Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways

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Accepted 27 April 2005

Development 132, 3027-3043
Published by The Company of Biologists 2005
doi:10.1242/dev.01881

Summary

In the developing vertebrate retina, progenitor cells initially proliferate but begin to produce postmitotic neurons when neuronal differentiation occurs. However, the mechanism that determines whether retinal progenitor cells continue to proliferate or exit from the cell cycle and differentiate is largely unknown. Here, we report that histone deacetylase 1 (Hdac1) is required for the switch from proliferation to differentiation in the zebrafish retina. We isolated a zebrafish mutant, *ascending and descending* (*add*), in which retinal cells fail to differentiate into neurons and glial cells but instead continue to proliferate. The cloning of the *add* gene revealed that it encodes Hdac1. Furthermore, the ratio of the number of differentiating cells to that of proliferating cells increases in proportion to Hdac activity, suggesting that Hdac proteins regulate a crucial step of retinal neurogenesis in zebrafish. Canonical Wnt signaling promotes the proliferation of retinal cells in zebrafish, and Notch signaling inhibits neuronal differentiation through the activation of a neurogenic inhibitor, Hairy/Enhancer-of-split (Hes). We found that both the Wnt and Notch/Hes pathways are activated in the *add* mutant retina. The cell-cycle progression and the upregulation of Hes expression in the *add* mutant retina can be inhibited by the blockade of Wnt and Notch signaling, respectively. These data suggest that Hdac1 antagonizes these pathways to promote cell-cycle exit and the subsequent neurogenesis in zebrafish retina. Taken together, these data suggest that Hdac1 functions as a dual switch that suppresses both cell-cycle progression and inhibition of neurogenesis in the zebrafish retina.

Key words: Cell cycle, *Danio rerio*, Histone deacetylase, Neurogenesis, Notch, Retina, Wnt, Zebrafish

Introduction

The vertebrate retina contains six major classes of neurons and one class of glial cells, which are organized into distinct layers (Dowling, 1987). In the vertebrate retina, neuroepithelial cells are initially mitotic and undergo cell division to generate two mitotic daughter cells. At the stage when neuronal differentiation occurs, progenitor cells start cell division producing postmitotic progeny, which exits from the cell cycle and differentiates into retinal neurons and glial cells. Cell fate decisions in the developing retina are independent of cell lineage; therefore, it seems likely that multipotent progenitor cells change their competence to generate different retinal cell types in response to position- and stage-dependent environmental cues (Livesey and Cepko, 2001). Although the generation of postmitotic cells is the first step of neuronal differentiation, it is not fully understood how mitotic retinoblasts determine when they should generate postmitotic progenies.

In various cell types, the progression of the cell cycle is regulated by different combinations of cyclins and cyclin-dependent kinases (Cdks) (Galderisi et al., 2003). However, three major types of Cdk inhibitor, Cip/Waf, Kip and Ink4 family proteins, are important for the exit from the cell cycle (Galderisi et al., 2003). In the vertebrate retina, cyclin D1 promotes the entry into the S phase (Fantl et al., 1995; Sicinski et al., 1995), while the Kip family Cdk inhibitor, p27, plays a central role in the exit from the cell cycle by suppressing cyclin D1 functions (Nakayama et al., 1996; Geng et al., 2001). Several cell-extrinsic and -intrinsic factors regulating the cell cycle have been identified (Ohnuma and Harris, 2003; Levine and Green, 2004; Yang, 2004). Wnt and Notch are the cell-extrinsic factors of the cell cycle, which functions upstream of cyclin D1 and p27 (Kubo et al., 2003; Ohnuma et al., 2002). In the chick retina, a Wnt family protein, Wnt2b, is expressed in mitotic progenitor cells and promotes cell proliferation (Kubo et al., 2003). It has been reported that cyclin D1 is a target of β-catenin/Lef-1, a component of canonical Wnt signaling (Shtutman et al., 1999; Tetsu and McCormick, 1999). Notch signaling promotes the exit of retinal progenitor cells from the cell cycle in *Xenopus* (Ohnuma et al., 2002), although Notch plays a role in the maintenance of neural stem cells in the brain (Ross et al., 2003).

In zebrafish, postmitotic cells are initially generated in the ventronasal retina adjacent to the optic stalk, and neuronal

production progresses to the entire neural retina (Hu and Easter, 1999). Our previous study suggested that neuronal production is initiated by the interaction between the optic stalk and the neural retina, and that its progression to the entire neural retina is regulated by the relay of short-range signaling (Masai et al., 2000). Several studies suggested that a candidate of this short-range signaling is Hedgehog (Hh) (Neumann and Nuesslein-Volhard, 2000; Stenkamp and Frey, 2003). Recently, we reported that the activation of cAMP-dependent protein kinase (PKA) effectively inhibits the cell-cycle exit of retinoblasts in zebrafish, and that continuous proliferation induced by the activation of PKA depends on canonical Wnt signaling (Masai et al, 2005). As PKA is an inhibitor of Hh signaling (reviewed by Ingham and McMahon, 2001) and the blockade of the Hh signaling pathway shows severe defects in cell-cycle exit of retinoblasts in zebrafish (Stenkamp and Frey, 2003; Masai et al., 2005), these data support that Hh acts as a short-range signal to induce cell-cycle exit of retinoblasts in the zebrafish retina. However, mechanisms underlying the cell-cycle exit and subsequent neurogenesis after the reception of Hh signals are largely unknown.

The acetylation and deacetylation of core histones of chromatin are among the most important histone modifications and are essential for many biological processes, including proliferation, differentiation and gene silencing (reviewed by Roth et al., 2001; Kurdistani and Grunstein, 2003; Ahringer, 2000). Histone deacetylases (Hdacs) are enzymes that remove acetyl groups from histone lysine tails, resulting in the compaction of the chromatin structure (de Ruijter et al., 2003; Marks et al., 2003). Hdacs are recruited to the multiprotein complex of transcription repressors and co-repressors, and facilitate the inhibition of target gene transcription by altering the acetylation states of chromatin (Jepsen and Rosenfeld, 2002; Yang and Seto, 2003). The Hdac family proteins are classified into three major classes: class I (Hdac1, 2, 3, 8), class II (Hdac4, 5, 6, 7, 9, 10) and class III (Sir2-like NAD-dependent Hdac), each of which plays different roles in cellular and developmental processes (de Ruijter et al., 2003; Marks et al., 2003). However, the division of labor among Hdacs for various biological processes is largely unknown.

It is generally accepted that co-repressor complexes containing N-CoR, Bmi-1 and NRSF/REST are important for the maintenance of neural stem cells (Hermanson et al., 2002; Molofsky et al., 2003; Kuwabara et al., 2004). However, there are some reports that the deacetylation and subsequent methylation of histones promote the differentiation of neural stem cells into neurons and glial cells. It has been reported that Hdac activity is necessary for oligodendrocyte differentiation from rat cortical progenitor cells (Marin-Husstege et al., 2002). In rat cortical progenitor cells, the methylation states of Lys4 and Lys9 of histone H3 bound to the astrocyte-specific gene promoter is essential for ciliary neurotrophic factor-induced astrocyte differentiation (Song and Ghosh, 2004). These observations suggest that the chromatin state of regulatory genes is dynamically regulated during development and modulates the behavior of neural progenitors.

To clarify the mechanism that underlies the switch from proliferation to differentiation in the developing retina, we identified zebrafish mutants showing defects in neuronal differentiation. Here, we describe a zebrafish mutant, *ascending and descending* (*add*), in which retinal progenitor cells fail to exit from the cell cycle but instead continue to proliferate. The identification of the *add* mutational locus indicates that the *add* gene encodes Hdac1. The ratio of the number of differentiating cells to the total number of cells increases in proportion to Hdac activity, suggesting that Hdac is an essential component of the switch from proliferation to differentiation of retinal cells in zebrafish. Canonical Wnt signaling promotes proliferation and Notch signaling inhibits neurogenesis in zebrafish retina. We found that both the Wnt and Notch signaling pathways are activated in the *add* mutant retina. The cell-cycle progression and the expression of the neurogenic inhibitor in the *add* mutant retina are suppressed by the blockade of Wnt and Notch signaling pathways. These data suggest that Hdac1 antagonizes these signaling pathways to promote retinal neurogenesis in zebrafish. Taken together, these data suggest that Hdac1 functions as a dual switch molecule that suppresses the cell-cycle progression and the inhibition of neurogenesis in the zebrafish retina.

Materials and methods

Fish strains

RIKEN wild type and WIK were used as wild-type strains for mutagenesis and mapping, respectively. A transgenic line carrying green fluorescent protein (GFP) under the control of *ath5* enhancers, Tg(ath5:GFP) (Masai et al., 2003), was used to monitor *ath5* expression. The TOPdGFP transgenic line (Dorsky et al., 2002) carries GFP under the control of a β-catenin-responsive promoter and was used to monitor the activation of the canonical Wnt signaling. The transgenic lines carrying hsp:Gal4 and UAS:the intracellular domain of Notch1a (NICD) were used to induce a constitutive active form of Notch conditionally (Scheer et al., 2001). *add* ^{rw399} and *mind bomb*^{ta52b} (*mib*^{ta52b}) (Jiang et al., 1996) were used in this study.

Histological analysis, immunohistochemistry and whole-mount in situ hybridization

Histological analysis, immunohistochemistry and whole-mount in situ hybridization were performed as previously described (Masai et al., 2000). The antibodies used in this study were zpr1 (Oregon Monoclonal Bank, 1:500), zn5 (Oregon Monoclonal Bank, 1:50), anti-glutamine synthetase (Chemicon, 1:250), anti-5-bromo-2′-deoxyuridine (BrdU) (Roche, 1:100), anti-Myc (Medical & Biological Laboratories, 1:100), anti-GFP (Santa Cruz Biotechnology, 1:100) and anti-phosphorylated histone H3 (Upstate, 1:500) antibodies. Sytox Green Nucleic Acid Stain (Molecular probes) was used at 1:50,000.

BrdU incorporation

Dechorionated embryos were soaked for 30 minutes in Ringer's solution containing 10 mM BrdU (Sigma) and 15% dimethylsulfoxide (DMSO) at 6°C. Alternatively, Ringer's solution with 10 mM BrdU was injected into the yolk of embryos. After BrdU treatment, the embryos were washed, incubated for at least 1 hour in water at 28.5°C and fixed with 4% paraformaldehyde (PFA).

The ratio of the number of dividing cells to total number of retinal cells

Embryos were labeled with anti-phosphorylated histone H3 antibody and Sytox Green Nucleic Acid Stain. These double-labeled embryos were scanned under a LSM510 laser-scanning microscope (Carl Zeiss) to acquire images of a 1.7 μm thick plane corresponding to the central retina. The numbers of dividing cells and total cells were counted using one image per eye.

Cell transplantation, mutagenesis, mapping and cloning

Cell transplantation at the late blastula stage, mutagenesis and mutant locus mapping were carried out as described previously (Masai et al., 2003).

Morpholino oligo injection

Morpholino oligos (Gene Tools) targeted against *hdac1* (TTGTTCCTTGAGAACTCAGCGCCAT) and a five-base mismatch containing control morpholino (TTcTTgCTTGAcAACTCAGg-GCgAT) were injected at the one-cell stage at a concentration of 0.25 mg/ml.

