STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

Thyroid Hormone-Induced Activation of Notch Signaling Is Required for Adult Intestinal Stem Cell Development During *Xenopus Laevis* Metamorphosis

TAKASHI HASEBE,[a] KENTA FUJIMOTO,[a] MITSUKO KAJITA,[b] LIEZHEN FU,[c] YUN-BO SHI,[c] ATSUKO ISHIZUYA-OKA[a]

Key Words. Intestinal remodeling • Notch signaling • Thyroid hormone • Amphibian metamorphosis • Epithelial stem cell

[a]Department of Biology, Nippon Medical School, Musashino, Tokyo, Japan; [b]Department of Molecular Biology, Institute for Advanced Medical Sciences, Nippon Medical School, Kawasaki, Kanagawa, Japan; [c]Section on Molecular Morphogenesis, Cell Regulation and Development Affinity Group, Division of Molecular and Cellular Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), Bethesda, Maryland, USA

Correspondence: Takashi Hasebe, Ph.D., Department of Biology, Nippon Medical School, 1-7-1 Kyonan-cho, Musashino, Tokyo 180-0023, Japan. Telephone: +81-422-34-3438; Fax: +81-422-34-1120; e-mail: hasebet@nms.ac.jp

Received June 9, 2016; accepted for publication October 28, 2016; first published online in STEM CELLS *EXPRESS* November 21, 2016.

1066-5099/2016/$30.00/0

http://dx.doi.org/10.1002/stem.2544

ABSTRACT

In *Xenopus laevis* intestine during metamorphosis, the larval epithelial cells are removed by apoptosis, and the adult epithelial stem (AE) cells appear concomitantly. They proliferate and differentiate to form the adult epithelium (Ep). Thyroid hormone (TH) is well established to trigger this remodeling by regulating the expression of various genes including Notch receptor. To study the role of Notch signaling, we have analyzed the expression of its components, including the ligands (DLL and Jag), receptor (Notch), and targets (Hairy), in the metamorphosing intestine by real-time reverse transcription-polymerase chain reaction and in situ hybridization or immunohistochemistry. We show that they are up-regulated during both natural and TH-induced metamorphosis in a tissue-specific manner. Particularly, Hairy1 is specifically expressed in the AE cells. Moreover, up-regulation of Hairy1 and Hairy2b by TH was prevented by treating tadpoles with a γ-secretase inhibitor (GSI), which inhibits Notch signaling. More importantly, TH-induced up-regulation of LGR5, an adult intestinal stem cell marker, was suppressed by GSI treatment. Our results suggest that Notch signaling plays a role in stem cell development by regulating the expression of Hairy genes during intestinal remodeling. Furthermore, we show with organ culture experiments that prolonged exposure of tadpole intestine to TH plus GSI leads to hyperplasia of secretory cells and reduction of absorptive cells. Our findings here thus provide evidence for evolutionarily conserved role of Notch signaling in intestinal cell fate determination but more importantly reveal, for the first time, an important role of Notch pathway in the formation of adult intestinal stem cells during vertebrate development. STEM CELLS *2017;35:1028–1039*

SIGNIFICANCE STATEMENT

During thyroid hormone (TH)-dependent metamorphosis of *Xenopus laevis*, the development of intestinal adult epithelial stem (AE) cells takes place, resembling the formation of the self-renewing adult intestinal crypt-villus axis during mammalian postembryonic development. We show that Notch signaling is activated in response to TH in the metamorphosing intestine. More importantly, we experimentally demonstrate, for the first time, that Notch signaling is required for the AE cell development during intestinal remodeling. Our findings further suggest that the amphibian intestine, with many TH response genes already identified, be a highly valuable animal model for clarifying the molecular bases of the stem cell regulation.

