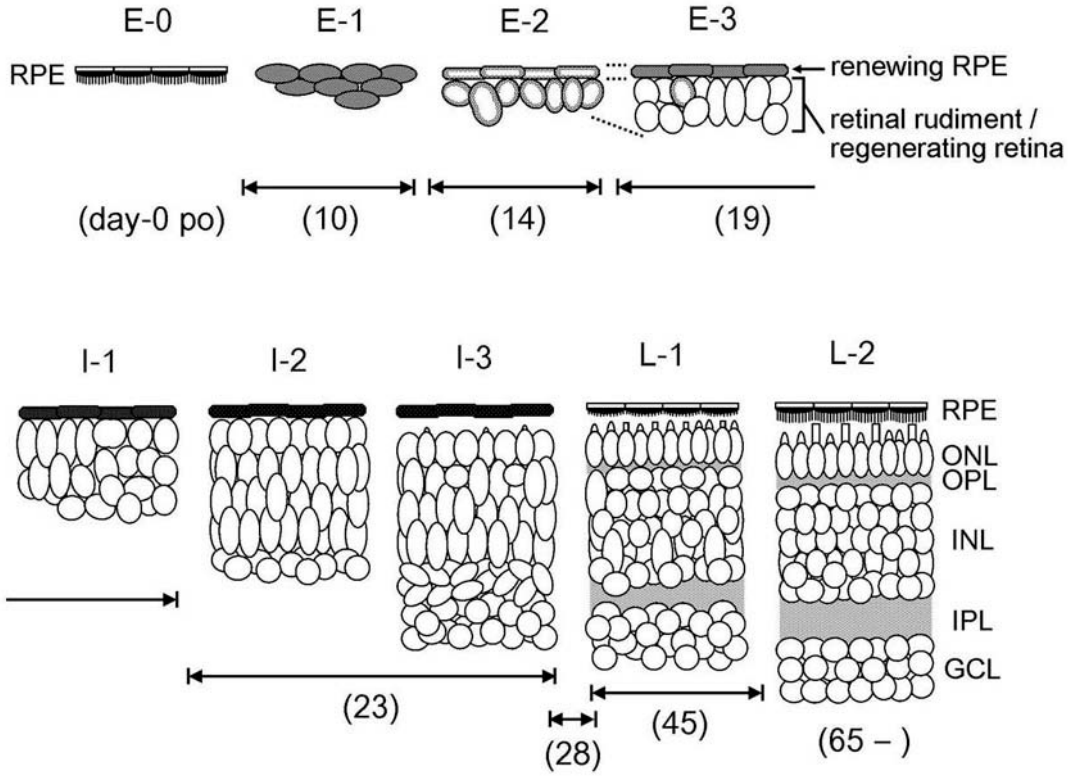

E-0
E-1
E-2
E-3
RPE
renewing RPE
retinal rudiment /
regenerating retina
(day-0 po)
(10)
(14)
(19)
I-1
I-2
I-3
L-1
L-2
RPE
ONL
OPL
INL
IPL
GCL
(23)
(28)
(45)
(65 –)

Figure 4. Molecular mechanism of notch signaling. Upon activation by Notch ligands like Delta from differentiating cells, the Notch Intra-Cellular Domain (NICD) is cleaved off from transmembrane region by gamma-secretase and translocated into nucleus to form a complex with the DNA-binding protein RBP-J. This complex induces expression of genes such as Hes-1 and Hes-5. They are inhibit bHLH neuronal activators, thus this cell remain its progenitor/precursor state. On the other hand, in differentiating cell, Hes-1/5 expression is off; allowing bHLH activators to induces expression of neural specific genes.

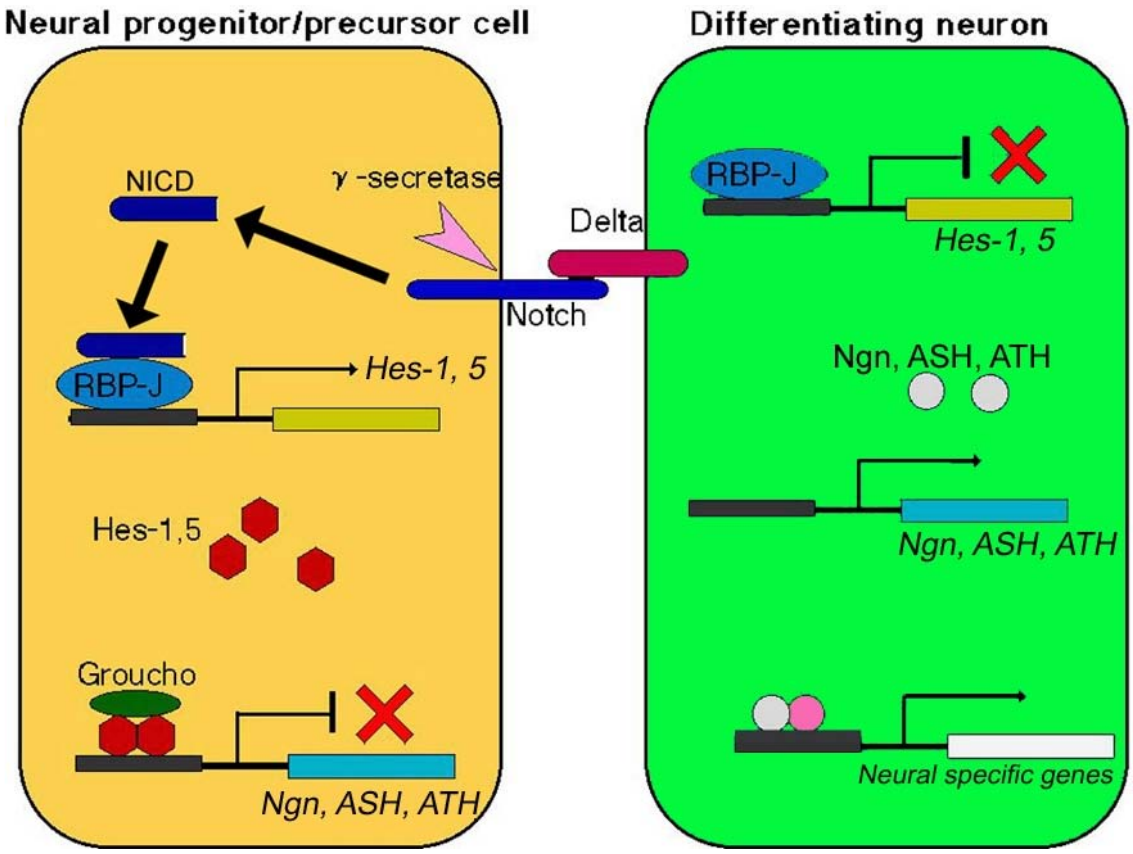
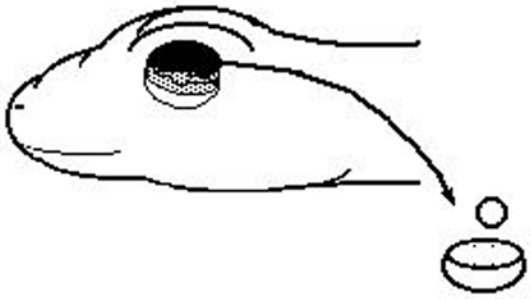

Neural progenitor/precursor cell
Differentiating neuron
NICD
γ -secretase
Delta
Notch
RBP-J
Hes-1, 5
Hes-1,5
Groucho
Ngn, ASH, ATH
RBP-J
Hes-1, 5
Ngn, ASH, ATH
Ngn, ASH, ATH
Neural specific genes

Figure 5. Outline of retinectomy. Deeply anesthetized animal was placed on a chamber, the dosal half of eye was cut open. The retina and lens were removed, and then the cornea and iris were gentry replaced at its original position.

A

B

C



D

Figure 6. Procedures for molecular cloning.

Amplifying a small cDNA fragment from cDNA library

of newt embryos

↓

synthesizing PCR- DIG probes

↓

screening from the cDNA library

↓

partial cDNA clone

↓

5’-RACE PCR (SMART RACE cDNA Amplification Kit)

↓

end-to-end PCR

↓

obtaining full length clone

Figure 7. Procedures for synthesis of Notch-1 DIG-RNA probes.

amplifying full length *Notch-1* cDNA clone

↓

inserting into the pCRII plasmid (in both directions)

↓

cutting 10 μg clones by Not I (37°C, 3 hr)

↓

PCI-CIA treating

↓

ethanol precipitation

↓

synthesizing DIG-labeled RNA probe (SP6, 37°C, 2 hr)

↓

hydrolyzing (60°C, 50 min)

Figure 8. Procedures for ISH.

Washing tissues in 80% PBT (RT, 10 min)

↓

proteinase K treatment (10 µg/ml in 80% PBT, 5 min, 37°C)

↓

fixation in 4% paraformaldehyde for 30 min, RT

↓

2 mg/ml glycine for 5 min

↓

washing 2 times in 80% PBT (RT, 5 min each)

↓

0.1 M TEA (RT, 10 min)

↓

0.1 M TEA with 0.5% acetic anhydride (RT, 10 min)

↓

washing 4 times in 80% PBT (RT, 5 min each)

↓

incubation in 2 x SSC/50% formamide/0.1% Tween 20 (RT, 30 min)

↓

incubation 100 ng/ml DIG RNA probe in hybridization mixture (70°C, 30 min)

↓

overnight incubation (50°C)

↓

washing tissues in 2 x SSC/50% formamid/0.1% Tween 20 (45°C, 30 min)

↓

washing 2 times in Tris-buffer

↓

RNase treatment (37°C, 30 min)

↓

2 x SSC/50% formamid/0.1% Tween 20 for 30 min at 45°C

↓

1 x SSC/50% formamid/0.1% Tween 20 for 30 min at 45°C

↓

1 x SSC/50% formamid/0.1% Tween 20 for 30 min at RT

↓

washing 6 times in 100 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.1% Tween 20 (RT, 15 min each)

↓

Blocking for 30 min

↓

anti-DIG alkaline phosphatase antibody treatment (1:2000, 4°C, overnight)

↓

washing in 100 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.1% Tween 20 (6 times, RT, 15 min each)

↓

incubating 2 times in 2 mM levamisole in 100 mM Tris-HCl (pH 9.5)/100 mM NaCl/50 mM $MgCl_2$ (RT, 5 min each)

↓

NBT/BCIP treatment

Figure 9. Procedures for double labeled IHC.