DNA construction and expression from constructs

To induce ectopic expression of zebrafish *hdac1*, its coding region was inserted into the pCS2-MT expression vector, resulting in the addition of the Myc-tag to N terminus of the coding region of Hdac1. The plasmid was modified to generate the expression construct, pCS2[hsp:myc-hdac1], by replacing the CMV promoter with zebrafish heat-shock promoter (hsp) (Halloran et al., 2000). The coding regions of *Xenopus* p27, ΔN-Tcf3 and Δ47-β-catenin was fused to myc-tag (*Xenopus* p27, ΔN-Tcf3) or GFP (Δ47-β-catenin) and inserted into a modified CS2 expression vector, in which the CMV promoter is replaced by hsp, to generate pCS2[hsp:myc-p27], pCS2[hsp:myc-ΔN-Tcf3] and pCS2[hsp:Δ47-β-catenin-GFP], respectively. The detailed procedures for generating these constructs have been described previously (Masai et al., 2005). These constructs were injected into zebrafish fertilized eggs, and embryos were incubated in water at 39°C for 1 hour repeatedly at 18, 26 and 42 hours-post-fertilization (hpf). After the heat-shock treatment, embryos were reared at 28.5°C and fixed at 33 hpf (Hdac1, p27, ΔN-Tcf3) or 48 hpf (Δ47-β-catenin). To introduce the co-expression of Hdac1 and Δ47-β-catenin, a mixture of pCS2[hsp:myc-hdac1] (30 μg/ml) and pCS2[hsp:Δ47-β-catenin-GFP] (30 μg/ml) was co-injected into embryos. To introduce ectopic expression of NICD, transgenic embryos carrying hsp:GAL4; UAS:NICD were incubated in water at 39°C for 1 hour repeatedly at 18, 26 and 42 hpf, and fixed at 48 hpf.

Quantification of the ratio of BrdU incorporation

By injecting DNA constructs pCS2[hsp:myc-hdac1], pCS2[hsp:myc-p27], pCS2[hsp:myc-ΔN-Tcf3] and pCS2[hsp:Δ47-β-catenin-GFP], ectopic expression was usually introduced in a mosaic manner and retinal cells expressing these genes produced their progeny forming a columnar cluster. To elucidate whether these genes promote the cell-cycle exit of retinal cells, we counted the number of cell clusters that contain BrdU-positive cells, and calculated the ratio of the number of BrdU-positive clusters to the total number of clusters.

To examine the ratio of the number of BrdU-labeled cells to total number of cells in wild-type and *add* mutant retinas, two adjacent serial sections corresponding to the central retina were prepared using a cryostat. One of sections was labeled with anti-BrdU antibody and used for counting the number of BrdU-labeled cells. As HCl treatment in BrdU labeling makes nucleic acid staining with Sytox Green inefficient, the other adjacent section was stained with Sytox Green and used for counting the number of nuclei. The percentage of BrdU-positive cells to total cells in the retina was approximated as the percentage of the number of BrdU-labeled cells in one section to the number of total cells in the other adjacent section.

In the experiment of Trichostatin A (TSA) treatment, BrdU signals scanned by a laser-scanning microscope were converted using NIH Image to a binary scale with two digits, 0 (negative) and 1 (positive), by which the BrdU positive area is adjusted to the outline of BrdU-positive cells. This procedure approximated the ratio of BrdU-positive area to total area to the ratio of the number of BrdU-positive cells to total number of cells. The ratio of BrdU-positive area to total area was calculated as the ratio of the number of pixels corresponding to 1 to total number of pixels corresponding to 0 and 1. The detailed procedures have been described previously (Masai et al., 2005).

Western blot analysis

Yolk-extirpated embryos (24 hpf) were homogenized using an extraction buffer (50 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 12% 2-mercaptoethanol) to extract proteins. The extracted proteins were subjected to SDS-PAGE and blotted onto a PVDF membrane (BioRad). After blocking with 5% skim milk and 0.1% Triton X in TBS, membrane filters were incubated with an anti-acetylated histone H4 antibody or an anti-histone H4 antibody (Upstate) at 1:1,000. Immunosignals were visualized with an alkali-phosphatase (AP)-conjugated anti-rabbit IgG antibody (Promega) at 1:5,000.

The treatment with a Hdac inhibitor, Trichostatin A (TSA)

TSA stock solution (Sigma, 1 mg/ml in DMSO) was diluted to appropriate concentrations for use. Embryos were soaked in a TSA-containing solution until an appropriate stage.

Labeling for apoptosis

Embryos were fixed with 4% PFA and sectioned on a microtome cryostat. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using an in situ cell death detection kit (Roche).

Results

Retinal progenitor cells fail to exit from the cell cycle in *add* mutant embryos

To elucidate mechanisms underlying neuronal differentiation in the retina, we screened zebrafish mutants by visualizing retinal layers with an anti-acetylated α tubulin antibody (Masai et al., 2003). By this screening, we identified *add*, a mutant in which retinal lamination is disrupted (data not shown). The size of the optic cup seems normal in *add* mutant embryos (Fig. 1A). However, the neural retina was markedly multifolded at 2 days postfertilization (dpf) in *add* mutant embryos (Fig. 1B,C), suggesting that retinal cells undergo hyperproliferation. In contrast to the neural retina, the overgrowth of retinal pigmented epithelium was not observed at least until 5 dpf. The overgrowth of the neural retina in the *add* mutant raises at least two possibilities. One possibility is that the rate of proliferation (the number of new cells produced per a given period) is higher in the *add* mutant retina. Another possibility is that retinal cells fail to exit from the cell cycle and instead continue to proliferate in the *add* mutant retinas. To elucidate which is the case in the *add* mutant retinas, we examined retinal phenotypes in the *add* mutant.

Our previous study showed that a zebrafish homolog of *Drosophila atonal*, *ath5*, is expressed transiently in retinoblasts undergoing the final mitosis and also in their postmitotic daughter cells (Masai et al., 2000). In wild type, GFP expression under the control of the *ath5* retinal enhancer (ath5:GFP) (Masai et al., 2005) spread in the large region of the neural retina at 34 hpf (Fig. 1D). However, ath5:GFP expression was not observed in the *add* mutant retina, except in the ventronasal region, from which retinal neurogenesis is initiated in zebrafish (Hu and Easter, 1999) (Fig. 1E). The labeling of 2 dpf wild-type and *add* mutant retinas with zn5 antibody revealed that the number of retinal ganglion cells markedly decreases in the *add* mutant retina (Fig. 1G). At 3 dpf, double-cone type of photoreceptors differentiated to form the outer photoreceptor layer in wild type (Fig. 1H), but the differentiation of photoreceptors was not observed in the *add* mutant (Fig. 1I). At 5 dpf, Müller glial cells differentiated in

wild type (Fig. 1J), but did not differentiate in *add* mutant embryos (Fig. 1K). In addition to severe blockade of neuronal and glial differentiation, apoptosis gradually increased in the *add* mutant retinas after 3 dpf, and most retinal cells were TUNEL positive at 5 dpf (data not shown). These data suggest that most of retinal cells in the *add* mutant fail to differentiate

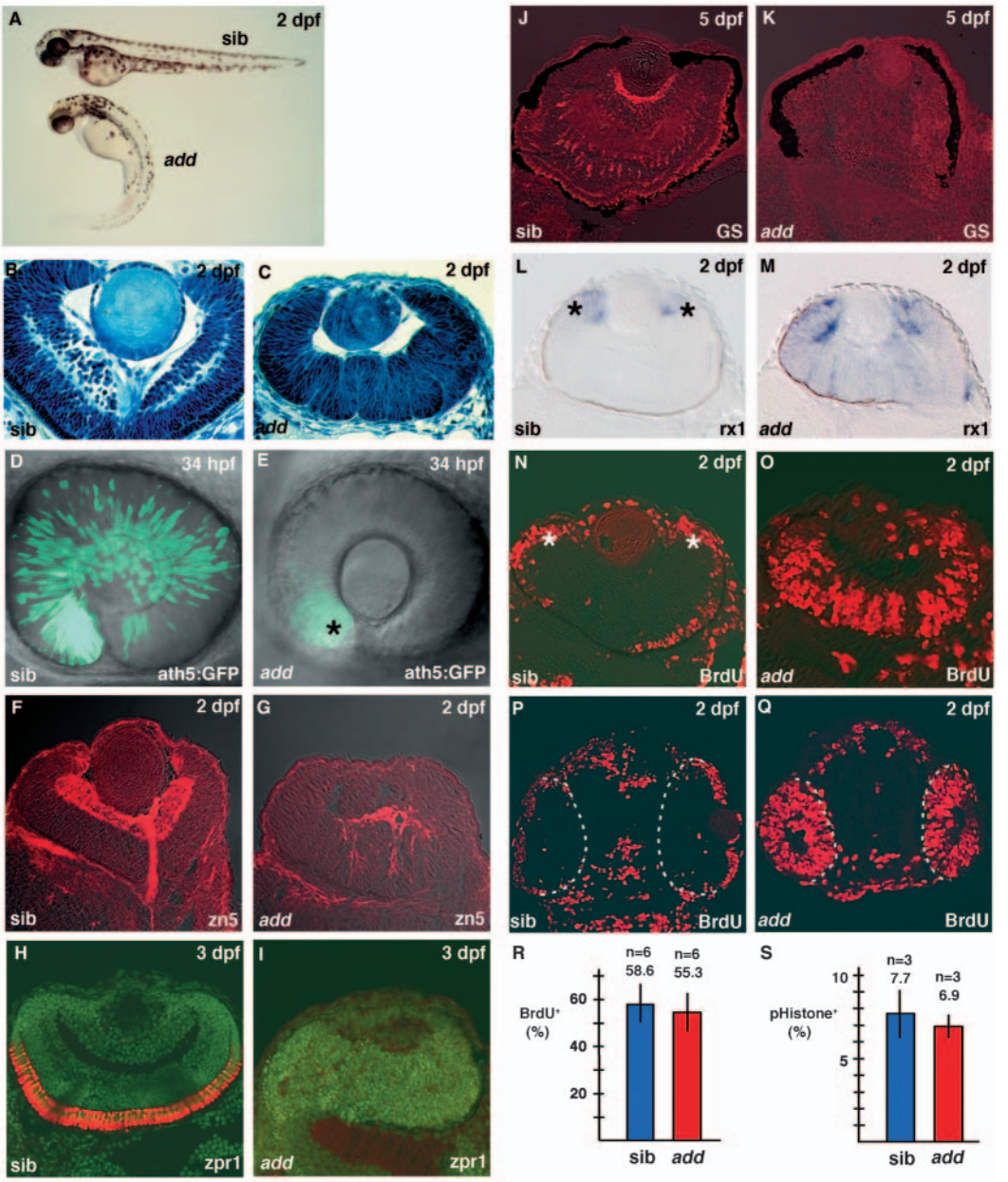

Fig. 1. Retinal mitotic cells fail to exit from the cell cycle in *add* mutant embryos. (A) Morphology of *add* mutant and wild-type sibling embryos. (B,C) Plastic sections of wild-type (B) and *add* mutant (C) retinas. *add* mutant embryos show a markedly multifolded retinal epithelium. (D,E) ath5:GFP expression in wild-type (D) and *add* mutant (E) retinas. ath5:GFP expression is markedly delayed in *add* mutant embryos and very faint in the ventronasal retina (E, asterisk). (F,G) Labeling of wild-type (F) and *add* mutant (G) retinas with the zn5 antibody, which stains retinal ganglion cells (red). (H,I) Labeling of wild-type (H) and *add* mutant (I) retinas with the zpr1 antibody, which stains double cone photoreceptors (red). All nuclei were counterlabeled with Sytox Green (green). (J,K) Labeling of wild-type (J) and *add* mutant (K) retinas with the anti-glutamine synthetase antibody, which stains Müller glial cells (red). (L,M) In situ hybridization of wild-type (L) and *add* mutant (M) retinas with *rx1* RNA probe. *rx1* expression is observed in nearly the entire neural retina of *add* mutants, whereas it is downregulated and localized within a proliferating region called the CMZ (L, asterisks) in wild type. (N,O) BrdU labeling of wild-type (N) and *add* mutant (O) retinas. BrdU-positive cells are located within the CMZ (N, asterisks) and a part of the outer photoreceptor layer in wild type, whereas many retinal cells incorporate BrdU even in the central retina of *add* mutant embryo. (P,Q) BrdU labeling of wild-type (P) and *add* mutant (Q) heads. Broken white lines show the interface between the brain and retina at 24 hpf. (R) The percentage of the number of BrdU-positive cells to total number of cells in wild-type (blue bar) and *add* mutant (red bar) retinas. (S) The percentage of the number of mitotic cells labeled with anti-phosphorylated Histone H3 antibody in wild-type (blue bar) and *add* mutant (red bar) retinas at 27 hpf.

into neurons and glial cells, and eventually undergo apoptosis at the later stages. As apoptosis was rarely observed in *add* mutant retinas at 2 dpf, we focus on retinal phenotypes before 3 dpf in this study.