INTRODUCTION

During metamorphosis of the anuran amphibians, the intestine is extensively remodeled to adapt from herbivorous to carnivorous life [1]. In the *Xenopus laevis* intestine, the larval epithelial cells are removed by apoptosis, and replaced by the adult epithelium (Ep) analogous to the mammalian one [2, 3]. This process, which is totally dependent on thyroid hormone (TH), involves the de novo development of the AE cells that originate from the larval Ep through dedifferentiation [4]. Amphibian metamorphosis resembles mammalian postembryonic development, because in both cases, TH levels peak as organ remodeling/maturation including the development of organ-specific adult stem cells takes place [1, 5–7]. Whereas it is difficult to manipulate uterus-enclosed mammalian embryos, tadpoles are easily manipulated and are independent of any maternal influence. The intestinal remodeling can be reproduced by

adding TH to the rearing water of premetamorphic tadpoles in vivo or to the medium of tadpole intestinal organ cultures in vitro [8]. These advantages, together with the similarities to mammalian postembryonic intestinal maturation, make intestinal metamorphosis an excellent model to study the mechanisms of TH-dependent adult stem cell development.

TH binds to its receptors (TRs), which form heterodimers with 9-cis retinoid acid receptors (RXRs). In the presence of the ligand, the TR/RXR complexes bound to the TH response elements (TREs) to activate the expression of direct TH response genes [9–14]. The products of these direct targets in turn affect the expression of downstream genes. Thus, to clarify the molecular basis of larval-to-adult intestinal remodeling, it is important to study the TH target genes regardless of the mechanisms of their response to TH. To this end, a number of TH response genes have been identified by several approaches [15–17]. Among them, Notch1, a member of Notch family of transmembrane receptors for Delta-like (DLL) and Jagged/Serrate (Jag) ligands, has been found to be up-regulated in the metamorphosing intestine [15].

The Notch signaling pathway is activated through a ligand-receptor interaction between adjacent cells. This interaction induces proteolytic cleavage of Notch receptor by the ADAM family and γ-secretase complex to produce the Notch intracellular domain (NICD) [18]. NICD subsequently translocates into the nucleus and forms a transcriptional activator complex with CSL/RBP-J. This complex then activates the expression of downstream target genes such as hairy and enhancer of split (Hes) family of genes [18–21]. Hes1, perhaps the best-studied Notch downstream target, is a bHLH-O transcription factor and shown to repress the expression of target genes including Atoh1 (Math1) and Ngn3 [22–24].

Through molecular mechanisms described above, the Notch signaling pathway is considered to play multiple roles in animal development and homeostasis in adult organs/tissues, including neural differentiation, vascular morphogenesis, somitogenesis, hematopoiesis, and so on. [25–28]. In particular, in the intestinal Ep of adult mammals, Notch signaling controls a binary cell fate determination between absorptive and secretory cells, which originate from common stem cells located in the intestinal crypt [22, 23, 29]. Inhibition of Notch signaling in the intestinal Ep by using conditional gene targeting of RBP-J or with pharmacologic γ-secretase inhibitors (GSI), which block the release of NICD, results in the loss of the proliferative crypt compartment and conversion of progenitor cells into postmitotic goblet cells through Hes1 repression [23, 30]. Thus, the precise control of Notch signaling is important for the cell fate determination and maintenance of the stem cells in the adult intestine [31]. However, the role of Notch signaling in the development of the adult stem cells in vertebrates remains largely unknown.