AT antibody treatment (1:2000, 4°C, overnight)

↓

washing

↓

secondary antibody treatment (FITC conjugated goat anti-mouse IgG, 1:150, RT, 4hr)

↓

washing

↓

incubation in 10% normal mouse serum (RT, 2hr)

↓

washing

↓

non-labeled goat anti-mouse IgG antibody (Fab fragment) treatment (1:100, 4°C, overnight)

↓

Cy3-GFAP antibody treatment

Figure 10. The sequence of *Cp-Notch-1*. Under lines, indicate the initiation codon and termination codon respectively.

```
   1 acgcggggaatggcgcctaagccaagaggaaggcaagataccgaactgctgcgaggagacacccgggtgc  70
  71 ccggagcgactgagacacccgcctcggaccggagcttatttatttacgcacctgagagagatacttacct 140
 141 acaggacacttacagtagctgcttcatcctcgcttggaacctatgacaccttaaaacggacacttgcagc 210
 211 ctaaggatgcggataccgggcttcgcggcactgctactgacgctgccctctctggcaccagcgctacgat 280
 281 gtactctacagagtgaaaaatgcatgaatggtggaaagtgcgaagcccttcccaacgggacttccgtctg 350
 351 cacgtgtagcagcagtgcatttgtaggagaaaggtgtcaatactccaacccatgccttagctctccatgt 420
 421 aggaactccggaacgtgccaagttgacatccgtggaaacaccgtggattatagctgtatctgccgcttgg 490
 491 gatattctgacaagctgtgcctgactcccatggataatgtttgcttgaacaacccttgtcgaaatggtgg 560
 561 gacgtgcgaactgctgtctagcctcacccagcataagtgcagatgtcccccgggatggacaggtgaaact 630
 631 tgccagcaggcggatccctgtgcctcaaacccttgcggcaatgggggacagtgtgtgcccttcgaagccc 700
 701 agtatgtctgcaagtgccctccagcttatcatggacccacctgcaaactggacatcaatgaatgtacttc 770
 771 tgtcccatgcaagaatggaggtacatgcgtgaacgaggtgggatcataccagtgcacctgcagaccggaa 840
 841 tacacgggacgcaactgcgaaaatctctacgtgccctgctacccttccccatgccagaatggtggaacct 910
 911 gccgccagacaggggataccacctatgaatgtgcttgtcttccaggtttcgattctcagaactgtgaggt 980
 981 gaacattgacgactgtcccggtaacacctgtaagaacggagggacctgtgtcgatggggtgaacacttac 1050
1051 aactgccagtgccccccagagtggacagggcagtactgtactgaggatgttgacgaatgccagctgatgc 1120
1121 caaatgcctgccagaatgggggcacatgccacaataaccatggaggatacaactgcgtgtgcgtgaatgg 1190
1191 ttggactggcgaggactgcagtgagaacattgacgattgtgccaacgccgcctgccacagtggggccacc 1260
1261 tgtcatgacagagtggcatctttctactgcgagtgcccgcatggacgaacagggctcctctgccacctca 1330
1331 atgacgcctgcatcagtagcccttgcaatgagggtgccaactgtgataccaacccagtgaatggaaaggc 1400
1401 catctgcacctgtccttctggctacatggggccggcatgcattcaagatgtcgacgagtgctctcttggt 1470
1471 gctaacccctgtgagcatgccggcaggtgcctgaacaccctaggatctttccagtgccaatgctccccag 1540
1541 gatacactggtccccgctgtgagatggatgtcaacgaatgcctgtccaatccatgcaagaatgatgccac 1610
1611 ttgcctggaccagatcggagaattccactgcatctgcatgccaggttacgaaggtgttttctgtcaaaca 1680
1681 aacacagatgagtgcgcgagcagcccttgcttgcacaacggccgctgcattgacaagatcaatgagtttc 1750
1751 attgcgagtgccccataggttttaatggccccctctgccagtacgacattgatgaatgtgccagtacccc 1820
1821 ttgcaagaatggagccaaatgtctggacggtgcaaattcatatacgtgtgactgtgcagaaggatattct 1890
1891 ggcttccactgtgagacggacatcgatgaatgtgatcctgatccctgccactacgggacctgctccgatg 1960
1961 gcatcgcaggattcacctgtcactgcgagcccggctacactggtcaccgatgtgaaatcgacgtcaacga 2030
2031 gtgccagagcatgccctgccagaacggaggagagtgccaggaccgcaaaaacagctattcttgcaggtgc 2100
2101 cccaaaggaaccacaggggtcaattgtgaaatcaacatggatgactgtgccagcagcccttgcgactacg 2170
```

2171 gcaaatgcatcgacaggataaatggctatgagtgcgcgtgcgaacctggatacacagggatcatgtgtaa 2240

2241 catcaacattgatgagtgcgactcgaacccctgtcacaatggcggcacgtgcaaggacggcatcaatggc 2310

2311 ttcacctgtgtttgcccgcagggctaccaggatccgacctgcctttcagaagtcaacgagtgcaacagca 2380

2381 acccctgcattcacggaagatgccatgatggcatcaacggataccgctgtgactgcgacccgggctggag 2450

2451 cggtacaaactgtgacataaataacaacgaatgtgactccaatccctgcatgaacggaggaacctgcaaa 2520

2521 gacatgaccagcggatacctctgcgcctgccgggacggattcagtggccccaattgtcaaactaacatta 2590

2591 atgagtgtgcttcaaacccgtgcctgaatcagggaacctgcattgatgacgtggcagggtacaactgtaa 2660

2661 ctgcctgctgccgtatactggacccacatgcggggaggtcttggctccatgctctgataacccatgcaag 2730

2731 aacggaggcgaatgcggggaatctgaagattatgagagcttctcctgctcctgtcctgctggctggcaag 2800

2801 gccaaacatgtgagattgatatcaacgaatgtgtcaaaagcccctgcaggaatggcgcggtgtgtcagaa 2870

2871 cacagatgggagctaccggtgcaactgcaaagctggctactcaggccgccattgcgagacggacatcgat 2940

2941 gactgtctgccgaatccttgtcacagtggaggttcgtgctctgatggcatcaacgcgttcttctgcaact 3010

3011 gcctcgccgggttccgcgggcccaagtgtgaggaggacatcaacgagtgtgccagcaacccttgcaaaaa 3080

3081 cggggccaactgcaccgactgcgtcaatagctacacatgcacctgcccatcagggttcagcgggattcat 3150

3151 tgtgaaaacaacacccctgactgcactgagagctcttgcttcaatggtggcacttgcatcgatggcatta 3220

3221 acacgttcacctgccggtgcccagctggcttcataggtagttactgtgagcatgacgtcaacgagtgtga 3290

3291 ctccaagccgtgtctgaatggtgggacctgtcaggacagctatgggacctacaaatgcacctgtccccag 3360

3361 ggtttcaccggcatgaactgccagaatctcgtgcgctggtgtgaatcctccccatgcaagcatggaggca 3430

3431 agtgctggcaaaccaacaacctgtaccgctgcgagtgcaacagcggatggaccgggctctactgcgatgt 3500

3501 gcccagtgtgtcctgcgaggtagctgccaaacagcaaggagttgacgtggccaatctgtgccggaactct 3570

3571 ggcctttgcgaagacacgggcaacactcaccactgccgatgccaggcagggtacacggggagctactgcg 3640

3641 aggagcaagtggatgaatgctcgcccaacccttgccagaatggcgccacctgcacagactatctcggagg 3710

3711 ttattcttgtgagtgtgtagctggctatcacgggattaactgctcacaggagatcaatgaatgccagtct 3780

3781 cacccctgccagaacggaggaacctgcatcgacctcgtcaatacctataaatgctcttgtcccagaggaa 3850

3851 cacaaggggtgcactgtgaaatcaatgtcgacgactgcaatcctttcttcgatcctgtcacccatgagcc 3920

3921 gaagtgcttcaacaatggcaagtgtgttgaccgggttgggggttacaattgcaattgcctacctggcttc 3990

3991 gttggcgagcgctgtgaaggagatgtcaacgaatgcctgtccaacccctgcgacccccgaggaactcaga 4060

4061 actgcattcagctggttaatgattacaggtgcgagtgtcgacagggatattcaggacgacgctgcaacac 4130

4131 agtggtcgatggatgcaagggcaagccatgtagaaatggtggaacatgttctgtcgcgagtaacacagat 4200

4201 cgcggattcatctgtaaatgcccatctggctttgatggtgctatctgccaatacgattcacggtcctgtg 4270

4271 gcaatctcccctgcctgcatggcggatcctgcgtctccatcctcaaaacctcgcagtgcatgtgcaccgc 4340

4341 tgcctacaccggcccccagtgccagtaccccgtcagcagcccctgcaactcaagcccttgttacaacggg 4410
4411 ggcacatgcaaatttgttcctgaagccccgttttatcagtgtatgtgccccggaaaattcaatggcctct 4480
4481 actgccatattttggattatgagttcaatgggggcgttgggcaggacatcattccgcccgagattgaaga 4550
4551 gcagtgtgagatcccagtgtgcgcatcctcctccgggaacaagatttgcaacacccagtgcaacaaccat 4620
4621 gcatgcggctgggacggtggcgactgttccttaaatttcaatgatccctggaagaactgcactcagtccc 4690
4691 tacagtgctggaagtatttcaacgatggcaagtgtgactcgcagtgcaacaacgctggatgcctctacga 4760
4761 tggttttgactgtcagaaagtggaagttcagtgcaacccttttgtatgatcaatactgtagagatcatttt 4830
4831 caagatggccactgtgaccaaggatgtaataatgcagaatgtgaatgggacggtttggactgctccaaca 4900
4901 acatgccagagaagcttgcagatggcaccctggttatcgtagtcctcacacctcctgaactattgaagaa 4970
4971 taactcctttaacttcctgcgagagctgagtcgagtccttcacactaacgtagtgttcaagaaggacagc 5040
5041 aaaggcgagtacatgataatcccttactacggaagtgatgaagagctgactaaacaccacatcaagaggt 5110
5111 cgaccgaaacttggtcagacgtctccaccaatgtcttcaataaagtaaaaatgtccctctatacgtcatc 5180
5181 caatgggaggcagcgcagagagttggatcaaaatgaaatcaaagggtcaattgtgtatctggaaattgac 5250
5251 aaccgccagtgcttccagtcctcgttgcagtgtttccagagtgccaccgatgtggccgcctttcttggcg 5320
5321 ccctggcctcccacggcaacctgaacatcccttacaagatcgaagcagtcaaaagtgaaactggggagcc 5390
5391 ctccaaggggcctcctttgtatcttatgtacgtgctggtggtggccctggtcgtgctggctttcatcggc 5460
5461 gtgggggtcctggtgtctcgcaagcgacaccgggagcatgggcagctctggttcccagaaggcttcaaag 5530
5531 tgactgagaccaacaagagtaagcggcgaccgcccctgggggaggattctgtgggcctgaaacctctgaa 5600
5601 aaattccaccgacttaatggatgataatcagactgagtggggtgatgaagaaacattggacagcaaaaga 5670
5671 tttaggtttgaacaggaggcaatgctgcccgacatgaacgatcagacggatcgccgacagtggactcagc 5740
5741 agcacctggacgcggctgacctgcgcatctcctccatggccccgacaccaccccaaggcgagattgatcc 5810
5811 tgactgcctggatgttaacgtccgtggtcctgatggatttaccccattgatgatcgcttcttgcagtgga 5880
5881 ggaggactggaaacagggaacagcgaagaggaggaagacgcatctgccaacgtaatttctgacttcctct 5950
5951 accagggtgccaatctacacaaccaaaccgatagaacgggagagacggctctccacctggctgcacggta 6020
6021 tgcacgctcagatgccgctaaacgtctgctggaggcgagcgctaacgcaaatgttcaagacaacatggga 6090
6091 cgaactccgctccatgctgctgtggctgctgacgctcaaggtgtcttccagattttgatcaggaacagag 6160
6161 caactgagttggatgcccggatgcacgatggaacgaccccgttgatcctggcggctcgattggcagtgga 6230
6231 gggcatggtagaagaactcattaactgccatgcagatgtcaacgcagtggatgacctaggtaaatctgct 6300
6301 ctgcattgggctgctgctgtgaacaacgtggacgcggctatcgtgctcctgaagaacggcgccaacaaag 6370
6371 acatgcaggacaacaaggaggaaacaccactattccttgcagcgagagaaggaagctacgagactggcaa 6440
6441 agttctcttggaccacttcgcaaacagggacatcactgatcatatggaccggctgcctcgtgacattgct 6510