To determine whether undifferentiated retinal cells continue to proliferate in *add* mutant, we examined the marker of proliferative cells and BrdU incorporation. In 2 dpf wild-type retinas, *rx1* expression was downregulated in differentiating neurons and localized in the ciliary marginal zone (CMZ), which corresponds to the proliferating region (Chuang et al., 1999) (Fig. 1L). Unlike wild-type retinas, *rx1* expression was not downregulated and remained in the entire neural retina of *add* mutant embryos (Fig. 1M). The labeling of dividing cells with BrdU revealed that lots of retinal cells remain mitotic in the central retina of the *add* mutant embryos at 2 dpf (Fig. 1O), whereas mitotic cells are localized only in the CMZ in wild-type retinas (Fig. 1N). Interestingly, such continuous BrdU incorporation was observed only in the retina but not in the brain of *add* mutant embryos (Fig. 1P,Q). These data suggest that retinal cells fail to differentiate into neurons and glial cells but continue to proliferate in *add* mutant embryos.

It has been reported that the rate of proliferation in the zebrafish retina is regulated in a stage-dependent manner probably through the modulation of the cell-cycle length (Li et al., 2000). The length of the cell cycle is estimated between 32 and 49 hours within 16-24 hpf, but abruptly shortened to about 10 hours after 24 hpf in the developing zebrafish retina. We examined whether the rate of proliferation is altered in the *add* mutant retinas. The rate of proliferation correlates with the ratio of the number of BrdU-labeled cells or mitotic cells to total number of cells for a given period. When BrdU was incorporated within 90 minutes at 24 hpf, the ratio of the number of BrdU-labeled cells to total number of cells in the *add* mutant retinas was not significantly different from that in wild-type retinas (Fig. 1R), suggesting that the percentage of cells undergoing the S phase within 90 minutes is not significantly altered in *add* mutant retinas. We also observed that some of BrdU-positive cells undergo mitosis in wild-type and *add* mutant retinas (data not shown), suggesting that the length of the G2 phase is less than 90 minutes in both cases. The labeling of retinas with anti-phosphorylated histone H3 antibody revealed that the ratio of the number of dividing cells to total number of cells in the *add* mutant retina is not significantly different from that in wild-type retina at 24 hpf (data not shown) and 27 hpf (Fig. 1S), suggesting that the percentage of cells undergoing the M phase is not altered in the *add* mutant retinas. These data suggest that the rate of proliferation is similar between wild-type and *add* mutant retinas. As the rate of proliferation is governed by the number of proliferative cells and the length of the cell cycle, it seems unlikely that the cell-cycle length is significantly altered at 24 hpf in the *add* mutant retina. These data are consistent with the observation that the morphology of the neural retina in the *add* mutant seems normal at 24 hpf, when neuronal differentiation begins in wild-type retinas (data not shown). Although it is still possible that the activity of maternal Add compensates for the defect in the cell-cycle length in the *add* mutant retinas, these data suggest that the blockade of cell-cycle exit primarily causes the overgrowth of the neural retina in *add* mutant embryos.

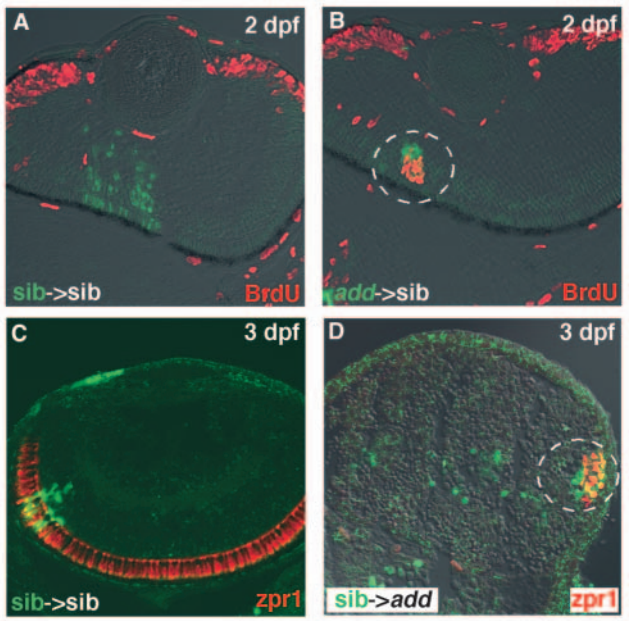

Fig. 2. *add* mutation behaves in a cell-autonomous manner. (A) Transplantation of wild-type cells into wild-type retinas. Wild-type donor cells (green) are BrdU-negative and differentiate into retinal neurons, which form retinal columns. (B) Transplantation of *add* mutant cells into wild-type retinas. *add* donor mutant cells (green) fail to form retinal columns and continue to incorporate BrdU (red in broken white circle) in the wild-type recipient retina. (C) Transplantation of wild-type cells into wild-type retinas. Wild-type donor cells (green) differentiate into double cone-type of photoreceptors, which are labeled with zpr1 antibody (red). (D) Transplantation of wild-type cells into *add* mutant retinas. Wild-type donor cells (green) express the photoreceptor marker, zpr1 (red in white dotted circle) in the *add*-recipient retina.

Add is required for the cell-cycle exit of retinal progenitor cells in a cell-autonomous manner

To elucidate whether the *add* mutation behaves in a cell-autonomous manner, we examined the behavior of *add* mutant cells transplanted into wild-type retinas and vice versa. When *add* mutant cells were transplanted into wild-type retinas, *add* mutant cells failed to exit from the cell cycle and continued to divide even in the wild-type environment (Fig. 2B). In the reserve case, when wild-type cells were transplanted into *add* mutant retina, wild-type cells exited from the cell cycle and some of them differentiated into photoreceptors in *add* mutant retinas (Fig. 2D). These data suggest that Add is required for the cell-cycle exit of retinal progenitor cells in a cell-autonomous manner.

add gene encodes Hdac1

To elucidate mechanisms underlying retinal phenotypes in the *add* mutants, we identified a gene responsible for the *add* mutation. The mapping of the *add* mutational locus using polymorphic markers revealed that the *add* mutational locus is located between the SSLP markers z11872 and z7235 at 40.6 cM on chromosome 19 (Fig. 3A). As the *hdac1* gene is mapped in this region, we sequenced the *hdac1* cDNA isolated from *add* mutant embryos and found that a nonsense mutation occurs in the conserved catalytic domain of Hdac1 (Fig. 3B,C). We examined the expression of *hdac1*, and found that *hdac1*

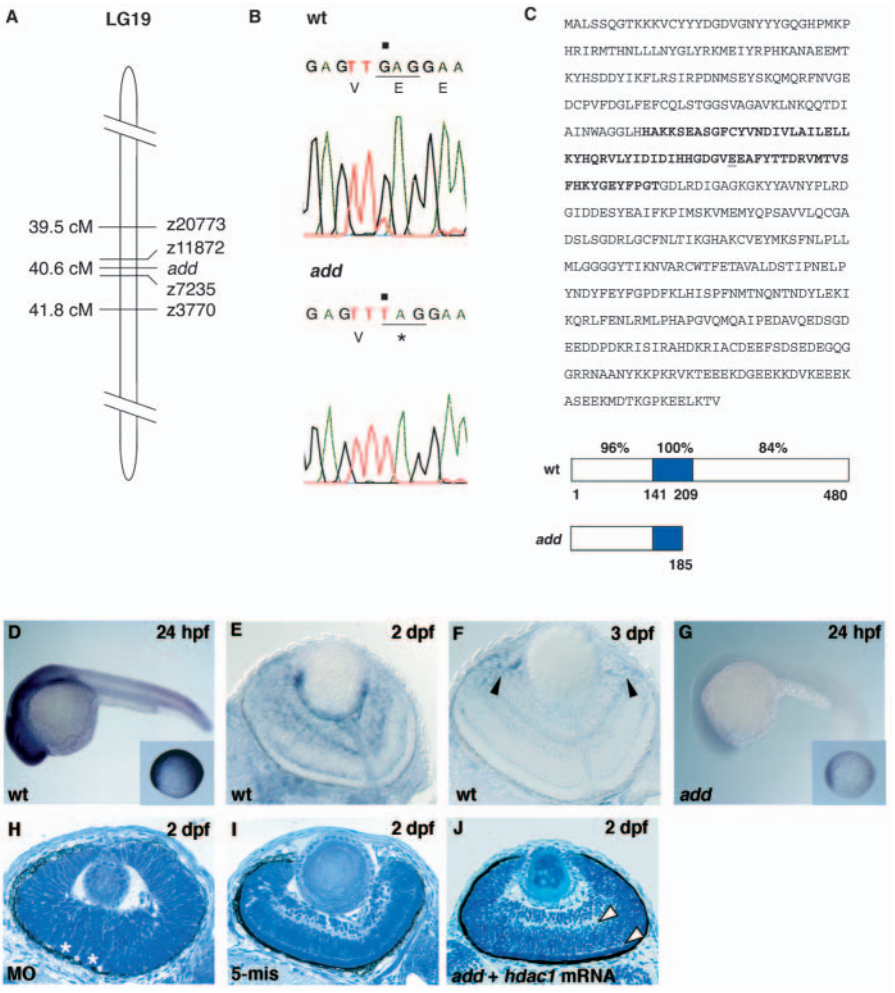

Fig. 3. *add* gene encodes Hdac1. (A) Schematic drawing of LG19. (B) Sequencing of *hdac1* DNA isolated from wild type and *add* mutants. G to T transversion (black squares) replaces 185 E with stop codon in *add* mutants. (C) Amino acid sequence of Hdac1 and its predicted structure of wild type and *add* mutant. Nonsense mutation occurs at 185 E (underline) within the Hdac catalytic region (bold letters in sequence, blue in schematic drawing below). Percentage of identical amino acids in zebrafish and human Hdac1 is indicated in each domain. (D-G) Expression of *hdac1* mRNA in wild-type (D-F) and *add* mutant (G) embryos. Arrowheads (F) indicate the expression in the CMZ. Insets (D and G) show embryos at 10 hpf. (H) Retina of embryos injected with *hdac1* morpholino oligos. Asterisks indicate folded retinal epithelium. (I) Retina of embryos injected with five mismatch control morpholino oligos. Retinal lamination is normal. (J) *add* mutant retinas expressing *hdac1* mRNA. The formation of the inner and outer plexiform layers are evident (arrowheads).

is expressed ubiquitously from one-cell stage until 18 hpf (inset in Fig. 3D), suggesting that there is a maternal transcription of *hdac1* mRNA. At 24 hpf, *hdac1* mRNA was predominantly expressed in the brain, including the retina (Fig. 3D). The expression was ubiquitous in the retina but gradually became confined to the CMZ (Fig 3E,F), indicating that *hdac1* is expressed in mitotic cells throughout retinal development. In *add* mutant embryos, *hdac1* expression level decreased during gastrulation and the expression could not be detected at 24 hpf (Fig. 3G). To confirm that the *add* gene encodes Hdac1, we carried out three sets of experiments. First, the injection with morpholino-antisense oligos of the *hdac1* gene into wild-type embryos phenocopied the *add* phenotypes, including the multifolded retina (Fig. 3H,I). Second, we examined whether the introduction of Hdac1 suppresses continuous BrdU incorporation in the *add* mutant retina. We injected the expression construct pCS2[hsp:myc-hdac1] into *add* mutant eggs. The heat-shock treatment induced ectopic expression of Hdac1 in the neural retina in a mosaic manner. We found that BrdU incorporation is significantly suppressed in *add* mutant cells expressing Hdac1, compared with that of *add* mutant cells expressing the control EGFP (Fig. 4). Third, retinal lamination is partially restored by the injection of *hdac1* mRNA into *add* mutant embryos (Fig. 3J). Taken together, these data suggest that the *add* gene encodes Hdac1.