In the present study, we focused mainly on *X. laevis* Hairy1 (Hes1 ortholog) and Hairy2b (Hes4 ortholog), two target genes of Notch signaling, and analyzed their spatiotemporal expression by real-time reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization (ISH). We also performed the expression analysis of other components of this signaling pathway, namely, the ligands (DLL1, DLL3, Jag1, Jag2) and receptor (Notch1). We show that all genes analyzed are up-regulated during both natural and TH-induced metamorphosis in a cell/tissue specific manner. Particularly, Hairy1 is specifically expressed in the AE/progenitor cells, whereas Hairy2b is expressed in the connective tissue (CT). In addition, TH-induced up-regulation of these Hairy genes was drastically suppressed by GSI treatment. Our results suggest that during intestinal remodeling, Notch signaling is activated to control the expression of its target genes in a tissue-specific manner. More importantly, TH-induced up-regulation of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), an intestinal stem cell marker gene [32, 33], was significantly inhibited by GSI treatment. These findings for the first time reveal an important role of Notch singling in the formation of organ-specific adult stem cells and/or their proliferation during vertebrate development. In addition, hyperplasia of Periodic acid-Schiff (PAS)-positive secretory cells was also observed in the cultured larval intestine treated with both TH and GSI, indicating the evolutionarily conserved role of Notch signaling in intestinal cell fate determination.

Materials and Methods

Animal and Treatment

X. laevis tadpoles and frogs were obtained and maintained as previously described [34]. The developmental stages were determined according to Nieuwkoop and Faber [35]. Premetamorphic tadpoles at stage 54 were treated with 10 nM 3,5,3′-triiodothyronine (T3) for 1-5 days.

Dibenzazepine (DBZ), a well-known γ-secretase inhibitor (GSI) [29], was purchased from Selleck Chemicals (Houston, TX). Premetamorphic tadpoles at stage 54 were pretreated with dimethyl sulfoxide (DMSO, vehicle) or 5 μM DBZ for 3 days. Then, the tadpoles were further treated with 10 nM T3 in the presence of DMSO or 5 μM DBZ for 3 or 5 days. The rearing water was changed everyday during these treatments.

At least four tadpoles were analyzed for each stage or day of treatment. Animal rearing and treatment were done according to the guidelines set by Nippon Medical School animal use and care committee.

Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA from the small intestine was extracted by using TRI Reagent (Molecular Research Center, Cincinnati, OH) followed by DNase treatment with DNA-free (Ambion, Austin, TX) to remove any DNA contamination. The integrity of RNA was checked based on 18S and 28S ribosomal RNAs by electrophoresis. Total RNA was mixed with GoTaq 1-Step RT-qPCR System (Promega, Madison, WI), and then quantitative real-time RT-PCR was performed by using PikoReal 96 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) according to the manufacture's instructions. The primer pairs used are: 5′-CCAGCGGACACTCTTCTTTC-3′ and 5′-AGCGCAATAGGTCCACAAAC-3′ for Hairy1 (GenBank: NM_001087927), 5′-AGTCTCCAGTGCAGGGACTC-3′, and 5′-TCCCATAGAACGGAACCAAC-3′ for Hairy2b (GenBank: NM_001088692), 5′-CGGCCATTTTTCCTGTGGTG-3′ and 5′-ATGCAGGCATCCTGGGTAAC-3′ for Delta-like 1 (DLL1 or X-delta1, GenBank: BC070634), 5′-TTGGGTATGGGGGCAAAGAC-3′ and 5′-TTCCCATAAAACCCGTGGGG-3′ for DLL3 (Delta-like protein C or X-delta2, GenBank: NM_001086082), 5′-GCATGCTCAAATCGCCTGAG-3′ and 5′-GAGGGCTCTCCTTGGGAAAC-3′ for Jagged1 (Jag1, GenBank: NM_001090307), 5′-TGCCCTCGCGGTTTCGTTGG-3′, and 5′-TCTGGCAAGGGTTGGGCTCAC-3′ for Jagged2 (Jag2, there is no accession number in GenBank. Gene locus: *X. laevis* chromosome 8S,