6511 caagagcgtatgcaccatgacattgtgcgacttctggatgaatataacttggtccggagcccacagatgc 6580

6581 acaacagtctgggggcacccacgttgtccccacagatctgctctcccagcagttacctgagcaatatgaa 6650

6651 acctgctgctcaagggaagaaagccagaaagcaaagtattaaaagtaatggctgcaatgggaaggagtcc 6720

6721 aaagacgtcaaagctaggaggaaaaaatcacaggatgggaaaaatcttctggatagctccggtggattat 6790

6791 cgccagtggactcacttgaatccccccatggctacatttcagatgtggcttccccacctctgatgacctc 6860

6861 tccatttcaacagtctccttcaatggctctgaacctcctgccaggcatgaccgatggtcacatgaccctc 6930

6931 aatcatcacaacctagcaggaaaacaagacatgacaatggctggctccaacagaatgacttttgagccag 7000

7001 tgcctccgcgtctttcccatctctctgtctcaagccccagtaccgtaatgagcagcggctctttgaactt 7070

7071 tactgtgggcggagcaccaaccatgaatgggcagtgtgattggctctcaaggttacaaaacgggatggtt 7140

7141 tcaaaccagtatagtgcactaagaaataatgcccagtcgagtgcccaccagcaagctcacagcatgatga 7210

7211 catctctgcagaacggcttgcccacaacaacattgtcccagttgatgagctaccaggcaatgccaaacac 7280

7281 aaggataggcagccagccccatttgatgcaggctcagcagctacagcaaatacaacagcagaacctgcaa 7350

7351 cagcaaattcagcagcagcaacaaactatgcagcaacaccacaattctagctcatctgctaataatcata 7420

7421 ttggtcaagtctttctcagcaacgatttaacccaggcagatctgcagcagatgaccagcaacagtatggc 7490

7491 agttcacacaattttgcctcaagatacccagcttctcacatcatccctaccatcttcactcacccagtct 7560

7561 atagcgactacacagtttttaacaccgccatcccaacacagttactcttctccaatggacaacactccta 7630

7631 gccaccaacttcaggtgtccgatcacccctttttgactccatctcctgaatccccagaccaatggtcaag 7700

7701 ctcttctcctcattccaacatttcagattggtcagaaggtatttctagcccccctacgagcatgcaagta 7770

7771 caaatagcacacatcccggaggcatttaagtaaaataatttgttctagagacagcggtacacagtggaac 7840

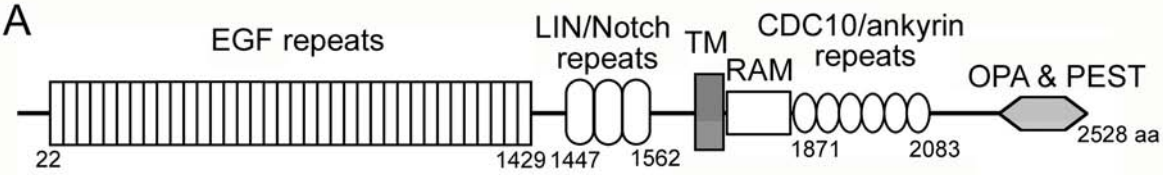
7841 ttaaaggttttacctaaatatataattaaaaaaaaaaaaaaaaaa 7885

Figure 11. The sequence of deduced amino acid of Cp-Notch-1.

```
   1 MRIPGFAALLLTLPSLAPALRCTLQSEKCMNGGKCEALPNGTSVCTCSSSAFVGERCQYSNPCLSSPCRNSGTCQVDIRG   80
  81 NTVDYSCICRLGYSDKLCLTPMDNVCLNNPCRNGGTCELLSSLTQHKCRCPPGWTGETCQQADPCASNPCGNGGQCVPFE  160
 161 AQYVCKCPPAYHGPTCKLDINECTSVPCKNGGTCVNEVGSYQCTCRPEYTGRNCENLYVPCYPSPCQNGGTCRQTGDTTY  240
 241 ECACLPGFDSQNCEVNIDDCPGNTCKNGGTCVDGVNTYNCQCPPEWTGQYCTEDVDECQLMPNACQNGGTCHNNHGGYNC  320
 321 VCVNGWTGEDCSENIDDCANAACHSGATCHDRVASFYCECPHGRTGLLCHLNDACISSPCNEGANCDTNPVNGKAICTCP  400
 401 SGYMGPACIQDVDECSLGANPCEHAGRCLNTLGSFQCQCSPGYTGPRCEMDVNECLSNPCKNDATCLDQIGEFHCICMPG  480
 481 YEGVFCQTNTDECASSPCLHNGRCIDKINEFHCECPIGFNGPLCQYDIDECASTPCKNGAKCLDGANSYTCDCAEGYSGF  560
 561 HCETDIDECDPDPCHYGTCSDGIAGFTCHCEPGYTGHRCEIDVNECQSMPCQNGGECQDRKNSYSCRCPKGTTGVNCEIN  640
 641 MDDCASSPCDYGKCIDRINGYECACEPGYTGIMCNINIDECDSNPCHNGGTCKDGINGFTCVCPQGYQDPTCLSEVNECN  720
 721 SNPCIHGRCHDGINGYRCDCDPGWSGTNCDINNNECDSNPCMNGGTCKDMTSGYLCACRDGFSGPNCQTNINECASNPCL  800
 801 NQGTCIDDVAGYNCNCLLPYTGPTCGEVLAPCSDNPCKNGGECGESEDYESFSCSCPAGWQGQTCEIDINECVKSPCRNG  880
 881 AVCQNTDGSYRCNCKAGYSGRHCETDIDDCLPNPCHSGGSCSDGINAFFCNCLAGFRGPKCEEDINECASNPCKNGANCT  960
 961 DCVNSYTCTCPSGFSGIHCENNTPDCTESSCFNGGTCIDGINTFTCRCPAGFIGSYCEHDVNECDSKPCLNGGTCQDSYG 1040
1041 TYKCTCPQGFTGMNCQNLVRWCESSPCKHGGKCWQTNNLYRCECNSGWTGLYCDVPSVSCEVAAKQQGVDVANLCRNSGL 1120
1121 CEDTGNTHHCRCQAGYTGSYCEEQVDECSPNPCQNGATCTDYLGGYSCECVAGYHGINCSQEINECQSHPCQNGGTCIDL 1200
1201 VNTYKCSCPRGTQGVHCEINVDDCNPFFDPVTHEPKCFNNGKCVDRVGGYNCNCLPGFVGERCEGDVNECLSNPCDPRGT 1280
1281 QNCIQLVNDYRCECRQGYSGRRCNTVVDGCKGKPCRNGGTCSVASNTDRGFICKCPSGFDGAICQYDSRSCGNLPCLHGG 1360
1361 SCVSILKTSQCMCTAAYTGPQCQYPVSSPCNSSPCYNGGTCKFVPEAPFYQCMCPGKFNGLYCHILDYEFNGGVGQDIIP 1440
1441 PEIEEQCEIPVCASSSGNKICNTQCNNHACGWDGGDCSLNFNDPWKNCTQSLQCWKYFNDGKCDSQCNNAGCLYDGFDCQ 1520
1521 KVEVQCNPLYDQYCRDHFQDGHCDQGCNNAECEWDGLDCSNNMPEKLADGTLVIVVLTPPELLKNNSFNFLRELSRVLHT 1600
1601 NVVFKKDSKGEYMIIPYYGSDEELTKHHIKRSTETWSDVSTNVFNKVKMSLYTSSNGRQRRELDQNEIKGSIVYLEIDNR 1680
1681 QCFQSSLQCFQSATDVAAFLGALASHGNLNIPYKIEAVKSETGEPSKGPPLYLMYVLVVALVVLAFIGVGVLVSRKRHRE 1760
1761 HGQLWFPEGFKVTETNKSKRRPPLGEDSVGLKPLKNSTDLMDDNQTEWGDEETLDSKRFRFEQEAMLPDMNDQTDRRQWT 1840
1841 QQHLDAADLRISSMAPTPPQGEIDPDCLDVNVRGPDGFTPLMIASCSGGGLETGNSEEEEDASANVISDFLYQGANLHNQ 1920
1921 TDRTGETALHLAARYARSDAAKRLLEASANANVQDNMGRTPLHAAVAADAQGVFQILIRNRATELDARMHDGTTPLILAA 2000
2001 RLAVEGMVEELINCHADVNAVDDLGKSALHWAAAVNNVDAAIVLLKNGANKDMQDNKEETPLFLAAREGSYETGKVLLDH 2080
2081 FANRDITDHMDRLPRDIAQERMHHDIVRLLDEYNLVRSPQMHNSLGAPTLSPQICSPSSYLSNMKPAAQGKKARKQSIKS 2160
2161 NGCNGKESKDVKARRKKSQDGKNLLDSSGGLSPVDSLESPHGYISDVASPPLMTSPFQQSPSMALNLLPGMTDGHMTLNH 2240
2241 HNLAGKQDMTMAGSNRMTFEPVPPRLSHLSVSSPSTVMSSGSLNFTVGGAPTMNGQCDWLSRLQNGMVSNQYSALRNNAQ 2320
2321 SSAHQQAHSMMTSLQNGLPTTTLSQLMSYQAMPNTRIGSQPHLMQAQQLQQIQQQNLQQQIQQQQQTMQQHHNSSSSANN 2400
2401 HIGQVFLSNDLTQADLQQMTSNSMAVHTILPQDTQLLTSSLPSSLTQSIATTQFLTPPSQHSYSSPMDNTPSHQLQVSDH 2480
2481 PFLTPSPESPDQWSSSSPHSNISDWSEGISSPPTSMQVQIAHIPEAFK                                  2528
```

Figure 12. *C. pyrrhogaster* newt Notch-1 (Cp-Notch-1). ***A,*** Domain structure. The cDNA clone (7885 bp; Accession No. AB095016) encoded an amino acid sequence (2528 aa) characteristic of Notch receptors: 36 EGF repeat region (residues 22-1429, 1408 aa), LIN/Notch repeat domain (1447-1562, 116 aa), transmembrane (TM) domain (1731-1753, 23 aa), RAM domain (1757-1811, 55 aa), CDC10/ankyrin repeat domain (1871-2083, 213 aa), OPA repeat region (2367-2390, 24 aa,) and PEST region (2480-2499, 20 aa). ***B,*** Homology. The percentage of amino acid identity of the EGF, LIN/Notch and CDC10/ankyrin repeat domains as well as the total length were calculated from multiple alignment of Cp-Notch-1 with known vertebrate Notch-1. In C-Notch-1 and Z-Notch-1, domains in the blanks have not been identified. ***C,*** Phylogenetic tree analysis. A multiple sequence comparison of Notch homologues was carried out. Cp-Notch-1 was classified into the Notch-1 family rather than the Notch-2 or Notch-3 one. Sequence data used herein were listed in Materials and Methods. H, human; M, mouse; R, rat; C, chick; X, *Xenopus laevis*; Z, zebrafish; Dm, *Drosophila melanogaster*. Note that Cp-Notch-like-protein (Kaneko et al., 2001) was quite different from Cp-Notch-1 (not shown).

A



B

Name	EGF repeats	LIN/Notch repeats	CDC10/ankyrin repeats	Total
Cp-Notch-1	100%	100%	100%	100%
H-Notch-1	78.3%	81.0%	92.5%	75.5%
M-Notch-1	77.7%	81.9%	92.0%	75.7%
R-Notch-1	77.2%	81.9%	92.5%	75.2%
C-Notch-1	-	88.8%	92.5%	-
X-Notch-1	80.0%	87.8%	91.5%	77.6%
Z-Notch-1	-	-	94.4%	-