The ratio of differentiation to proliferation in the zebrafish retina correlates with Hdac activity

To confirm that the absence or reduction of Hdac activity causes the defects in the cell-cycle exit of retinal cells, we examined the level of histone acetylation in *add* mutant embryos. Western blotting with an anti-acetylated histone H4 antibody revealed that the acetylation level of histone H4 is higher in *add* mutant embryos than that in the wild type (Fig. 5A). Trichostatin A (TSA) is a Hdac inhibitor, which binds specifically to the catalytic domain of both type I and type II Hdac proteins (Yoshida et al., 1995). When wild-type embryos were treated with 1200 nM TSA from 14 hpf, they showed retina-specific hyperproliferation, which was much more severe than that in *add* mutant embryos (Fig. 5B). This surprising severe phenotype of TSA-treated retinas suggests that Hdac activity derived from the maternal *hdac1* gene or other *hdac* genes partially compensates for the lack of zygotic Hdac1 activity in *add* mutant embryos. However, the morphology of the neural retinas treated with 1200 nM TSA from 14 hpf was not significantly affected at 24 hpf (data not shown), suggesting that the removal of residual Hdac activity by a high dose of TSA mainly enhances the defect in cell-cycle exit.

To elucidate the relationship between cell-cycle exit and Hdac activity, we applied TSA at different concentrations.

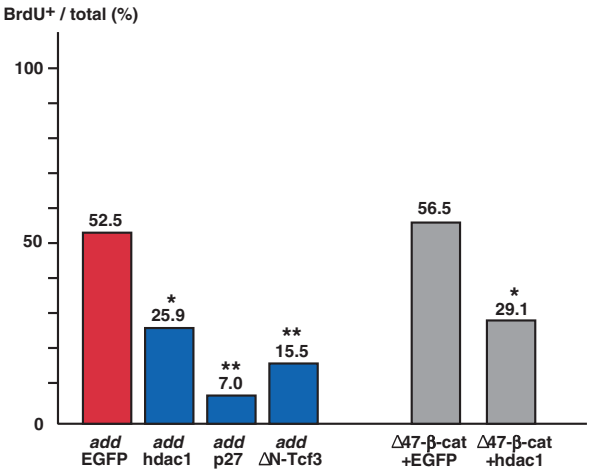

Fig. 4. Hdac1, p27 and ΔN-Tcf3 suppress *add*- or Wnt-mediated retinal proliferation. Left bars (red, blue) indicate the ratio of the number of BrdU-positive cell clusters to total number of cell clusters in *add* mutant retinal cells expressing EGFP (n=39), Hdac1 (n=58), p27 (n=43) and ΔN-Tcf3 (n=148). Right bars (grey) indicate the ratio of the number of BrdU-positive cell clusters to total number of cell clusters in wild-type retinal cells co-expressing Δ47-β-catenin and EGFP (n=23), and wild-type retinal cells co-expressing Δ47-β-catenin and Hdac1 (n=48). *P<0.05 and **P<0.01 versus control; χ^2-test.

Following the treatment of wild-type embryos with low concentrations of TSA, such as 400 nM, retinal cells differentiated and formed normal lamination at 3 dpf (Fig. 5C), and this phenotype was much milder than that in *add* mutant embryos. BrdU labeling of 2 dpf retinas treated with TSA at different concentrations revealed that the ratio of the number of proliferating cells to the total number of cells was proportional to the concentration of TSA (Fig. 5I). For example, almost all retinal cells were mitotic at 2 dpf in the treatment with 1200 nM TSA (Fig. 5D). A small number of postmitotic cells were generated in the presumptive retinal ganglion cell layer in the treatment with 800 nM TSA (Fig. 5E). The large population of retinal ganglion cells exited from the cell cycle in the treatment with 400 nM TSA (Fig. 5F). Almost all cells in the retinal ganglion cell and inner layers were postmitotic in the absence of TSA treatment (Fig. 5G). These observations suggest that the percentage of BrdU-positive cells correlates with the dose of TSA. Furthermore, the level of histone acetylation in these series of TSA-treated wild-type embryos correlated with the concentration of TSA applied (Fig 5H,I). Taken together, these data suggest that the ratio of the number of differentiating cells to that of proliferating cells correlates with Hdac activity.

Hdac1 functions upstream of the interaction between cyclin D1 and the Cdk inhibitor p27

To elucidate the relationship between Hdac activity and cell-cycle regulators such as cyclin D1 and p27, we examined the expressions of *cyclin D1* (*ccnd1*) (Yarden et al., 1995) and *p27b* (Masai et al., 2005) in *add* mutant embryos. In wild-type retinas, *cyclin D1* and *p27b* expressions were down- and up-regulated in differentiating neurons, respectively (Fig. 6A,C). However, *cyclin D1* expression remained and *p27b* expression

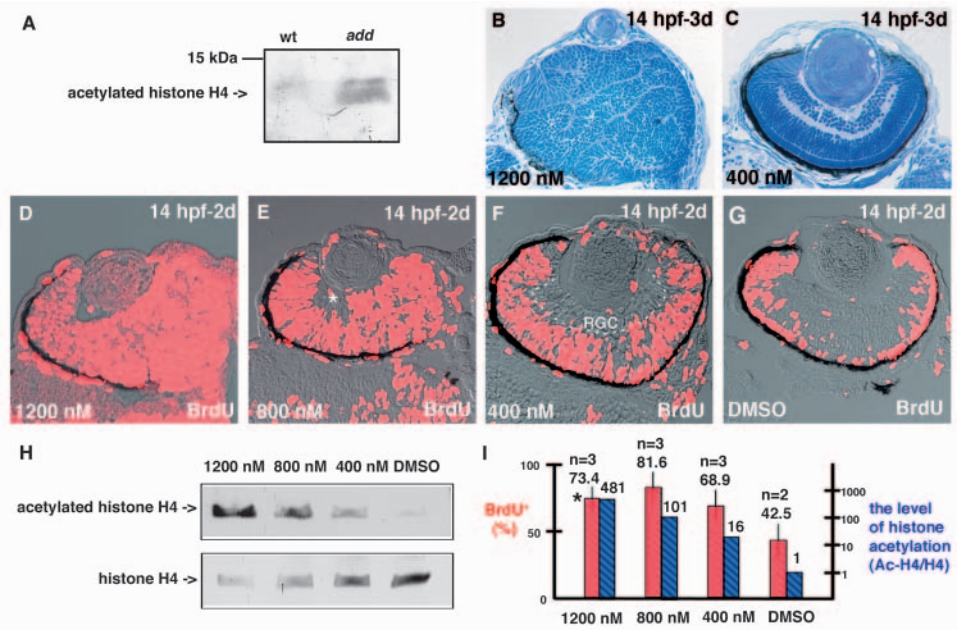

Fig. 5. The ratio of differentiating cells to proliferative cells correlates with the level of Hdac activity. (A) Western blot analysis of 24 hpf wild-type and *add* mutant embryos using anti-acetylated histone H4 antibody. (B) Treatment of wild-type embryos with 1200 nM TSA from 14 hpf. (C) Treatment of wild-type embryos with 400 nM TSA from 14 hpf. (D-G) BrdU labeling of 2 dpf wild-type embryos treated with TSA from 14 hpf at different concentrations: 1200 nM (D), 800 nM (E), 400 nM (F) and control DMSO (G). Asterisk (E) indicates BrdU-negative area in presumptive retinal ganglion cell layer. (H) Western blot analysis of 24 hpf wild-type embryos treated with TSA from 14 hpf at different concentrations using antibodies against acetylated histone H4 (upper panel) and total histone H4 (lower panel). (I) Histogram of percentage of the BrdU-positive retinal cells (left, red bars) and histone acetylation level (right, blue bars). The percentage of BrdU-positive cells in 1200 nM TSA treatment is underestimated because of massive cell death (asterisk). RGC, retinal ganglion cell layer.

was not detected in the *add* mutant retinas (Fig. 6B,D). To introduce p27 expression in the *add* mutant retina, we injected the expression construct of *Xenopus p27* (Ohnuma et al., 1999), pCS2[hsp:myc-p27], into fertilized eggs. Heat-shock treatment of injected embryos induced ectopic p27 expression in the neural retina in a mosaic manner. BrdU incorporation was significantly suppressed in p27-expressing cells of *add* mutant retinas (Fig. 4; Fig. 6E-F), suggesting that p27 inhibits the cell-cycle progression in the *add* mutant retinas. These data suggest that Hdac1 functions upstream of the interaction between cyclin D1 (Ccnd1) and p27. The proto-oncogene *cmyc* is implicated in cell growth, proliferation and loss of differentiation (Pelengaris and Khan, 2003). In the wild type, *myc* (Schreiber-Agus et al., 1993) was expressed in the most peripheral region of the CMZ where retinal stem cells are located (Fig. 6G). The expression of *cmyc* spread centrally in the *add* mutant retina (Fig. 6H) and was detected in a large region of the neural retina of embryos treated with 1200 nM TSA (Fig. 6H, inset), suggesting that the blockade of Hdac activity induces the upregulation of *cmyc* transcription.

Canonical Wnt signaling is required for hyperproliferation in *add* mutant embryos

It has been reported that *cyclin D1* and *cmyc* are downstream targets of the canonical Wnt signaling pathway (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999), and that the competition between β-catenin and Hdac proteins regulates the transition of a Wnt effector molecule, Tcf/Lef, from a transcription repressor to an activator (Billin et al., 2000). Thus, we examined the relationship between Wnt signaling and Hdac activity. In the TOPdGFP transgenic fish carrying GFP under the control of a β-catenin-responsive promoter (Dorsky et al., 2002), GFP was expressed in the CMZ at 2 dpf (Fig. 7A), suggesting that the canonical Wnt signaling pathway is activated in mitotic cells in zebrafish retinas. TSA treatment enhanced this GFP expression in the neural retina (Fig. 7B), suggesting that canonical Wnt signaling is enhanced in the absence of Hdac activity. The deletion mutant of Tcf3, ΔN-Tcf3, which lacks the β-catenin-binding site, functions as a dominant suppressor of canonical Wnt signaling (Kim et al., 2000). The introduction of ΔN-Tcf3 significantly suppressed BrdU incorporation in *add* mutant embryos (Fig. 4; Fig. 7C-D), suggesting that the activation of Wnt signaling is required for continuous progression of the cell cycle in *add* mutant embryos.

To elucidate whether the activation of Wnt signaling alone is sufficient to induce hyperproliferation in the zebrafish retina, we examined the phenotypes of retinas expressing Δ47-β-catenin, which lacks N-terminal phosphorylation sites and functions as an activated form of β-catenin (Chenn and Walsh, 2002). We injected the expression construct pCS2[hsp:Δ47-β-catenin-GFP] into wild-type embryos at one-cell stage. As GFP was fused to the C terminus of the coding region of Δ47-β-catenin, we monitored ectopic expression of Δ47-β-catenin by GFP fluorescence. After heat-shock treatment, we selected 2 dpf embryos that expressed GFP in a large region of the neural retina (inset in Fig. 7F). In this case, the neural retina was folded like that of *add* mutants (Fig. 7E) and retinal cells expressing Δ47-β-catenin incorporated BrdU (Fig. 7F). These data suggest that the activation of Wnt signaling inhibits the cell-cycle exit and instead promotes proliferation in the zebrafish retina.