nucleotide 9247347-9334515) [36], 5′-GCCTTGGGACCTTTATCCTC-3′ and 5′-TGAGTGCATCGAAGACCTTG-3′ for Notch1 (GenBank: NM_001087605), 5′-TGGGCTTGTCTAGCGGTGTGC-3′ and 5′-CCAC TTTCCCACCAGGCACCG-3′ for leucine-rich repeat containing G protein-coupled receptor (LGR) 5 (GenBank: NM_001199223), 5′-GCCTAGCCAACAGCTCTCTCCAC-3′ and 5′-ACCTTCAGATTGTCATGT GCCCC-3′ for intestinal fatty acid-binding protein (IFABP; GenBank: NM_001085877), 5′-GCCGTGGTGCTCCTCTTGCC-3′ and 5′-TGCCACAGTACACAAACTGTCCG-3′ for ribosomal protein L8 (rpL8; GenBank: NM_001086996). The level of specific mRNA was normalized against the level of rpL8 mRNA [37] for each sample. Samples were analyzed in duplicate for at least three times. The specificity of the amplification was confirmed by the dissociation curve analysis and gel electrophoresis. The results were analyzed by Student's *t*-test or ANOVA followed by Scheffe's post hoc test.

In Situ Hybridization

X. laevis Hairy1 and Hairy2b full-length cDNA clones in pCMVSPORT6 (IMAGE:4030543 and 4032838, respectively) were purchased from Open Biosystems (Huntsville, AL). These plasmids were used to synthesize the full-length probes. The constructs to synthesize the probes for IFABP and LGR5 have been described previously [33, 38].

All plasmids were linearized to synthesize sense and antisense probes with T3, T7, or SP6 RNA polymerase by using digoxigenin (DIG) RNA Labeling Mix (Roche Applied Science, Indianapolis, IN). Full-length probes were digested by alkaline treatment (40 mM $NaHCO_3$, 60 mM Na_2CO_3) into ca 200 base-long.

Intestinal fragments were isolated from the anterior part of the small intestine just after the bile duct junction and were fixed in MEMFA followed by cryosectioning at 7 μm. ISH was performed by using sense or antisense probes of Hairy1, Hairy2b, IFABP, and LGR5 as previously described [39, 40]. Photographs were taken by using a digital CCD color camera (DP70, Olympus, Tokyo, Japan) attached to an optical microscope (BX51, Olympus).

Organ Culture

The intestinal fragments were isolated from the anterior part of the small intestine of *X. laevis* tadpoles at stage 56/57, and cultured as previously described [40]. Briefly, the intestinal fragments were opened lengthwise and cut into small pieces (3-4 mm long). They were cultured on Transwell culture insert (Corning, Lowell, MA) set in a 6-well plate filled with the culture medium [41] without T3 in the presence of DMSO (control) or 10 μM DBZ at 26°C for 3 days (precultivation). After precultivation, the intestinal fragments were cultured with 20 nM T3 in the presence of DMSO or 10 μM DBZ for 5 or 7 days. They were then used for either ISH or immunohistochemistry (IHC).

Immunohistochemistry

The intestinal fragments were fixed in 95% ethanol on ice, embedded in paraffin and sectioned at 5 μm. Sections were incubated with the mouse anti-proliferating cell nuclear antigen (PCNA) antibody (1:100; Novocastra Laboratories, Newcastle, UK) or the rabbit anti-intestinal fatty acid-binding protein (IFABP) antibody to identify absorptive cells (1:500; [42]). They were then incubated with peroxidase-labeled streptavidin (Nichirei Biosciences, Tokyo, Japan) followed by 0.02% 3, 3′-diamino-benzidine-4HCl (DAB) and 0.006% H_2O_2. To identify the mucus-secreting goblet cells, some other sections were subjected to PAS staining followed by counter-staining with hematoxylin. Photographs were taken as mentioned above. The percentage of PCNA-positive cells was calculated and the results were analyzed by Student's *t*-test [43, 44].