C

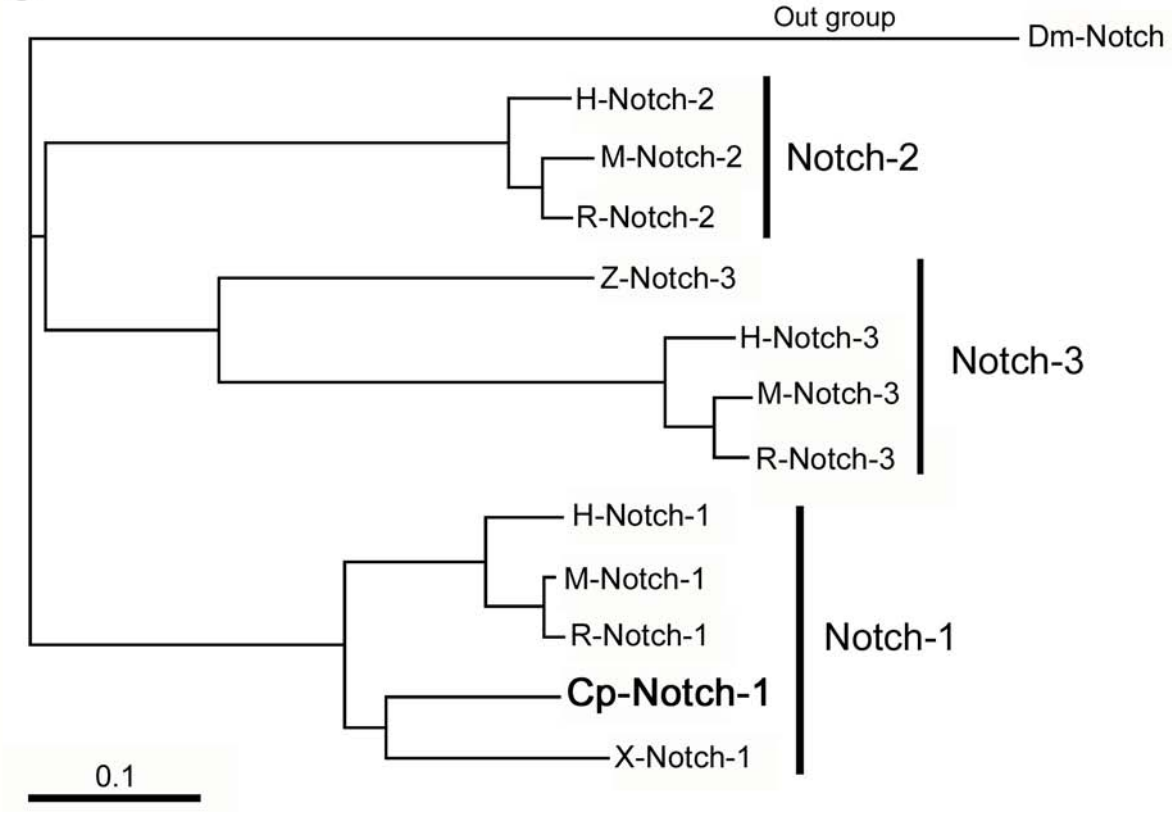


Figure 13. *C. pyrrhogaster* newt Delta-1 (Cp-Delta-1). *A,* Domain structure. The cDNA clone (3646 bp; Accession No. AB095017) encoded an amino acid sequence (726 aa) characteristics of Delta/Serrate/Lag-1 (DSL) ligands: DSL domain (residues 180-224, 45 aa) and 8 EGF repeat region (225-519, 295 aa). *B,* Homology. The percentage of amino acid identity was calculated from multiple alignment of Cp-Delta-1 with known vertebrate Delta-1 homologues. This molecule had the identity of 65-84% in total as compared with the Delta-1 homologues of other vertebrates. In the DSL domain and EGF repeat region, the identity was higher than 80% (DSL domain, 80.0-93.3%; EGF repeat region, 80.0-91.9%). *C,* Phylogenetic tree analysis. A multiple sequence comparison of Delta homologues was carried out. Cp-Delta-1 was classified into the Delta-1 family rather than the other Delta or other DSL ligands such as Serrate or Jagged (data not shown). The sequence data used here were taken from DDBJ (accession no. in parentheses): *Drosophila melanogaster* (Dm) Delta (X06289), human (H) Delta-like (Dll)1 (AF196571), H-Dll3 (AK075302), H-Dll4(AY358894), mouse (M) Dll1 (X80903), M-Dll3 (AB013440), M-Dll4 (BC042497), rat (R) Delta-1 (U78889), chick (C) Delta-1 (U26590), *Xenopus laevis* (X) Delta-1 (L42229), X-Delta-2 (U70843), zebrafish (Z) DeltaA (AF030031), Z-DeltaB (AF006488), Z-DeltaC (AF146429), Z-DeltaD (AF426384).

A

B

Name	DSL domain	EGF repeats	Total
Cp-Delta-1	100%	100%	100%
H-Dll1	91.1%	86.8%	77.8%
M-Dll1	88.9%	84.1%	74.5%
C-Delta-1	93.3%	91.9%	83.6%
X-Delta-1	80.0%	88.1%	77.7%
Z-DeltaA	88.9%	80.7%	65.7%
Z-DeltaD	93.3%	80.0%	72.3%

C

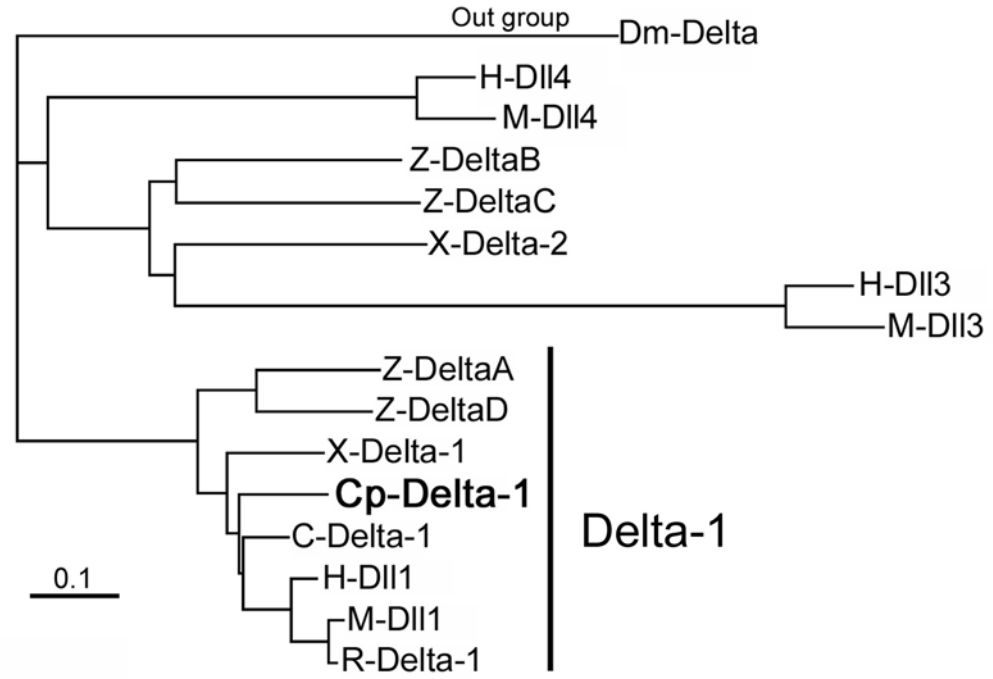
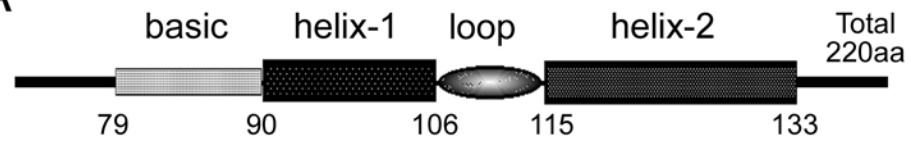


Figure 14. *C. pyrrhogaster* newt Ngn-1 (Cp-Ngn-1). *A,* Domain structure. The cDNA clone (1719 bp; Accession No. AB065284) encoded an amino acid sequence (220 aa) containing the b-HLH domain (residues 79-133, 55 aa). *B,* Homology. The percentage of amino acid identity was calculated from multiple alignment of Cp-Ngn-1 with known vertebrate Ngn-1 homologues. This molecule had the identity of 51-58% in total as compared with other vertebrate Ngn-1. In the b-HLH domain, the identity was higher than 83% (83.6-87.3%). *C,* Phylogenetic tree analysis. A multiple sequence comparison of Ngn or atonal homologues was carried out. Cp-Ngn-1 was classified into the Ngn-1 family rather than the other Ngn or other b-HLH factors such as the vertebrate NeuroD, atonal or achaete-scute homologues. The sequence data used here were taken from DDBJ (Accession No. in parentheses): human (H) Ngn-1 (BT019366), H-Ngn-2 (BC036847), H-Ngn-3 (AF234829), mouse (M) Ngn-1(U67776), M-Ngn-2 (BC055743), M-Ngn-3 (U76208), Math-3 (D85845), Math-5 (AF071223), Mash-1 (M95603), M-NeuroD (AK005073), rat (R) Ngn-1 (U67777), chiken (C) Ngn-1 (AF123883), C-Ngn-2 (AF303000), *Xenopus laevis* (X) ngnr-1a (U67778), X-ngnr-1b (U67779), Xath-3 (D85188), Xath-5a (U93170), Xath-5b (U93171), Xash-1 (M98272), X-NeuroD (U28067), zebrafish (Z) Ngn-1 (BC066427), Zath-3 (AY036624), Zath-5 (AB049457).

A



B

Name	bHLH	Total
Cp-Ngn-1	100%	100%
H-Ngn-1	87.3%	52.6%
M-Ngn-1	87.3%	51.4%
C-Ngn-1	85.5%	57.4%
Z-Ngn-1	83.6%	53.7%

C

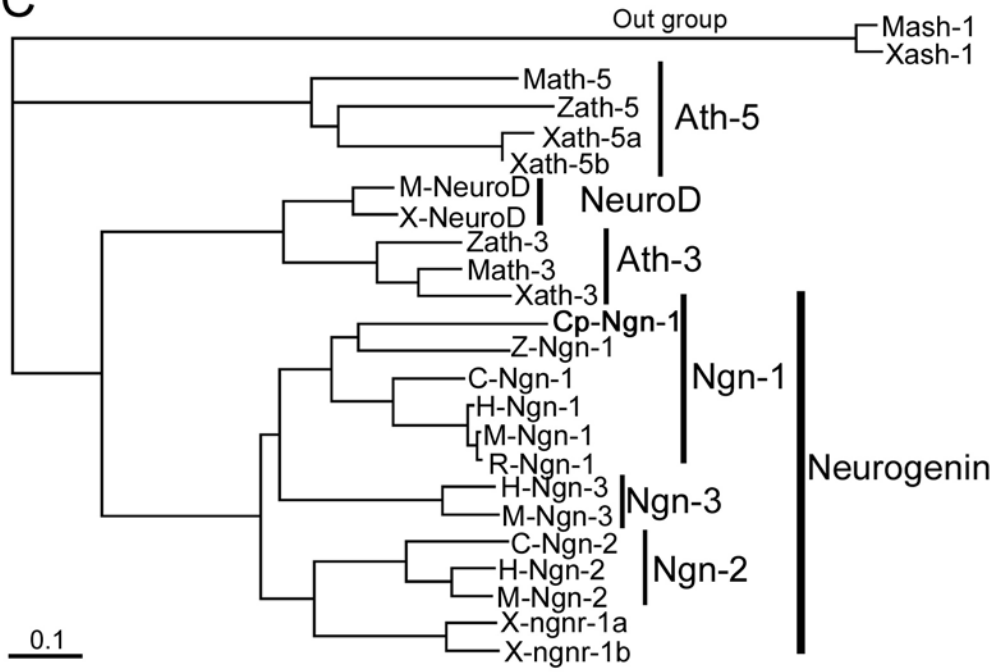


Figure 15. Expression pattern of *Notch-1* during retinal development. ***A,*** Stage 26. *Notch-1* expression was not detected in the optic vesicle. ***B,*** Stage 30. *Notch-1* was uniformly expressed in the central region of the inner cell layer of the optic cup. ***C,*** Stage 32. *Notch-1* expression was apparently decreased in the inner one third of the central retina (red asterisk). ***D,*** Stage 37. The *Notch-1* positive region extended toward the periphery, while both the inner half (presumptive ganglion cell layer) and the outer most cell layer (presumptive photoreceptor cell layer) became *Notch-1* negative (red asterisks). **E,** Stage 42. The retina had segregated into two synaptic layers. *Notch-1* expression was restricted in the peripheral immature retina (red arrowheads). ***F,*** Stage 55. The *Notch-1* positive region in the periphery became narrower (red arrowheads). ***G,*** Juvenile (1-2 weeks after metamorphosis). The *Notch-1* signal in the periphery was very low (red arrowhead). When the image in the square is enhanced (***g_1***) and traced (***g_2***), the *Notch-1* positive region is obvious (blue area in g_2). ***J,*** Adult. The *Notch-1* signal was not detected even in the peripheral margin of neural retina (i.e., *ora serrata*; OS) and non-pigmented region (NPR, green asterisk) of *pars ciliaris retinae*. Green asterisks in B-G indicate the *Notch-1* negative non-pigmented epithelium adjacent to the growing retina. For abbreviations, see Fig. 1. Scale bar: 150 μm.