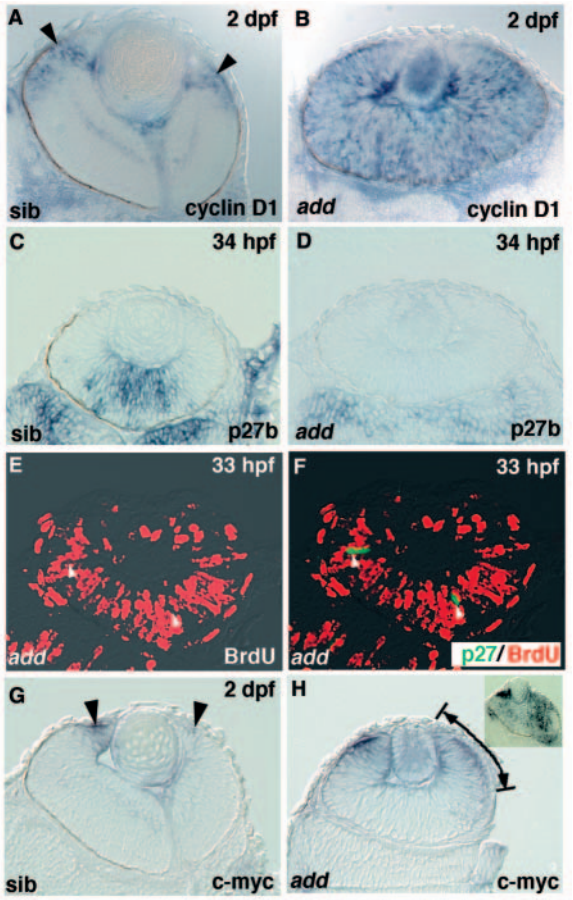

Fig. 6. Hdac1 functions upstream of the interaction between cyclin D1 and p27. (A,B) *cyclin D1* expression in wild-type (A) and *add* mutant (B) retinas. Almost all retinal cells express *cyclin D1* in *add* mutants, while in wild-type retinas, *cyclin D1* expression level decreases except in the CMZ (arrowheads). (C,D) *p27b* expression in wild-type (C) and *add* mutant (D) retinas. *p27b* expression is absent in the *add* mutant retinas. (E,F) *add* mutant retinas expressing Myc-tagged *Xenopus p27* labeled with anti-Myc antibody (F, green) and anti-BrdU antibody (red). The introduction of *Xenopus* p27 inhibits BrdU incorporation in the *add* mutant retinas (arrowheads). (G,H) *cmyc* expression in wild-type (G) and *add* mutant retinas (H). *cmyc* is expressed in the peripheral CMZ (arrowheads) in wild-type retinas, while *cmyc* expression spreads centrally in *add* mutant retinas (arrows/lines). *cmyc* expression in wild-type retina treated with 1200 nM TSA (H, inset) spreads over a large region.

To elucidate whether the introduction of Hdac1 inhibits the cell-cycle progression induced by Δ47-β-catenin, we examined the phenotypes of retinas co-expressing Δ47-β-catenin and Hdac1. A mixture of two DNA constructs, pCS2[hsp:Δ47-β-catenin-GFP] and pCS2[hsp:myc-hdac1], was injected into fertilized eggs. Double labeling with anti-Myc and anti-GFP antibodies confirmed that heat-shock treatment induced ectopic co-expression of Δ47-β-catenin and Hdac1 in the neural retina (data not shown). For a statistical analysis, we selected embryos in which GFP expression was introduced sparsely in the neural retina, and examined whether BrdU incorporation occurs in each of GFP-positive cells/cell clusters. As a control, retinal cells co-expressing Δ47-β-catenin and EGFP frequently

incorporated BrdU even in the wild-type post-mitotic environment (Fig. 7G-H). However, BrdU incorporation was significantly suppressed in retinal cells co-expressing Δ47-β-catenin and Hdac1, compared with that of co-expression of Δ47-β-catenin and EGFP (Fig. 4; Fig. 7I-J). These data suggest that Hdac1 antagonizes Wnt signaling to suppress the proliferation of retinal cells in zebrafish.

Notch-dependent Hes expression is enhanced in *add* mutant retinas

The introduction of ΔN-Tcf3 inhibited BrdU incorporation in *add* mutant retinas (Fig. 4), but did not effectively induce *ath5* expression in the *add* mutant retinas (Fig. 8B), suggesting that the blockade of Wnt signaling does not fully rescue neuronal differentiation in the *add* mutant retina. Notch inhibits the expression of proneural bHLH genes through the activation of the neurogenic inhibitors Hairy/Enhancer-of-split-related (Hes) (Artavanis-Tsakonas et al., 1999; Hatakeyama and Kageyama, 2004). It has been reported that the expression of a zebrafish ortholog of Hes1, *her6*, is upregulated in the hindbrain of zebrafish *hdac1* mutant (Cunliffe, 2004). However, we did not detect *her6* expression in the developing zebrafish retina (data not shown). Thus, we examined the expression of another zebrafish Hes gene, a zebrafish ortholog of Hes5, *her4* (Pasini et al., 2004), because Hes5 is involved in the maintenance of neural stem cells and the inhibition of neurogenesis in the vertebrates (Ross et al., 2003). In 2 dpf wild-type retinas, *her4* expression was localized within the central part of the CMZ, which corresponds to the intermediate zone between retinal stem cells and differentiated neurons (Fig. 8C). In *add* mutant embryos, *her4* expression expanded to the central region of the neural retina at 2 dpf (Fig. 8D). Similar upregulation of *her4* expression was also observed when NICD, which functions as a constitutive active form of Notch (Scheer et al., 2001), was ubiquitously introduced by heat-shock treatment of the transgenic embryos carrying hsp:Gal4 and UAS:NICD (Fig. 8F). These data raise the possibility that the Notch signaling pathway is activated in the *add* mutant retinas.

The Notch signaling pathway is severely inhibited in the *mib* mutant (Jiang et al., 1996) and the *mib* gene encodes a RING-domain containing E3-type ubiquitin ligase, which is required for Notch activation through its interaction with Delta (Itoh et al., 2003). *her4* expression was absent in the *mib* mutant retinas (Fig. 8G), suggesting that *her4* expression in the zebrafish retina is regulated by Notch signaling. Furthermore, the upregulation of *her4* expression in the *add* mutant retina is inhibited by the introduction of *mib* mutation (Fig. 8H). These data suggest that a high level of *her4* expression in the *add* mutant retina depends on the activation of Notch signaling. Taken together, these data suggest that Hdac1 antagonizes

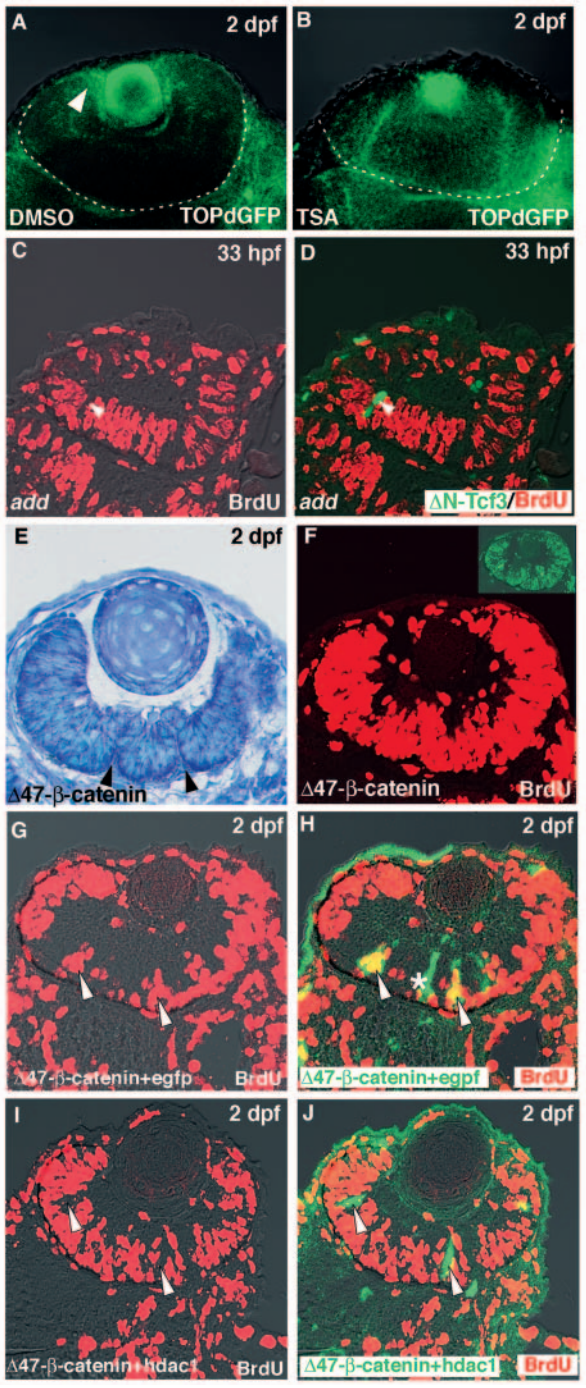

Fig. 7. Hdac1 antagonizes Wnt signaling to promote cell-cycle exit of retinal cells. (A,B) GFP expression in TOPdGFP transgenic retinas treated with DMSO (A) and 400 nM TSA from 10 hpf (B). Arrowhead indicates GFP expression in the CMZ. GFP expression level increases in the TSA-treated TOPdGFP transgenic retina. Broken white lines show the interface between the brain and retina. (C,D) *add* mutant retinas expressing Myc-tagged ΔN-Tcf3 labeled with anti-Myc antibody (D, green) and anti-BrdU antibody (red). The introduction of ΔN-Tcf3 inhibits BrdU incorporation in the *add* mutant retina (arrowhead). (E,F) Two-dpf wild-type retinas expressing GFP-tagged Δ47-β-catenin. The retinal epithelium is folded in the same way as that of *add* mutants (E, arrowheads). Nearly all of the retinal cells are GFP positive (inset, F) and BrdU positive (F, red), indicating that retinal cells expressing Δ47-β-catenin are mitotic. (G,H) Wild-type retinas injected with a mixture of the constructs pCS2[hsp:Δ47-β-catenin-GFP] and pCS2[hsp:EGFP]. BrdU incorporation (red) is observed in cells expressing Δ47-β-catenin (green) in the central retina. Arrowheads and asterisk indicate BrdU-positive (yellow) and BrdU-negative Δ47-β-catenin-expressing cells (green), respectively. (I,J) Wild-type retinas injected with a mixture of the constructs pCS2[hsp:Δ47-β-catenin-GFP] and pCS2[hsp:myc-hdac1]. BrdU incorporation (red) is suppressed in retinal cells co-expressing Hdac1 and Δ47-β-catenin (arrowheads, green).