The sections of the intestine from stage-62 tadpoles were double-immunostained with a mixture of the mouse anti-human cytokeratin 19 (CK19) (1:100; Novocastra), which is a predominant cytokeratin in the adult Ep including the stem cells [45], and the rabbit anti-human DLL1 (1:100; Abcam, Tokyo, Japan), the rabbit anti-human Jag1 (1:50; Santa Cruz Biotechnology, Dallas, TX), or the rabbit anti-human Notch1 (1:500; Rockland Inc., Gilbertsville, PA). They were then incubated with a mixture of Alexa Fluor 568-conjugated anti-mouse IgG (1:500; Molecular Probes, Eugene, OR) and 488-conjugated anti-rabbit IgG antibodies (1:500; Molecular Probes), counterstained with 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (10 μg/ml; Dojindo, Kumamoto, Japan) and analyzed by fluorescence microscopy. The immunogen sequences used to raise anti-DLL1, anti-Jag1 and anti-Notch1 antibodies were nearly identical (88%, 100%, and 86%, respectively) to the *X. laevis* counterparts (manufactures' information).

Results

Up-Regulation of the Genes Involved in Notch Signaling in the Intestine During Natural and TH-Induced Metamorphosis

To study the temporal gene regulation of Hairy1 and Hairy2b, two target genes of Notch signaling, during intestinal remodeling, we first carried out real-time RT-PCR using total RNA extracted from the small intestine at various metamorphic stages (Fig. 1). Hairy1 mRNA was expressed at low levels from stage 54 (premetamorphosis) to stage 61 (early metamorphic climax) but significantly up-regulated at stage 62, when the adult epithelial cells are actively proliferating [46]. Thereafter, its expression decreased toward stage 66 (the end of metamorphosis) (Fig. 1A). Similar results were obtained for Hairy2b mRNA (Fig. 1C). In addition, we examined the temporal expression of Notch ligands and receptor. Similar to the expression profiles of Hairy genes, DLL1 (Fig. 1E), DLL3 (Fig. 1G), Jag1 (Fig. 1I), Jag2 (Fig. 1K), and Notch1 (Fig. 1M) mRNAs were significantly up-regulated at metamorphic climax and down-regulated toward the end of metamorphosis.

As TH can induce precocious intestinal remodeling, if the Notch signaling is involved in this process, the expression profiles of genes analyzed above should be reproduced during TH-induced metamorphosis. Thus, we analyzed their expression in the intestine of premetamorphic tadpoles at stage 54 treated with 10 nM T3 for 1 to 5 days. The expression of Hairy1 mRNA (Fig. 1B) was significantly up-regulated after 1 day of T3 treatment. Thereafter, Hairy1 expression reached the highest level after 4 days. Similarly, Hairy2b expression (Fig. 1D) was significantly up-regulated and reached the highest level after 4 days. Their expression remained the peak levels after 5 days. DLL1 (Fig. 1F), DLL3 (Fig. 1H), Jag1 (Fig. 1J), Jag2 (Fig. 1L), and Notch1 (Fig. 1N) also showed similar expression profiles to those of Hairy genes. These results suggest that Notch signaling is activated in the intestine during TH-dependent metamorphosis.