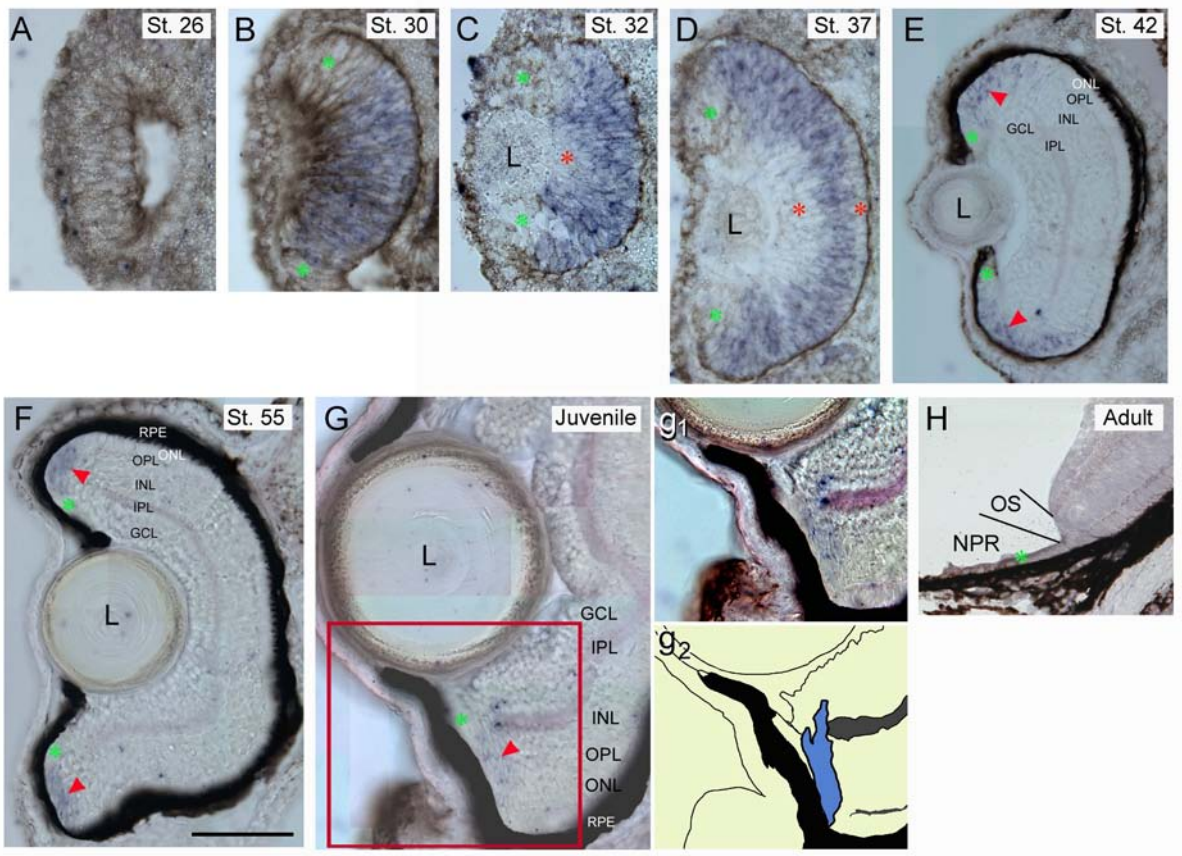

A
St. 26
B
St. 30
C
St. 32
L
D
St. 37
L
E
St. 42
ONL
OPL
INL
GCL
IPL
L
F
St. 55
RPE
OPL
ONL
INL
IPL
GCL
L
G
Juvenile
L
GCL
IPL
INL
OPL
ONL
RPE
g1
g2
H
Adult
OS
NPR

Figure 16. Expression pattern of *Notch-1* during retinal regeneration. ***A, B,*** Stage E-3. *Notch-1* positive cells appeared sparsely (arrows, A). B: a negative control with a sense probe. ***C, D,*** Stage I-1. *Notch-1* was expressed in the regenerating retina uniformly (C). D: a negative control. ***E-G,*** Stage I-2. *Notch-1* expression was declined in cells located in the most proximal region (asterisk, E) where a type of voltage-gated Na^+ channels *Cp-Na$_V$1* was complementarily expressed (F). Those cells are probably premature ganglion cells. G: a negative control with a sense probe for *Notch-1*. ***H-J,*** Stage I-3. *Notch-1* expression was declined not only in the inner two fifth (presumptive ganglion cell layer) of regenerating retina but also the outer most cell layer (presumptive photoreceptor cell layer) (asterisks, H). A small number of photoreceptor cells expressed *rhodopsin* (*Rho*) in their ellipsoid body (arrowhead, I). The image of a photoreceptor cell is enlarged in the square. J: a negative control with a sense probe for *Notch-1*. ***K,*** Stage L-1. *Notch-1* expression became restricted in a small number of cells in the inner nuclear layer; those look like Müller glia cell (arrow). ***L,*** Stage L-1 to L-2. *Notch-1* expression was not detected. Small horizontal bars in B, D, G and J indicate the most proximal margin of regenerating retina. For abbreviations, see Fig. 3. Scale bar: 50 μm.

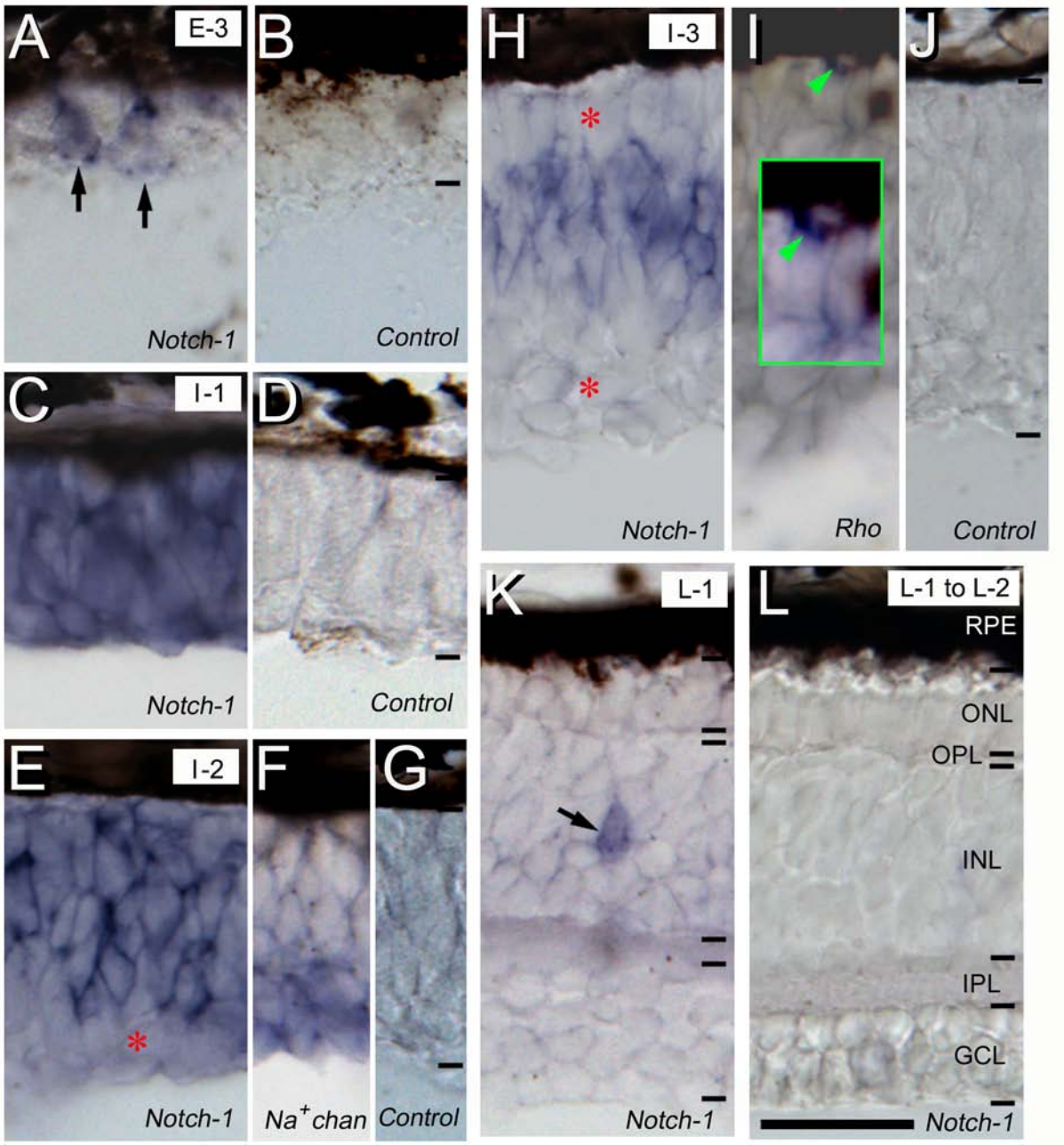

A
E-3
Notch-1
B
Control
C
I-1
Notch-1
D
Control
E
I-2
Notch-1
F
Na+chan
G
Control
H
I-3
Notch-1
I
Rho
J
Control
K
L-1
Notch-1
L
L-1 to L-2
RPE
ONL
OPL
INL
IPL
GCL
Notch-1

Figure 17. ***A-C,*** Expression pattern of *Notch-1* in the peripheral region of regenerating retina. A trace of image is shown under the image (***a-c***). The blue area in m-o shows the *Notch-1* positive region. M: 20 days po. N: 23 days po. A: 45 days po. *Notch-1* expression became restricted in the peripheral region of retina with progress of regeneration. Note that the *Notch-1* negative non-pigmented epithelium (green asterisks) adjacent to the peripheral regenerating retina was preserved throughout regeneration. Small horizontal bars in B, D, G and J indicate the most proximal margin of regenerating retina. For abbreviations, see Fig. 3. Scale bar: 200 μm.

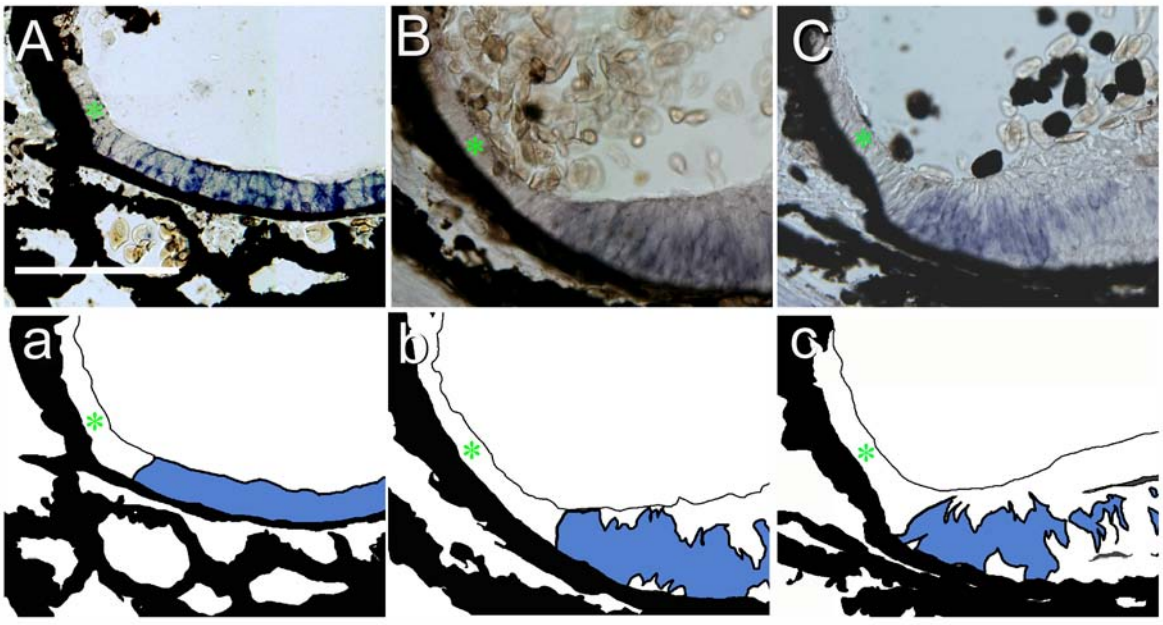
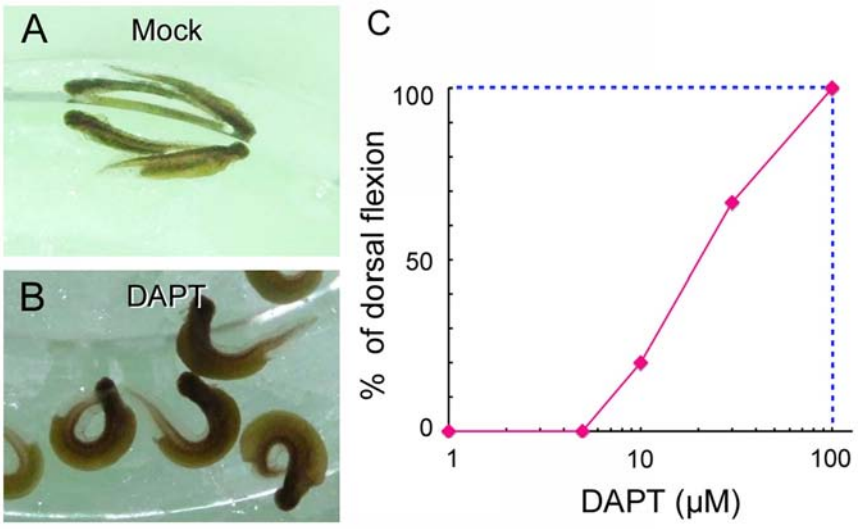

A
B
C
a
b
c

Figure 18. Culture of newt embryos in the presence of DAPT. Newt embryos of stage 26 were cultured in 0.1 x MMR containing 5-100 μM DAPT for 5 days. *A,* Embryos cultured in the presence of solvent DMSO (1%). They looked normal in morphology except that their developmental stage (39-42) was slightly delayed. In the absence of DMSO, embryos normally developed to stage 41-43. *B,* Embryos cultured in the presence of 100 μM DAPT/1% DMSO. They showed an abnormal 'dorsal flexion' phenotype consistently. *C,* Dose-dependent appearance of the 'dorsal flexion' phenotype. When embryos were cultured in the presence of 10 μM DAPT, they occasionally showed an abnormal 'dorsal flexion' phenotype. The appearance ratio of this phenotype increased in a dose dependent manner and reached 100% at 100 μM DAPT.