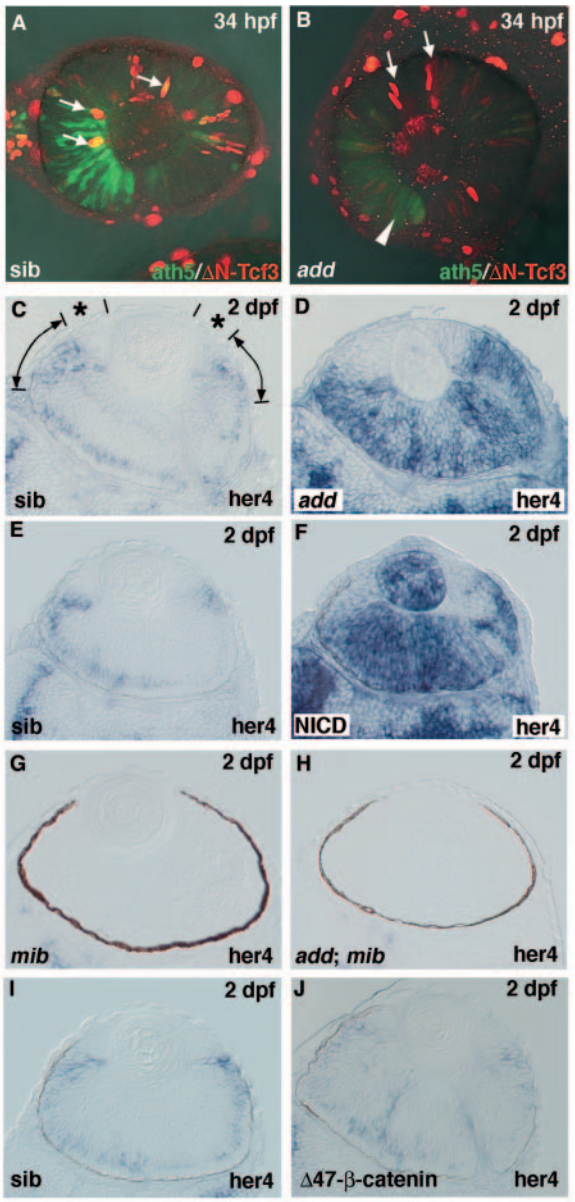

Fig. 8. Notch/Hes signaling pathway is activated in the *add* mutant retina. (A) Lateral view of 34 hpf wild-type retinas expressing ΔN-Tcf3. A confocal image of a 4.5 μm plane is shown. Although ath5:GFP expression (green) progresses to the large region of the neural retina in this stage (see Fig. 1D), ath5:GFP spreads to the dorsonasal retina in this plane. Some of ΔN-Tcf3-expressing cells (red) are ath5:GFP positive (arrows, yellow). (B) A confocal image of lateral view of 34 hpf *add* mutant retinas expressing ΔN-Tcf3. Very faint ath5:GFP expression is detected in the ventronasal retina (green, arrowhead). Cells expressing ΔN-Tcf3 (red) in the dorsal retina do not express ath5:GFP (arrows). (C-D) In situ hybridization of wild-type (C) and *add* mutant (D) retinas with *her4* RNA probe. In the wild-type retina, *her4* expression is localized in the central part of the CMZ (lines and arrows), which corresponds to the intermediate zone between retinal stem cells (asterisks) and differentiated neurons. *her4* expression is also observed in a part of the outer layer, where neurogenesis occurs at this stage. By contrast, *her4* expression remains in the large region of the neural retina in the *add* mutant (D). (E,F) In situ hybridization of wild-type (E) and wild-type retinas expressing NICD (F) with *her4* RNA probe. *her4* expression is upregulated in NICD-expressing retinas. (G,H) In situ hybridization of *mib* (G) and *add; mib* double mutant (H) retinas with *her4* RNA probe. *her4* expression is not observed in either case. (I,J) In situ hybridization of wild-type (I) and wild-type retinas expressing Δ47-β-catenin (J) with *her4* RNA probe. *her4* is expressed in the CMZ and the presumptive outer layer, the latter of which is undulated because of Δ47-β-catenin-induced folding of the neural retina.

Notch signaling to inhibit *her4* expression in the zebrafish retina. To examine whether the activation of Wnt signaling induces *her4* expression, the expression construct pCS2[hsp:Δ47-β-catenin-GFP] was injected into fertilized wild-type eggs. After heat-shock treatment, we selected 2 dpf embryos showing GFP expression in a large region of the neural retina. In the neural retina expressing Δ47-β-catenin, *her4* expression was observed in the central part of CMZ and the presumptive outer layer, the latter of which is undulated along the outline of folded retinas (Fig. 8J). It is unlikely that canonical Wnt signaling directly activates Notch-mediated *her4* expression.

Notch signaling inhibits the cell-cycle exit of retinal cells and neurogenesis

Previous studies suggested that Notch signaling is involved in the maintenance of cell proliferation and neural stem cells (Gaiano and Fishell, 2002; Ross et al., 2003). To elucidate whether Notch signaling plays a role in hyperproliferation in the *add* mutant or whether Notch signaling inhibits solely neurogenesis, we examined BrdU incorporation and *ath5* expression in the *add; mib* double-mutant retina. At 2 dpf, most retinal cells were BrdU-positive in *add* mutant retinas (Fig. 9B). However, BrdU incorporation was inhibited in the central region of the neural retina in the *add; mib* double mutant (Fig. 9D). These data suggest that the activation of Notch signaling is required for the maintenance of cell proliferation in the *add* mutant. *ath5* expression is severely inhibited in the 34 hpf *add* mutant retina (Fig. 1E and Fig. 9F), but it is partially restored in the *add; mib* double-mutant retina (Fig. 9H), suggesting that Notch signaling also inhibits *ath5* expression in the *add* mutant retina.

In the 2 dpf *add; mib* double mutant, BrdU incorporation still occurred in the CMZ (Fig. 9D), where Wnt signaling is highly activated (Fig. 7A). This observation raises the possibility that high activation of the Wnt signaling pathway promotes Notch-independent cell proliferation in the retina. To elucidate this possibility, we examined BrdU incorporation in *mib* mutant retinas with a highly activated Wnt signaling. We injected the expression construct pCS2[hsp:Δ47-β-catenin-GFP] into *mib* mutant eggs. After heat-shock treatment, we selected 2 dpf embryos that express GFP in a large region of neural retina (Fig. 9J, inset). We observed that cells expressing Δ47-β-catenin incorporate BrdU even in the central region of *mib* mutant retina (Fig. 9J), while cells expressing control EGFP were BrdU negative in the central retina of *mib* mutants (Fig. 9I). These data suggest that Notch signaling is dispensable for the maintenance of cell proliferation when Wnt signaling is highly activated. TSA treatment at high concentrations or for a longer period induced more severe hyperproliferation than the *add* mutation (Fig. 5B; Fig. 9K). We examined whether Notch signaling is required for cell proliferation induced by the treatment of TSA in a high dose. As in the case of the *mib* mutant injected with Δ47-β-catenin

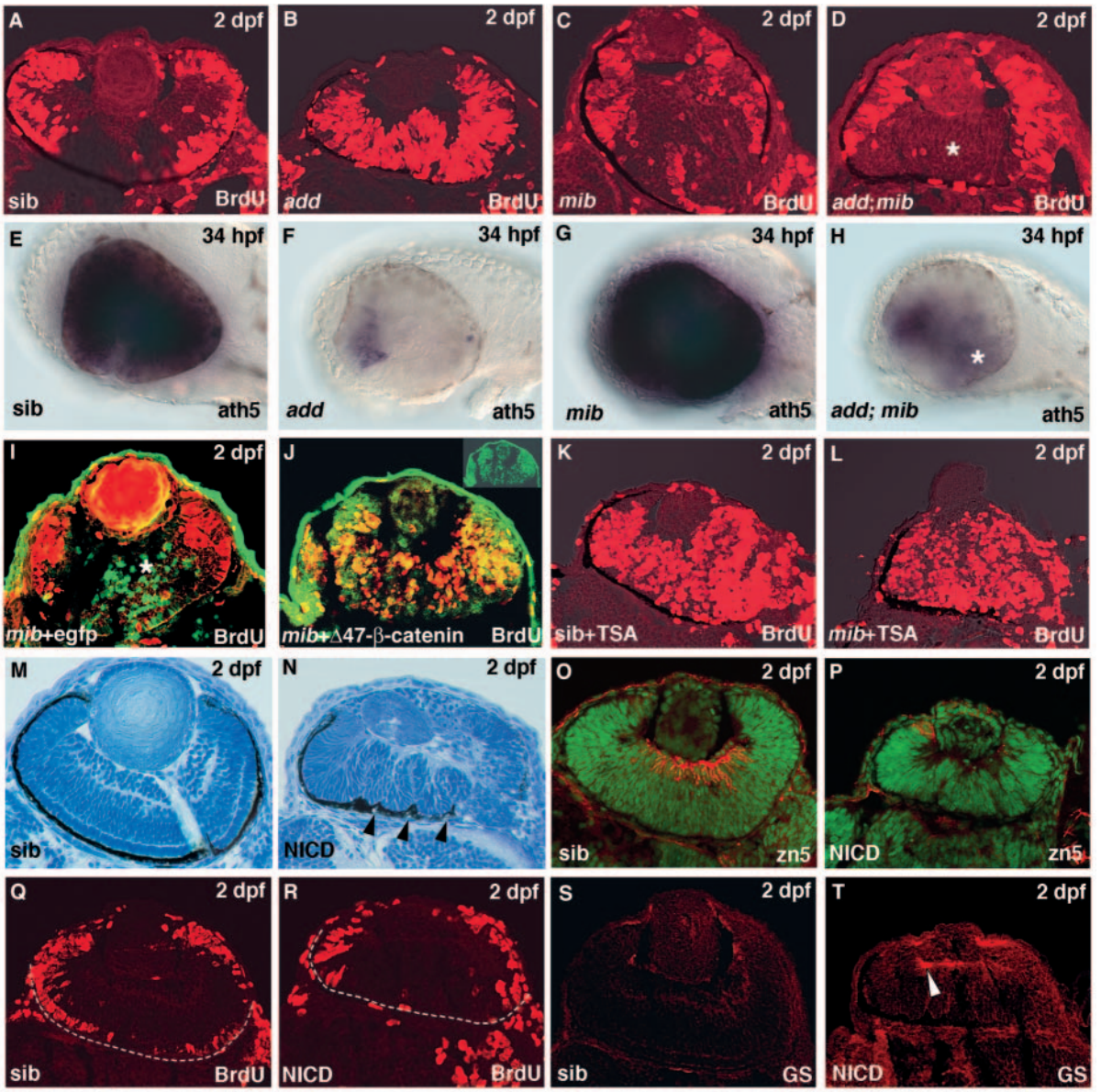

Fig. 9. Notch signaling inhibits both cell-cycle exit and neurogenesis in the retina. (A-D) BrdU labeling in wild-type (A), *add* (B), *mib* (C) and *add; mib* mutant retinas. BrdU incorporation is suppressed in the central region of the *add; mib* double mutant retina (D, asterisk). (E-H) *ath5* expression in wild-type (E), *add* (F), *mib* (G) and *add; mib* (H) mutant retinas. *ath5* expression is severely inhibited in *add* mutant retinas, is enhanced in *mib* mutant retinas and is partially restored in the *add; mib* double mutant retinas (H, asterisk). (I,J) BrdU labeling of *mib* mutant injected with the DNA construct pCS2[hsp:EGFP] (I) and with pCS2[hsp:Δ47-β-catenin-GFP] (J). Cells expressing Δ47-β-catenin (green, inset in J) are BrdU-positive (red), even in the central region of *mib* mutant retinas (J), while cells expressing EGFP are BrdU negative in the central region of *mib* mutant retinas (I). (K,L) BrdU labeling of wild-type retina treated with TSA (K) and *mib* mutant retina treated with TSA (L). Treatment with 400 nM TSA from 10 hpf induces more severe phenotypes than that from 14 hpf shown in Fig. 5F. (M,N) Plastic sections of retinas of wild-type retina (M) and wild-type retina expressing NICD (N). Wild-type retina expressing NICD is folded in the same way as the *add* mutant retina (arrowheads). (O,P) Labeling of wild-type (O) and NICD-expressing (P) retinas with zn5 antibody, which labels retinal ganglion cells (red). Nuclei were counterlabeled with Sytox Green (green). (Q,R) BrdU labeling of wild-type (Q) and NICD-expressing retinas (R). In both cases, BrdU incorporation is not observed in the central retina. Broken white lines show the interface between the brain and retina. (S,T) Labeling of wild-type (S) and NICD-expressing retinas (T) with anti-glutamine synthetase antibody, which labels Müller glia (red). Glial cells differentiate precociously in the NICD-expressing retina (arrowhead).

(Fig. 9J), BrdU incorporation was not inhibited in the central retina of *mib* mutants treated with TSA (Fig. 9L), suggesting that Notch signaling is also dispensable for the proliferation induced by the severe blockade of Hdac activity. These data suggest that retinal proliferation is maintained independent of the activity of Notch signaling, when Wnt signaling is highly activated or Hdac activity is severely inhibited.

To elucidate whether the activation of Notch signaling alone is sufficient to induce hyperproliferation, we examined retinal phenotypes in wild-type embryos expressing NICD. When

NICD was ubiquitously introduced by the heat-shock treatment of transgenic embryos carrying hsp:Gal4; UAS:NICD, the neural retina was folded similarly to the *add* mutant at 2 dpf (Fig. 9N) and the differentiation of retinal ganglion cells was inhibited (Fig. 9P). However, NICD did not significantly enhance BrdU incorporation (Fig. 9R) and the expression of *cyclin D1* at 2 dpf (data not shown). These data suggest that Notch activation is insufficient to maintain the proliferation of retinal cells at least at 2 dpf in zebrafish. Consistent with a previous report (Scheer et al., 2001), the activation of Notch signaling promotes the precocious differentiation of Müller glia even at 2 dpf (Fig. 9T).

Discussion

The mechanism underlying the timing and rate of neurogenesis in the developing brain is one of the important issues in neurobiology. The zebrafish retina is an excellent system with which to elucidate this mechanism, because neurogenesis propagates across the neural retina in a spatially and temporally regulated wave-like fashion (Hu and Easter, 1999). Our previous studies and others revealed that Hh signaling regulates the progression of retinal neurogenesis in zebrafish (Masai et al., 2000; Neumann and Neusslein-Volhard, 2000; Stenkamp and Frey, 2003; Masai et al., 2005). However, the mechanism that determines whether retinal progenitor cells continue to proliferate or differentiate still remains to be elucidated. In this study, we report a zebrafish retinal mutant, *add*, in which retinal neurogenesis is severely inhibited. In this mutant, retinal cells fail to exit from the cell cycle but instead continue to proliferate, suggesting that Add is required for the switch from proliferation to differentiation in the zebrafish retina. The cloning of the *add* gene revealed that it encodes Hdac1. The treatment with the Hdac inhibitor TSA revealed that the ratio of the number of differentiating cells to the number of proliferating cells correlates with Hdac activity, suggesting that Hdac1 may play a rate-limiting role in retinal neurogenesis in zebrafish. The canonical Wnt signaling promotes cell proliferation and the Notch signaling pathway inhibits neurogenesis in the zebrafish retina. We found that both the Wnt and Notch signaling pathways fail to be suppressed in the *add* mutant retinas, suggesting that Hdac1 suppresses both pathways to promote the cell-cycle exit of retinoblasts and the subsequent neurogenesis. Taken together, these data suggest that Hdac1 functions as a dual switch that suppresses both cell-cycle progression and inhibition of neurogenesis in the zebrafish retina (Fig. 10B).

Hdac1 antagonizes Wnt signaling to promote the cell-cycle exit of retinoblasts

Members of the Wnt growth factor family are involved in the regulation of multiple processes during development in flies and vertebrate animals (Wodarz and Nusse, 1998). Recent studies suggested that the canonical Wnt signaling promotes the proliferation of various types of stem cell population (Morin, 1999; van de Wetering et al., 2002; Reya et al., 2003), including neural progenitor cells (Chenn and Walsh, 2002; Megason and McMahon, 2002). In contrast to the role of Wnt signaling in the maintenance of progenitor cells, it also promotes neuronal differentiation from neural progenitor cells (Hirabayashi et al., 2004; Israsena et al., 2004), suggesting that Wnt signaling modulates the behavior of stem cells in a stage-dependent manner. In the developing vertebrate retina, Wnt signaling regulates both proliferation and neuronal differentiation. A recent study has suggested that Wnt2b promotes the cell-cycle progression in the chick retina (Kubo et al., 2003). It has been reported that a Wnt

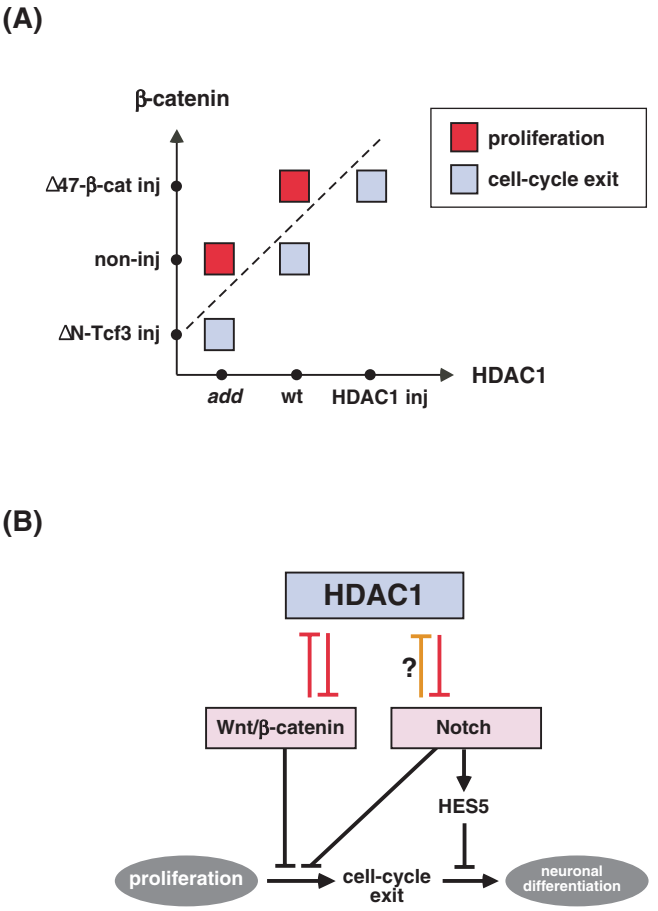

Fig. 10. A signaling network of Hdac1, Wnt and Notch in zebrafish retinal neurogenesis. (A) Retinal phenotypes produced by different combinations of Wnt and Hdac1 activities. Squares that represent 2 dpf retinal phenotypes (red, proliferation; blue, cell-cycle exit) are plotted at positions where combinations of Hdac and Wnt activities intersect. The broken line is a theoretical borderline between 'proliferation' and 'cell-cycle exit'. (B) The canonical Wnt signaling promotes the cell-cycle progression and Notch signaling inhibits neurogenesis through the activation of her4/Hes5 in the zebrafish retina. In this study, we found that Notch signaling is involved in the maintenance of proliferation of retinal cells. Hdac1 antagonizes Wnt and Notch signaling pathways to promote the cell-cycle exit and subsequent neurogenesis in the zebrafish retina. Because a balance between Wnt signaling and Hdac activity correlates with the ratio of proliferation to differentiation, one theory is that the competition between β-catenin and Hdac1 determines whether retinal cells continue to proliferate or exit from the cell cycle. Although it is generally accepted that, following Notch activation, the CSL co-repressor complex containing Hdac is displaced by a co-activator complex coordinated by NICD (Lai, 2004), it remains to be elucidated whether a balance between NICD and Hdac1 activities regulates Notch-mediated proliferation and neurogenic inhibition in the zebrafish retina.

signaling molecule, glycogen synthase kinase 3β, modifies the activity of *Xenopus* neuroD in a post-translational manner, and controls the timing of neuroD functions (Moore et al., 2002).

In this study, we show that the introduction of Δ47-β-catenin induces hyperproliferation in the zebrafish retina, suggesting that the activation of Wnt signaling promotes proliferation of retinal cells in zebrafish. The hyperproliferation phenotype induced by Δ47-β-catenin is morphologically similar to that of *add* mutant retinas, raising the possibility that Wnt signaling is activated in the *add* mutant retinas. Indeed, the expression of targets of canonical Wnt signaling, such as *cyclin D1* and *cmyc*, is elevated in the *add* mutant or TSA-treated retinas. Furthermore, the hyperproliferation of retinal cells in *add* mutant retina is suppressed by the introduction of ΔN-Tcf3, which functions as a dominant suppressor of canonical Wnt signaling. These data support the possibility that aberrant activation of Wnt signaling causes the hyperproliferation in the *add* mutant retinas. We also show that the introduction of Hdac1 significantly suppresses the cell-cycle progression induced by Δ47-β-catenin. These data suggest that Hdac1 antagonizes Wnt signaling to promote the cell-cycle exit of retinal progenitor cells in zebrafish.

TSA treatment of wild-type embryos in different concentrations revealed that the ratio of differentiation to proliferation in the zebrafish retina correlates with the level of Hdac activity, raising the possibility that a balance between Wnt signaling and Hdac activity is important to determine whether retinal cells exit from the cell cycle or re-enter it. In this study, we examined retinal phenotypes in four different combinations of Hdac and Wnt activities: the *add* mutant (Hdac1, low; Wnt, intact), the *add* mutant injected with ΔN-Tcf3 (Hdac1 low; Wnt, low), the wild type injected with Δ47-β-catenin (Hdac1, intact; Wnt, high), and the wild type co-injected with Δ47-β-catenin and Hdac1 (Hdac1, high; Wnt, high). The phenotypes in these four combinations clearly showed that the balance between Hdac1 and β-catenin correlates with retinal cell exit from or re-entry into the cell-cycle, respectively (Fig. 10A). This is reminiscent of previous in vitro and genetic experiments demonstrating that the expression of Tcf target genes is regulated by a balance between β-catenin and Hdac/co-repressors (Cavallo et al., 1998; Roose et al., 1998; Billin et al., 2000). Hdac activity may increase a threshold level of β-catenin, at which retinal cells respond to Wnt signals. It is interesting that the manipulation of only two factors, Hdac1 and β-catenin, can regulate the ratio of the number of differentiating cells to the number of proliferating cells. This may imply that Hdac1-mediated suppression of Wnt signaling is an essential component of the mechanism determining the rate of neurogenesis in the zebrafish retina.

How does Hdac1 suppress the Wnt signaling pathway? As Hdac proteins are recruited by transcription repressors and suppress the transcription of target genes, it is possible that Hdac1 switches off the transcription of genes that are activated by Wnt signaling and important for cell proliferation. One of the candidates is *cyclin D1*. It has been reported that *cyclin D1* is a target of canonical Wnt signaling (Shtutman et al., 1999; Tetsu and McCormick, 1999), and that the competition between β-catenin and Hdac proteins regulates the transition of Tcf/Lef from a transcription repressor to an activator (Billin et al., 2000). We showed that the *cyclin D1* expression is not downregulated in the *add* mutant retinas. Furthermore, the introduction of p27 inhibits the cell-cycle progression in the *add* mutant retinas, suggesting that Hdac1 functions upstream of the interaction between cyclin D1 and p27. These data suggest that Hdac1 antagonizes Wnt signaling to suppress the transcription of *cyclin D1* in the zebrafish retina. If this is the case, Hdac1 or a Hdac1-associated co-repressor directly competes with β-catenin to interact with the bipotential transcription factor Tcf/Lef. Such a direct competition model between Hdac1/co-repressor and β-catenin seems to agree with the observation that a balance between Hdac1 and β-catenin correlates with the ratio of differentiating cells to proliferating cells in the zebrafish retina.

Recent study revealed that various types of Wnt ligands are expressed in the murine neural retina and that there is a dynamic pattern of Wnt receptor (Frizzled) and Wnt antagonist (Secreted-frizzled-related protein, Sfrp) gene expression in the murine neural retina (Liu et al., 2003), raising the possibility that retinal cells may receive a variety of Wnt signals that are secreted from different sources and modulated in different ways. The most recent study using *Xenopus* retinas showed that Frizzled 5-mediated canonical Wnt signaling is involved in the maintenance of the potential of progenitor cells to generate neurons as well as their proliferation rate (Van Raay et al., 2005). The blockade of Frizzled 5 in the *Xenopus* retina does not influence the expression of progenitor markers such as Rx and Chx10, but biases progenitor cells toward a non-neural fate through the inactivation of Sox2. Hdac1-mediated inhibition of Wnt signaling may influence not only the entry of the cell cycle but also different aspects of retinal progenitor cells, such as the potential to generate neurons in the zebrafish retina.

Hdac1 antagonizes Notch-mediated activation of HES in the zebrafish retina

ΔN-Tcf3 suppresses the cell-cycle progression in *add* mutant retinas. However, *add* mutant cells expressing ΔN-Tcf3 fail to express neurogenic markers such as *ath5*. This observation indicates that the inhibition of Wnt signaling does not fully restore neuronal differentiation in the *add* mutant retina. These data suggest that Hdac1 also regulates Wnt-independent targets during retinal neurogenesis. Candidates are neurogenic inhibitors such as Hes (Hatakeyama and Kageyama, 2004). In the Hes1 knockout mouse, retinal neurons differentiate precociously (Tomita et al., 1996). In the absence of Notch signaling, a transcription factor of the Cbf1/Su(H)/Lag1 (CSL) family associates with Hdac proteins and actively keeps the transcription of Hes genes switched off (Kao et al., 1998). In this study, we show that the expression of a zebrafish ortholog of murine Hes5, *her4*, is upregulated in the *add* mutant retina, suggesting that Hdac1 negatively regulates the transcription of neurogenic inhibitors such as Hes. We found that this upregulation of *her4* expression in the *add* mutant retina is inhibited by the introduction of the *mib* mutation, suggesting that *her4* expression in the *add* mutant retinas requires the activation of Notch signaling. NICD, an active form of Notch, which is produced by ligand-dependent proteolytic cleavages, disrupts the association of CSL with co-repressors, resulting in the conversion of CSL from a transcription repressor to an activator (Lai, 2004). The decrease in Hdac activity may

facilitate the interaction between CSL and NICD to induce *her4* expression.