A
Mock
B
DAPT
C
100
50
0
% of dorsal flexion
1
10
100
DAPT (μM)

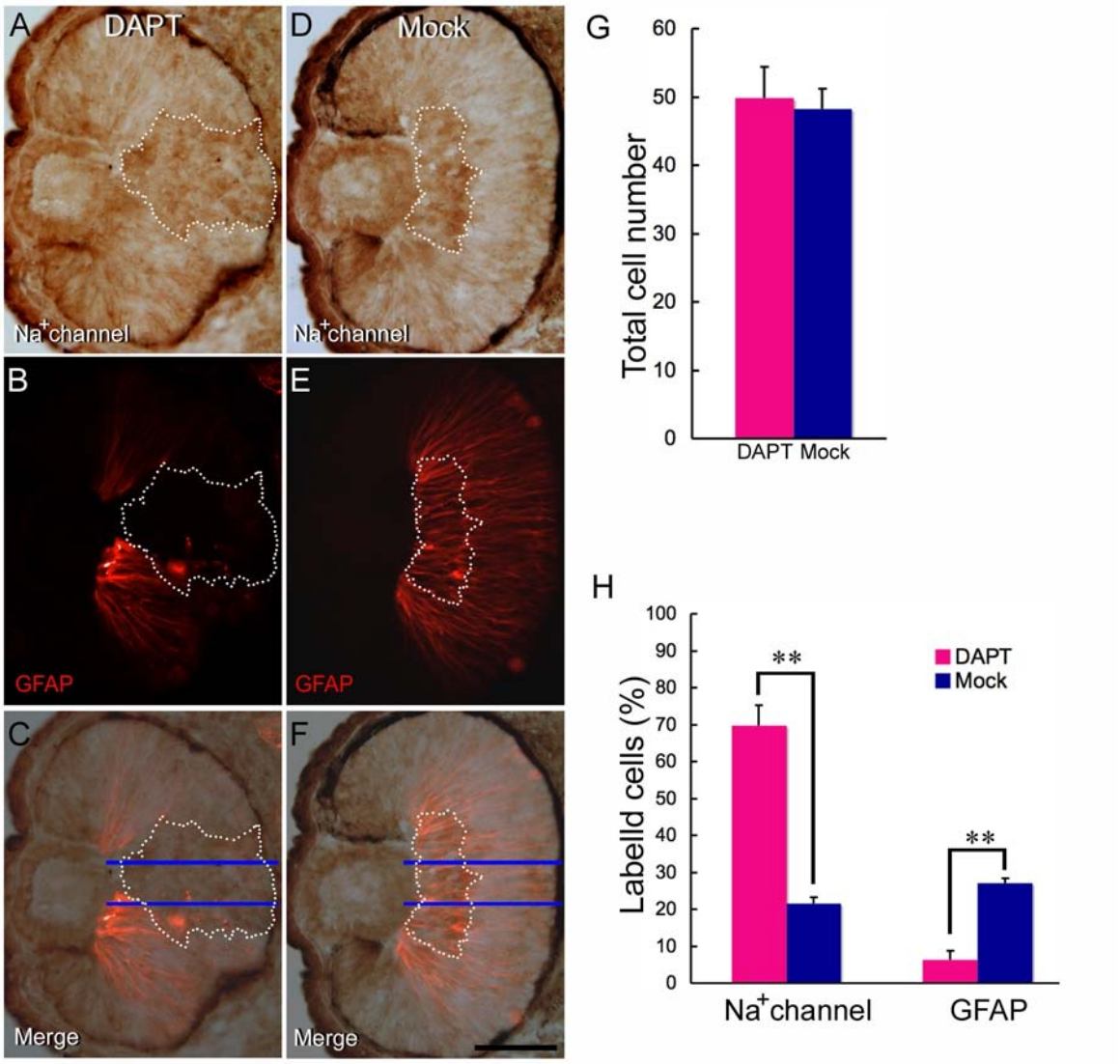

A
DAPT
Na+channel
B
GFAP
C
Merge
D
Mock
Na+channel
E
GFAP
F
Merge
G
Total cell number
60
50
40
30
20
10
0
DAPT Mock
H
Labelld cells (%)
100
90
80
70
60
50
40
30
20
10
0
**
**
DAPT
Mock
Na+channel
GFAP

Figure 19. Effects of DAPT on retinal development. ***A-C,*** An ocular section of newt embryo cultured in the presence of 100 μM DAPT. ***D-F,*** A mock control. The culture medium contained the solvent 1% DMSO only. ***A, D,*** Immunoperoxidase labelling image with the voltage-gated Na^+ channel antibody. In the presence of DAPT Na^+ channel expressing neurons increased in number and enlarged their territory to the most distal margin of retina (the area enclosed by a dotted line in A) as compared to the mock control where those are restricted in the inner one third of the central retina, i.e., presumptive ganglion cell layer (the area enclosed by a dotted line in D). ***B, E,*** Immunofluorescence labelling image with the GFAP antibody. In the presence of DAPT (B), the GFAP-labelled cells almost disappeared from the Na^+ channel-labelled area, while in the mock control (E) the retinal progenitor cells, which elongate longitudinally throughout the thickness of retina were intensely labelled even in the central retina. Note that in the newt the GFAP antibody labels not only Müller glia cells (Umino and Saito, 2002) but also retinal progenitor cells in a region corresponding to the Notch(+) region (compare E to Fig. 3C). ***C, F,*** A merge of both images. Scale bar: 100 μm. ***G,*** Comparison of the total cell number between the DAPT and mock treatment. Cells were counted in the area of 50 μm in width (between blue bars in C and F) in the central retina. The total cell number per section (49.8 ± 4.5, n=6) in the DAPT treatment had no apparent difference from that (48.3 ± 2.9, n=7) in the mock treatment. ***H,*** Comparison of the ratio of Na^+ channel/GFAP-labelled cells to the total cell number between the

DAPT and mock treatment. The ratio (69.7 ± 5.5%) of Na^+ channel-labelled cells in the DAPT treatment was significantly larger than that (21.6 ± 1.7%) in the mock treatment (**p<0.01, *t*-test). On the other hand, the ratio (6.3 ± 2.4%) of GFAP-labelled cells in the DAPT treatment was significantly smaller than that (27.0 ± 1.3%) in the mock treatment (**p<0.01, *t*-test).

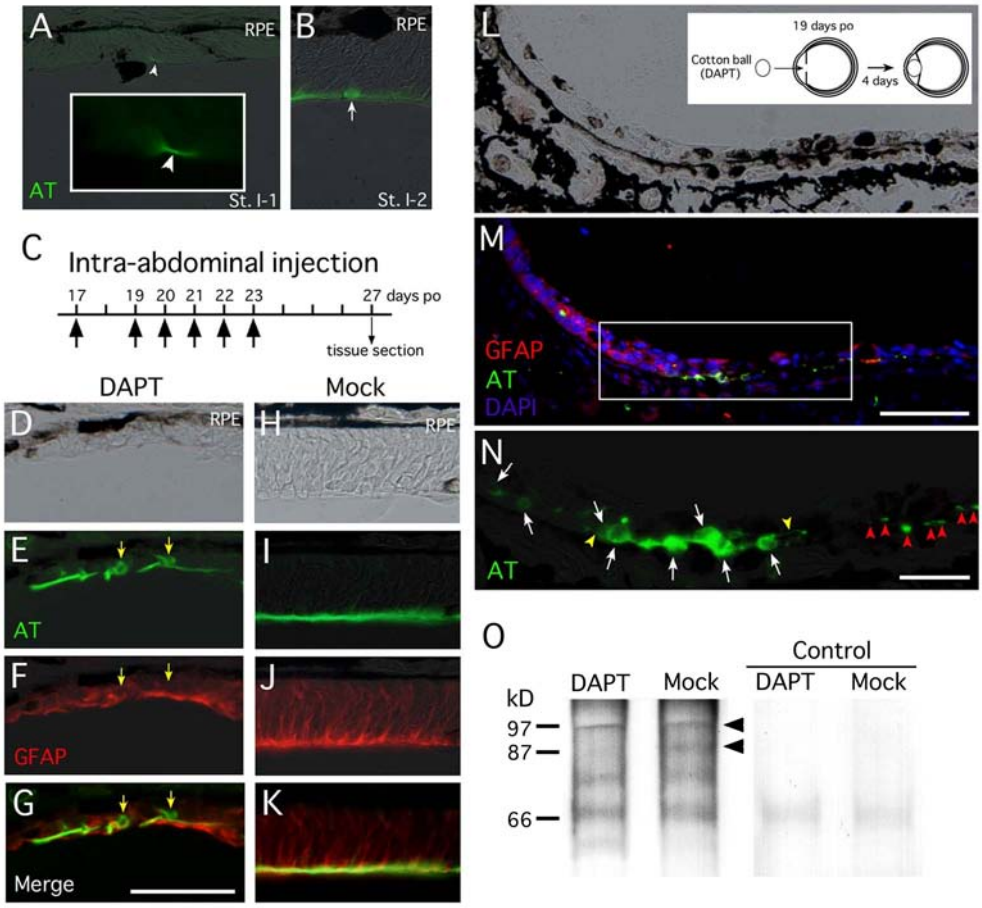

A
RPE
AT
St. I-1
B
RPE
St. I-2
C
Intra-abdominal injection
17
19
20
21
22
23
27 days po
tissue section
DAPT
Mock
D
RPE
H
RPE
E
AT
I
F
GFAP
J
G
Merge
K
L
19 days po
Cotton ball
(DAPT)
4 days
M
GFAP
AT
DAPI
N
AT
O
Control
DAPT
Mock
DAPT
Mock
kD
97
87
66