It has been reported that the expression of a zebrafish Hes1 ortholog, *her6*, is upregulated in the hindbrain of the zebrafish *hdac1* mutant (Cunliffe, 2004), which was isolated by the insertional mutagenesis (Golling et al., 2002). This previous study, carried out by Cunliffe (Cunliffe, 2004), also showed that *her6* expression is not inhibited in *mib* mutants injected with morpholino-antisense oligos of the *hdac1* gene, suggesting that the upregulation of *her6* expression in the *hdac1* mutant hindbrain seems independent of the activation of Notch signaling. This contrasts with our observation that the activation of Notch signaling is required for the upregulation of *her4* expression in the *add* mutant retina. In *mib* mutants injected with *hdac1* morpholino oligos, both Notch signaling and Hdac1 activities are attenuated, but the residual Notch signaling mediated by maternal Mib may counter residual Hdac activity derived from other Hdac proteins to induce *her6* expression in the hindbrain. By contrast, it is possible that maternal Hdac1 or other Hdac proteins counter Notch signaling mediated by maternal Mib to inhibit *her4* expression in the *add; mib* mutant retina. However, *her4* expression was not observed in the *mib* mutant treated with TSA, by which almost all Hdac activity, including maternal Hdac1 and other Hdac proteins, could be inhibited (M.Y. and I.M., unpublished). This observation suggests that maternal Mib-mediated Notch signaling in the retina may be too low to activate *her4* expression even in the severe blockade of Hdac activity. Previous studies have suggested that the Notch signaling pathway is modulated by several Notch-modifying proteins, such as Numb, Deltex and Mastermind-like (reviewed by Hansson et al., 2004). Such modifications of the Notch signaling pathway may elevate the Notch signaling activity in the hindbrain and contribute to *her6* expression in *mib* mutants injected with *hdac1* morpholino oligos.

Notch signaling is involved in the maintenance of proliferation in the retina

Notch signaling participates in a wide variety of cellular processes, including the maintenance of stem cells, specification of cell fate, differentiation, proliferation and apoptosis (reviewed by Artavanis-Tsakonas et al., 1999; Radtke and Raj, 2003). Several recent reports have found that Notch signaling promotes the maintenance of neural stem cells by enhancing the self-renewal of neural stem cells or inhibiting their differentiation into neuronal and glial progenitors (Nakamura et al., 2000; Chambers et al., 2001; Hitoshi et al., 2002; Chojnacki et al., 2003) (reviewed by Gaiano and Fishell, 2002; Ross et al., 2003). In contrast to these studies, it has been reported that the activation of Notch signaling promotes the cell-cycle exit of retinal progenitor cells in *Xenopus* retina (Ohnuma et al., 2002). A previous study using the zebrafish retina showed that the introduction of NICD using the Gal4/UAS system guides retinal cells into one of two fates: that of Müller glia or of seemingly undifferentiated cells (Scheer et al., 2001). Although Scheer et al. (Scheer et al., 2001) reported that the introduction of NICD reduces cell proliferation in the zebrafish retina, a role for Notch in cell-cycle regulation in the zebrafish retina is still not clarified.

In this study, we showed that the Notch signaling pathway fails to be suppressed in the *add* mutant retinas, and that the introduction of *mib* mutation inhibits continuous proliferation in the *add* mutant retinas. These data suggest that Notch signaling is involved in the maintenance of cell proliferation in the zebrafish retina. As p27 effectively inhibits the proliferation in the *add* mutant retina, Notch signaling functions upstream of the interaction between cyclin D1 and p27. It has been reported that NICD activates the transcription of cyclin D1 probably through a CSL-dependent pathway (Ronchini and Capobianco, 2001). However, we observed that NICD did not effectively induce *cyclin D1* expression in the retina (data not shown). Alternatively, p27 may be a target of Notch signaling. It has been reported that Hes1 promotes cell proliferation by suppressing the expression of the Cdk inhibitor $p21^{CIP}$ (Castella et al., 2000; Kabos et al., 2002). The upregulation of *her4* expression in the *add* mutant retinas may negatively regulate Cdk inhibitors such as p27. A recent study has revealed that Notch signaling triggers the onset of proliferation during *Drosophila* eye development (Baonza and Freeman, 2005). In the *Drosophila* eye, cells located in the morphogenetic furrow are arrested in G1 phase of the cell cycle and Notch signaling promotes the G1-S transition of these arrested cells by multiple pathways including the activation of dE2F, a member of E2F transcription factors, and cyclin A. It will be interesting to investigate whether a similar mechanism is involved in Notch-mediated proliferation in the zebrafish retina.

As both Wnt and Notch signaling pathways are involved in the maintenance of cell proliferation in the zebrafish retina, it is important to elucidate the relationship between Wnt and Notch signaling pathways. We showed that the activation of Notch signaling does not maintain proliferation in the zebrafish retina at 2 dpf. This contrasts with the observation that Wnt signaling is sufficient to induce hyperproliferation in the zebrafish retina. One possibility is that Notch signaling is one of downstream pathways activated by Wnt signaling. However, the upregulation of *her4* expression is not observed in the retinas injected with Δ47-β-catenin. Thus, it is unlikely that Wnt signaling directly activates the Notch signaling pathway in the zebrafish retina. In this study, we found that the activation of Notch signaling is dispensable for retinal proliferation, when Wnt signaling is highly activated. It is possible that a strong Wnt signal activates the transcription of cell-cycle regulators such as *cyclin D1*, which may overcome the impairment of Notch signaling. Recent study showed that the integration of Notch and Wnt signaling is important for the maintenance of hematopoietic stem cells (Duncan et al., 2005). In hematopoietic stem cells, Notch signaling is required for the maintenance of an undifferentiated state but not for the cell-cycle progression, both of which are mediated by Wnt signaling. It is possible that Notch signaling is required for the maintenance of an undifferentiated state of retinal progenitor cells, rather than for cell-cycle progression in zebrafish. Although the activation of Wnt signaling upregulates the expression of the Notch target Hes1 in hematopoietic stem cells (Duncan et al., 2005), it would be interesting to examine whether a common mechanism underlies the integration of Wnt and Notch signaling pathway in both zebrafish retinal progenitor cells and mouse hematopoietic stem cells.

Does Hdac1 interact with the Hh/PKA pathway?

In this study, we show that TSA treatment decreases the

number of postmitotic differentiating cells in a dose-dependent manner. In the treatment with TSA in a high dose, a small number of postmitotic cells are generated to form small BrdU-negative area (Fig. 4E,F). Because the size of BrdU-negative area represents the degree of the progression of neuronal production, these data suggest that Hdac activity influences the progression of neuronal production in the zebrafish retina. Our previous study suggested that the progression of neuronal production in the zebrafish retina is regulated by Hh-PKA signaling pathway (Masai et al., 2005). PKA effectively inhibits the cell-cycle exit of retinal progenitor cells, and PKA-mediated proliferation depends on canonical Wnt signaling (Masai et al., 2005). These data raise the possibility that Hdac1 antagonizes PKA signaling in retinal neurogenesis. One of the major PKA substrates, cAMP response element-binding protein (CREB), binds to the promoter of cell-cycle regulators such as cyclin D1 (reviewed by Mayr and Montminy, 2001; Lonze and Ginty, 2002). PKA phosphorylates Ser133 of CREB and this phosphorylated form of CREB recruits p300/CBP histone acetyltransferase (HAT) to activate the transcription of target genes (reviewed by Mayr and Montminy, 2001; Lonze and Ginty, 2002), including *cyclin D1* (D'Amico et al., 2000; Pradeep et al., 2004). It has been reported that protein phosphatase 1 interacts with Hdac1 to promote both the dephosphorylation of Ser133-CREB and the deacetylation of histones bound to target genes, leading to the attenuated transcription (Canettieri et al., 2003). Hdac1 may antagonize PKA-mediated CREB phosphorylation in zebrafish retinal neurogenesis. However, our previous study demonstrated that, unlike Hdac1, a dominant-negative form of PKA (dnPKA) does not significantly inhibit Δ47-β-catenin-induced hyperproliferation (Masai et al., 2005). Furthermore, we observed that the introduction of dnPKA did not significantly suppress the cell-cycle progression in *add* mutant retinas (M.Y. and I.M., unpublished). Thus, it seems unlikely that the interaction between Hdac1 and the PKA-CREB pathway is a major pathway for suppression of the transcription of cyclin D1 in the zebrafish retina.

Recently, it has been reported that Hdac1 is required for Hh-mediated induction of brachiomotor neurons in the zebrafish hindbrain (Cunliffe, 2004). Although the expression of *sonic hedgehog* (*shh*) and *patched1* in the ventral CNS are relatively normal, exogenous *shh* expression fails to induce the differentiation of brachiomotor neurons in the zebrafish *hdac1* mutant, suggesting that *hdac1*-deficient cells lose the competence to respond to Hh signals. Our previous study and others revealed that retinal neurogenesis is delayed in a zebrafish *smoothened* mutant, *slow muscle omitted* (*smu*) (Stenkamp and Frey, 2003; Masai et al., 2005). The common defect in the cell-cycle exit of retinoblasts between the *smu* and the *add* mutant raises the possibility that Hdac1 is involved in the Hh signaling pathway. However, the delay of retinal neurogenesis in the *smu* mutant was not enhanced in the presence of heterozygous *add* mutation, and hyperproliferation in the *add* mutant retina was not influenced by the introduction of heterozygous *smu* mutation (M.Y. and I.M., unpublished), suggesting that there is no genetic interaction between Smoothened and Hdac1 in retinal neurogenesis. Although Hdac1 activity influences the progression of retinal neurogenesis, a relationship between Hh/PKA signaling pathway and the Hdac1 pathway is essentially unknown. In the future, it will be important to elucidate how these two pathways are integrated to promote the cell-cycle exit and subsequent neurogenesis in the zebrafish retina.

Other issues that should be addressed in the future

In this study, we showed that Hdac1 plays an essential role in the switch from proliferation to differentiation in the zebrafish retina. This anti-proliferative function of Hdac is unexpected, because it was generally accepted that Hdac inhibitors efficiently suppress the proliferation of tumor cells (Johnstone and Licht, 2003). Furthermore, Hdac1-deficient mice show severe defects in cell proliferation due to the aberrant activation of Cdk inhibitors (Lagger et al., 2002). The roles of Hdacs may be diverse among species and cell types. As Hdac1-deficient mice die before retinal neurogenesis begins, it is important to examine the retinal phenotypes of conditional Hdac1-knockout mice, in order to elucidate whether the role of Hdac1 in retinal neurogenesis is conserved throughout vertebrate animals. Furthermore, we found that hyperproliferation occurs exclusively in the neural retina but not in the brain in the *add* mutants. As the treatment of embryos with TSA from 14 hpf induces retina-specific hyperproliferation (Fig. 5E,F), it is unlikely that this retina-specific defect in the cell-cycle exit is due to the redundancy of Hdac genes in the brain. At present, the mechanism that underlies the retina-specific defect in the cell-cycle exit is unknown. In the future, the identification of a molecule that modulates a signaling network of Hdac1, Wnt, Notch and Hh-PKA pathways will be definitely important to understand the mechanism underlying the retina-specific hyperporliferation in the *add* mutant retinas.

We thank Dr Shin-ichi Ohnuma for providing the plasmid of *Xenopus* p27, Dr Ajay Chitnis for ΔN-Tcf3, and Dr Randall T. Moon for TOPdGFP transgenic fish. We thank Ms. Naoko Kume for technical support and all the members of Masai and Okamoto's laboratories for helpful discussion. The zebrafish lines carrying hsp: Gal4 and UAS: myc-NICD were purchased from Zebrafish International Resource Center (Oregon). This work was supported by grants from RIKEN BSI, and CREST, JST (H.O.), and RIKEN IRU (I.M.).

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