Figure 20. Effects of DAPT on retinal regeneration. ***A, B,*** AT-IR during normal retinal regeneration. At stage I-1 (A) a weak immunofluorescence was rarely observed in short processes, probably the axon of premature ganglion cell (arrowhead). An enlargement is shown in the inset. At stage I-2 (B) the fluorescence was observed in fibers along the most proximal margin of regenerating retina (presumptive optic fiber layer) and only rarely in round cell bodies in the most proximal region, probably those of premature ganglion cells (arrow). ***C,*** Time schedule of intra-abdominal injection. DAPT or the solvent DMSO was injected into the abdominal space on 17 and 19-23 days po (once a day) and then tissue sections were prepared on 4 days after the last injection. ***D-G,*** Effects of intra-abdominal injection of 10 mM DAPT. D, a bright-light image; E and F, AT- and GFAP-immunofluorescence images; G, a merge of the images in E and F. AT-labelled neurons appeared in clusters along a region of one-two cells in thickness in the regenerating retina of which the most part was 4-5 cells in thickness (stage I-1 to I-2). The AT-IR was observed in both the cell body (arrows, E) and processes. Note that such an intense immunorecativity was not observed in other 4-5 cell regions of the same regenerating retina but only in the optic fibers. The AT-labelled cell bodies were also labelled with the GFAP antibody lightly (arrows, F). ***H-K,*** A mock control where DMSO with no dilution was injected as DAPT. Images were displayed as in D-G. Morphological abnormalities of regenerating retina were not recognized except a delay of regeneration. The regenerating retina was typically 4-5

cells in thickness (stage I-1 to I-2), being more immature than the normally regenerating retina (a trasitional stage from I-3 to L-1; see Fig. 1). The AT-IR was observed only in the optic fibers (I). The GFAP-immunoreactivity was observed in the retinal progenitor cells that elongate longitudinally throughout the thickness of regenerating retina (J) as in the developing retina (see Fig. 5E). ***L-N,*** Intraocular implantation of cotton ball containing DAPT. A cotton ball containing 10mM DAPT was implanted into the anterior chamber of the eyeball of 19 days po, and tissue sections were prepared on 4 days after implantation (inset in L). L, a bright-light image of the ventral part of regenerating retina (the pupil is on the upper left side); M, a merge of AT- and GFAP-immunofluorescence images and DAPI counterstaining image of nuclei; N, enlargement of the AT-immunofluorescence image in the square in M. A cluster of AT-labelled neurons appeared along the regenerating retina of 1-3 cells in thickness, which was comprised of pigmented or non-pigmented cells. The AT-IR was observed in both the cell body (arrows in N) and processes (yellow arrowheads in N). The cell bodies of neurons were present along the border between the regenerating retina and RPE layer, and extended processes along the most distal margin of regenerating retina. Varicosity-like dots were also observed lining along the most distal margin of regenerating retina (red arrowheads in N). The cell bodies were also labelled with the GFAP antibody (M). Scale bar: 100 μm in G (applies to A, B, D-L); 150 μm in M (applies to L); 60 μm in N. ***O,*** Immunoblot analysis. Eye-cups of 19 days po were cultured in the presence of

100 μM DAPT for 20 hr and then the protein extract from 3 eye-cups was immunoblotted with the Notch-1 antibody which recognizes the intracellular domain of Cp-Notch-1 (lane *DAPT* on the left-hand side). The protein band (~97kD) corresponding to the membrane-tethered intracellular domain (NTM) was evident but that (~87kD) of the Cp-Notch-1 intracellular domain (Cp-NICD) was too pale to detect. In the mock control where the eye-cups were cultured in the absence of DAPT (the second lane *Mock* from the left-hand side), both protein bands were detected (arrowheads). Other bands lower than 87kD are non-specific. The right-hand two lanes (*Control*) were the negative control without the primary antibody.

Figure 21. A morphological abnormality of regenerating retina caused by intra-abdominal injection of 10 mM DAPT. A cluster of neurons was observed around a crack of regenerating retinas of 4-5 cells in thickness. ***A,*** Na^+ channel immunoperoxidase labeling. A large number of neurons expressing voltage-gated Na^+ channels were generated throughout the thickness of regenerating retina (arrows). Arrowheads indicate Na^+ channel-labeled neurons, probably premature ganglion cells, appeared in the conventional region. ***B,*** GFAP-immunofluorescence labeling. The fluorescence image was merged on the bright-light image in A. The outlines of the cell bodies of displaced neurons were labeled with the GFAP antibody. Those neurons still had GFAP-labeled processes extending longitudinally, suggesting a hasty production of neurons from progenitor cells. The cell bodies of conventional neurons were also labeled with the GFAP antibody. Scale bar: 50 μm.

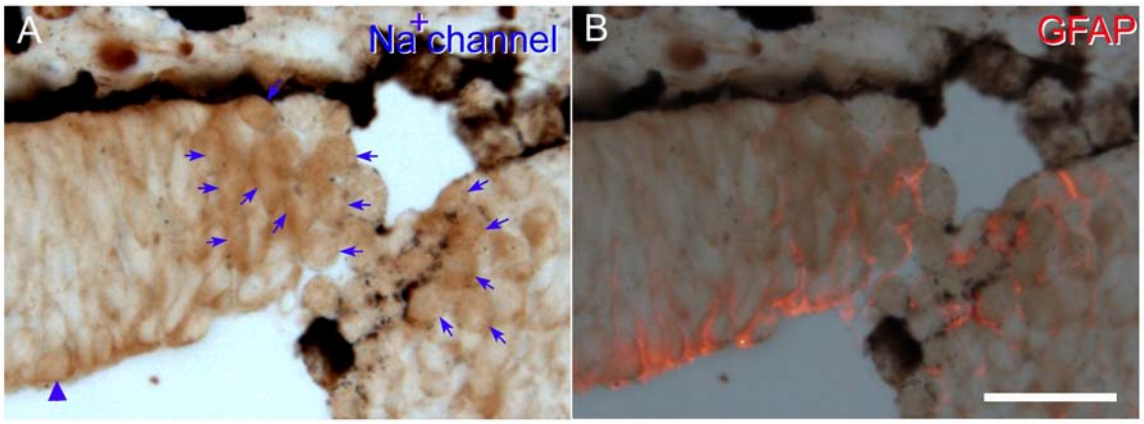

A
Na+ channel
B
GFAP

Figure 22. Intraocular implantation of a cotton ball containing DMSO (Mock control for Figure 20L-N). ***A***, immunofluorescence of AT. ***B,*** immunofluorescence of GFAP. ***C,*** merge. In mock control, regenerating retina did not have the cells which abnormally AT-IR. Scale bar: 100 μm.

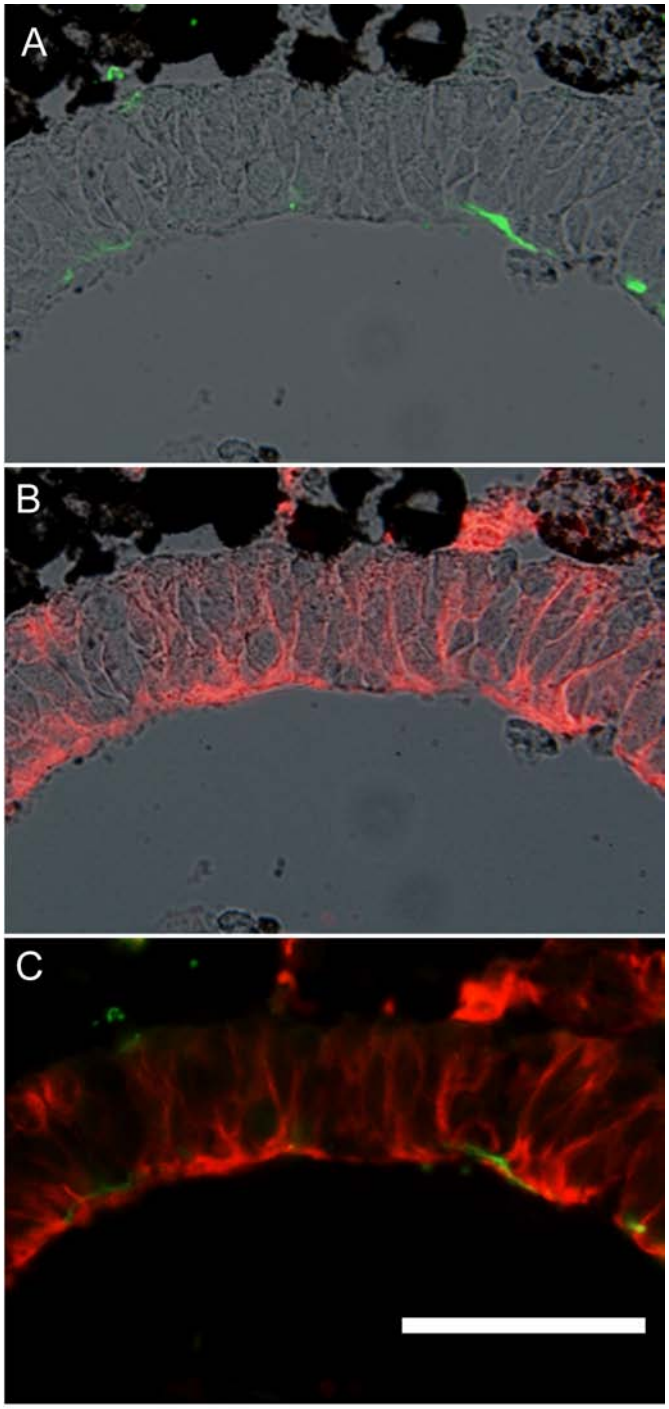

A
B
C

Figure 23. Expression patterns of genes involved in Notch signaling and neural marker genes in association with retinal regeneration. ***A,*** PCR analysis with eye-cups. Template cDNAs were prepared from six eye-cups of 0, 10, 14, 19, 23 and 45 days po, and from six normal neural retinas (*N*). Genes analyzed were *Notch-1*, *Delta-1*, *Hes-1*, *Ngn-1*, *N-CAM*, *voltage-gated* Na^+ *channel* $CpNa_V1$ and *Opsin Cp-L* (for each accession number, see Materials and Methods; for PCR conditions, see Supplemental Table 1). Arrowhead indicates an RNA-spliced variant of *N-CAM*, *N-CAM-180*. *Ef1-α* was also examined as an internal control. The number in parenthesis indicates the cycle number of PCR. The amount of *Ef1-α* PCR product of 14 days po was slightly lower than that of 10 days po. This might reflect errors during the process of tissue collection, and responsible for a transient decrease of band intensity on 14 days po in *Hes-1* and *Ngn-1*. ***B,*** Single-cell RT-PCR analysis. One solitary RPE cell acutely isolated from adult eyeballs was carefully picked up for RT-PCR (see Materials and Methods). Photomicrograph shows an RPE cell. Scale bar: 20 μm. Genes analyzed were *RPE65*, *Hes-1*, *Ngn-1*, *Delta-1*, *Notch-1* and *Pax6* (for each accession number, see Materials and Methods; for PCR conditions, see Supplemental Table 2). In this cell, the intense product band of *RPE65* was detected by the first series of PCR. The product bands of *Hes-1* and *Ngn-1* were detected by the first series of nested PCR, and that of *Delta-1* was detected by the second series of nested PCR while those of *Notch-1* and *Pax-6* were not.

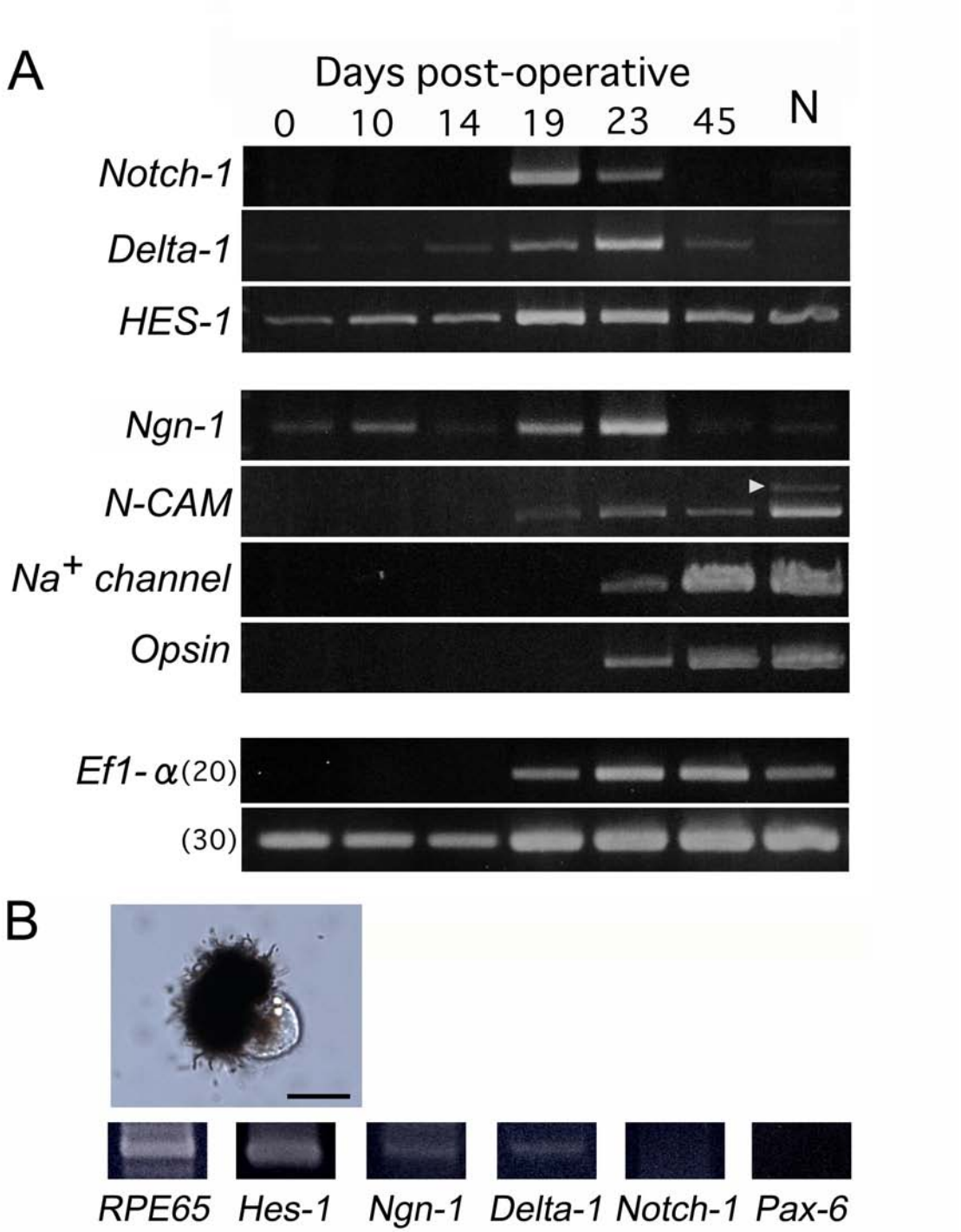
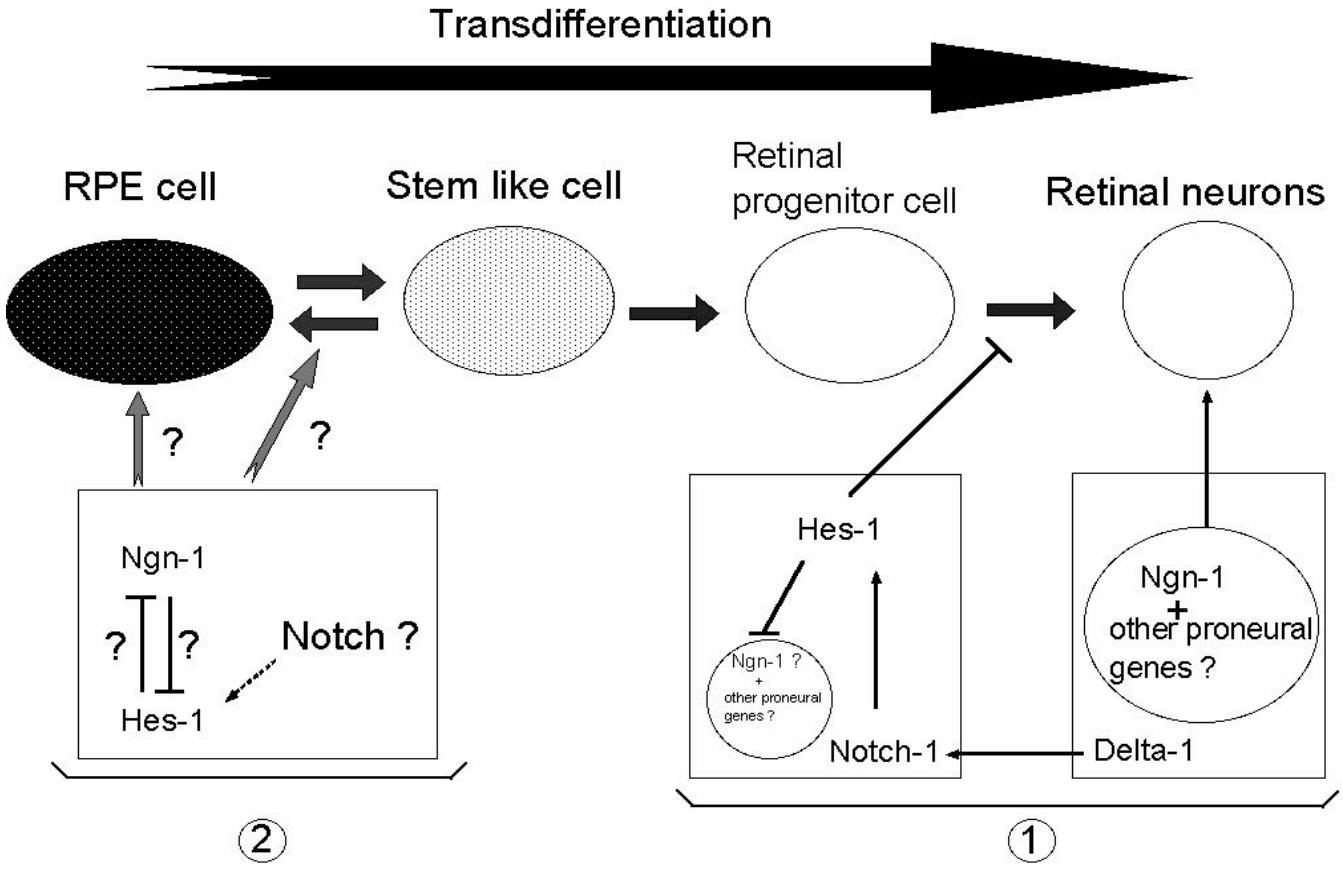

A
Days post-operative
0
10
14
19
23
45
N
Notch-1
Delta-1
HES-1
Ngn-1
N-CAM
Na+ channel
Opsin
Ef1-α(20)
(30)
B
RPE65
Hes-1
Ngn-1
Delta-1
Notch-1
Pax-6

Figure 24. Two points of Notch signaling involvement in retinal regeneration through transdifferentiation. 1), classical lateral inhibition system. Notch signaling may be involved in maintenance of retinal progenitor cells and inhibition of differentiations of retinal neurons. 2), Notch signaling has a possibility to be involved in the early process of transdifferentiation or RPE cell state itself.


Transdifferentiation
RPE cell
Stem like cell
Retinal progenitor cell
Retinal neurons
?
?
Ngn-1
?
?
Notch ?
Hes-1
2
Hes-1
Ngn-1 ? + other proneural genes ?
Notch-1
Ngn-1 + other proneural genes ?
Delta-1
1

8. Tables

Table 1. PCR primers for analysis of retinal regeneration

Gene Name (Ac. No.)	Primer set	Product size (bp)	PCR cycles
Notch-1 (AB095016)	Sense 5'-atgatcgcttcttgcagtggaggaggactggaa-3' Antisense 5'-atctggaagacaccttgagcgtcagcagccaca-3'	287	35
Delta-1 (AB095017)	Sense 5'-gaagggtggggtggactctt-3' Antisense 5'-ctcctgtatcatgctcttgg-3'	760	35
Hes-1 (AB019516)	Sense 5'-ccccggacaagcccaagagtgccagcga-3' Antisense 5'-ccgccgtacactttgggggggctctggtg-3'	466	35
Neurogenin (AB065284)	Sense 5'-atacagcacagcatcaagaagaaccggc-3' Antisense 5'-agcagcatctccttccctgacttttcct-3'	239	45
N-CAM (D85084)	Sense 5'-agactggcaatgctgcaaactcag-3' Antisense 5'-ggatcatgccttgctctcattctc-3'	N-CAM-140: 2551 [1)] N-CAM-180: 3315	25
Na^+ channel (AF123593)	Sense 5'-atgagggtggttgtgaatgcccttgtag-3' Antisense 5'-tctggtcatccgtttctatcatcatcgttacc-3'	700	35
Opsin cp-L (AB043891)	Sense 5'-ctcggtacaagggaagtcaagaggga-3' Antisense 5'-gatggaaagaggcagaatacagcaggtg-3'	736	35
Ef1-α (AB005588)	Sense 5'-gacctttgccccccagtaacgtaaccac-3' Antisense 5'-actgggtgttgctggcgctacttcttg-3'	573	20 or 30

Table 2. Nested PCR for single cell RT-PCR

Gene Name		Nested PCR	
	1st Primer set {Product size (bp)}	1st Primer set {Product size (bp)}	2nd Primer set {Product size (bp)}
Notch-1	S, 5'-cagcaaaggcgagtacatga-3'	S, 5'-cctcccacggcaacctgaac-3'	S, 5'-cgaagaggaggaagacgcat-3'
(AB095016)	A, 5'-ggttgtgatgattgagggtc-3'	A, 5'-tcttcaggagcacgatagcc-3'	A, 5'- tcttcaggagcacgatagcc-3'
	(1907)	(1028)	(452)
Delta-1	S, 5'-aggggatatggtggattgaactgcc-3'	S, 5'- agccccaagagcatgatacaggagacaaca-3'	S, 5'-gttagtgtcattggggcga-3'
(AB095017)	A, 5'-aaacgatacacttcaggcag-3'	A, 5'-aaccaaccgtccatagtgc-3'	A, 5'-ctggtactttgtctcttttg-3'
	(1026)	(879)	(299)
Hes-1	S, 5'- gttcgagatttgcagagccctaca-3'	S, 5'-gccaactcaagacgctcatcct-3'	none
(AB019516)	A, 5'- cgttatagtcagtgtgtcgtcctc-3'	A, 5'-gaaggcgaattgtccgtcagtg-3'	
	(1147)	(413)	
Neurogenin	S, 5'-aacttgggcggacagcacaactttacga-3'	S, 5'-atacagcacagcatcaagaagaaccggc-3'	none
(AB065284)	A, 5'-agcagcatctccttccctgacttttcct-3'	A, 5'-agcatctccttccctgacttttcctg-3'	
	(482)	(236)	
RPE65	S, 5'-tgctgaaaaactgacgggggaatacctc-3'	none	none
(AB095018)	A, 5'-aatctgtggaaactcaaaggctaggcgc-3'		
	(400)		
Pax-6	S, 5'-ataagggagacacaggggatc-3'	S, 5'-tcatagcggagtcaaccagct-3'	S, 5'-tgagcaagatcgcgcagtaca-3'
(D88741)	A, 5'-tgcttgcttgcctcctctggtt-3'	A, 5'-ttcccttctccactttgccct-3'	A, 5'-gcagctagtctctctcttgca-3'
	(875/919)[1)]	(793/835)[2)]	(484)

[1), 2)] These primer sets were designed to amplify four variants of Pax-6 (SL, SS, LL. and LS), so that, this reaction has two predictive product sizes. All PCRs were performed 45 cycles each.

9.Acknowledgements

First of all, I must express to Associate Professor Chikafumi Chiba my deepest gratitude for giving me the opportunity to study for being scientist. He taught me all the things necessary to be a researcher, offered many helpful criticisms over the years and allowed me to finish my dissertation. There are no appropriate words to express my appreciation to him, and therefore, I repeatedly would like to express my sincere, thank to him.

I am grateful to Prof. Takehiko Saito and Prof. Masahiro Yamada for giving me opportunity to learn in their laboratory.

I wish to thank to Dr. Yuko Kaneko of Saitama Medical School for her valuable technical advices of ISH methods.

I wish to express my gratitude to Prof. Hiroshi Yamagishi and Associate Prof. Kei Nakatani, Associate Prof. Kenjiro Yoshimura, Assistant Prof. Kazunori Oami, and Prof. Mihoko Takahashi of A1 group of Functional Biosciences, Graduate School of Life and Environmental Sciences, University of Tsukuba for their critical reading of manuscript and their valuable advices.

I wish to express my graduate to Associate Prof. Katsuo Furukubo-Tokunaga for his critical reading of the manuscript and his valuable comments.

I would like to thank my laboratory colleagues for their helps and valuable discussions.

I especially thank to all members of the Wild Animal Researching Club of the University of Tsukuba, they always encouraged me for a decade.

Finally, I especially thank my parents, Akira and Ryoko, my brothers Taro and Gakuta, for ensuring my education for very long time.