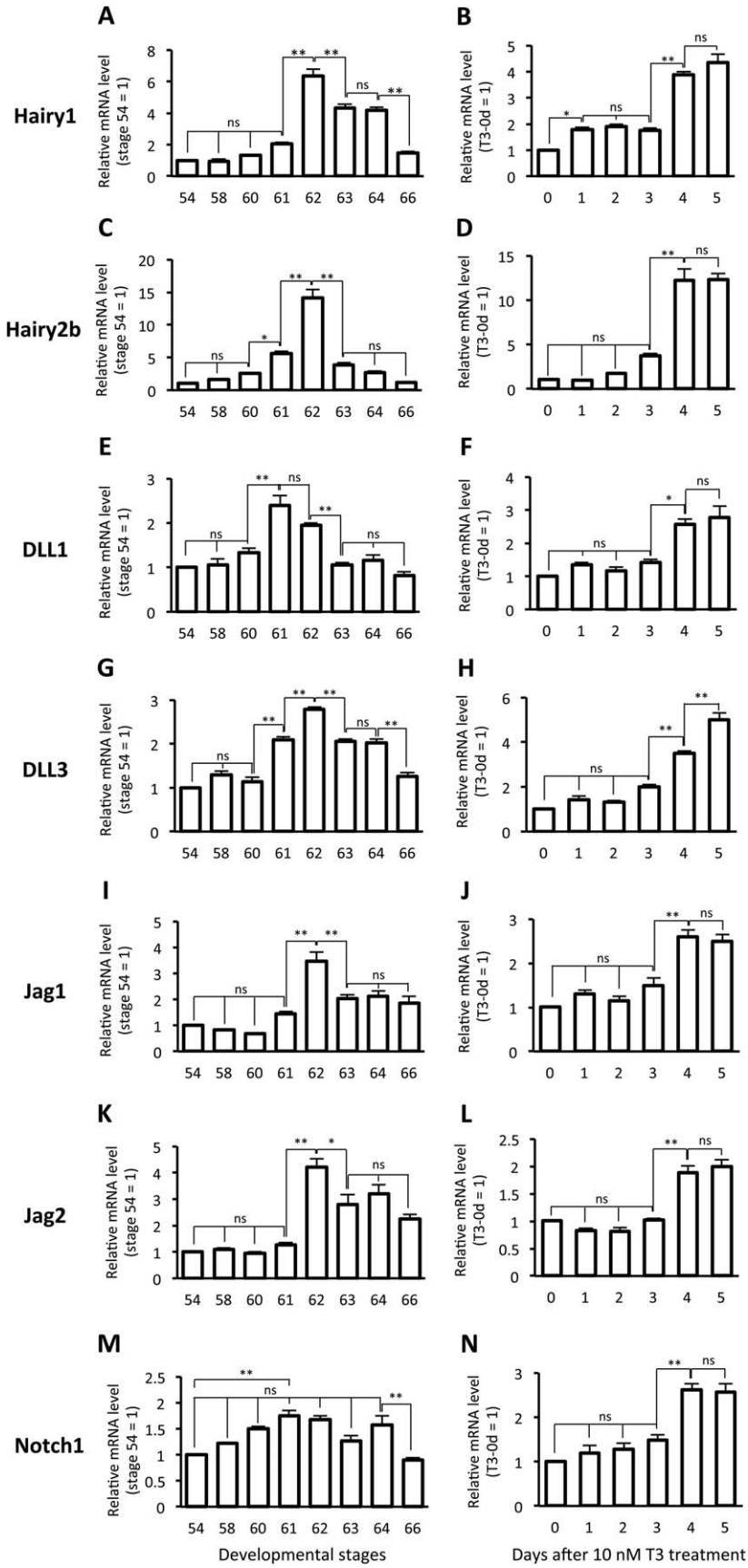

Figure 1. Expression profiles of genes involved in Notch signaling in *X. laevis* intestine during natural and T3-induced metamorphosis. Quantitative real-time RT-PCR was performed using total RNAs prepared from the intestine of animals at indicated developmental stages (**A, C, E, G, I, K, M)** or stage-54 tadpoles after 10 nM T3 treatment (**B, D, F, H, J, L, N**). Levels of Hairy1 (A, B), Hairy2b (C, D), DLL1 (E, F), DLL3 (G, H), Jag1 (I, J), Jag2 (K, L), and Notch1 (M, N) mRNAs are shown relative to those of ribosomal protein L8 (rpL8) mRNA, with the values at stage 54 or 0-day treatment set to 1. Error bars represent the SEM ($n = 7$ for A–D; $n = 3$ for E–H, J, M, N; $n = 8$ for I, L; $n = 5$ for K). The values were analyzed by ANOVA followed by Scheffe's post hoc test whose results are shown only for the adjacent stages or days except for Notch1 (M). Asterisks indicate that the mRNA levels are significantly different. *, $p < .05$, **, $p < .01$; ns: not significant.

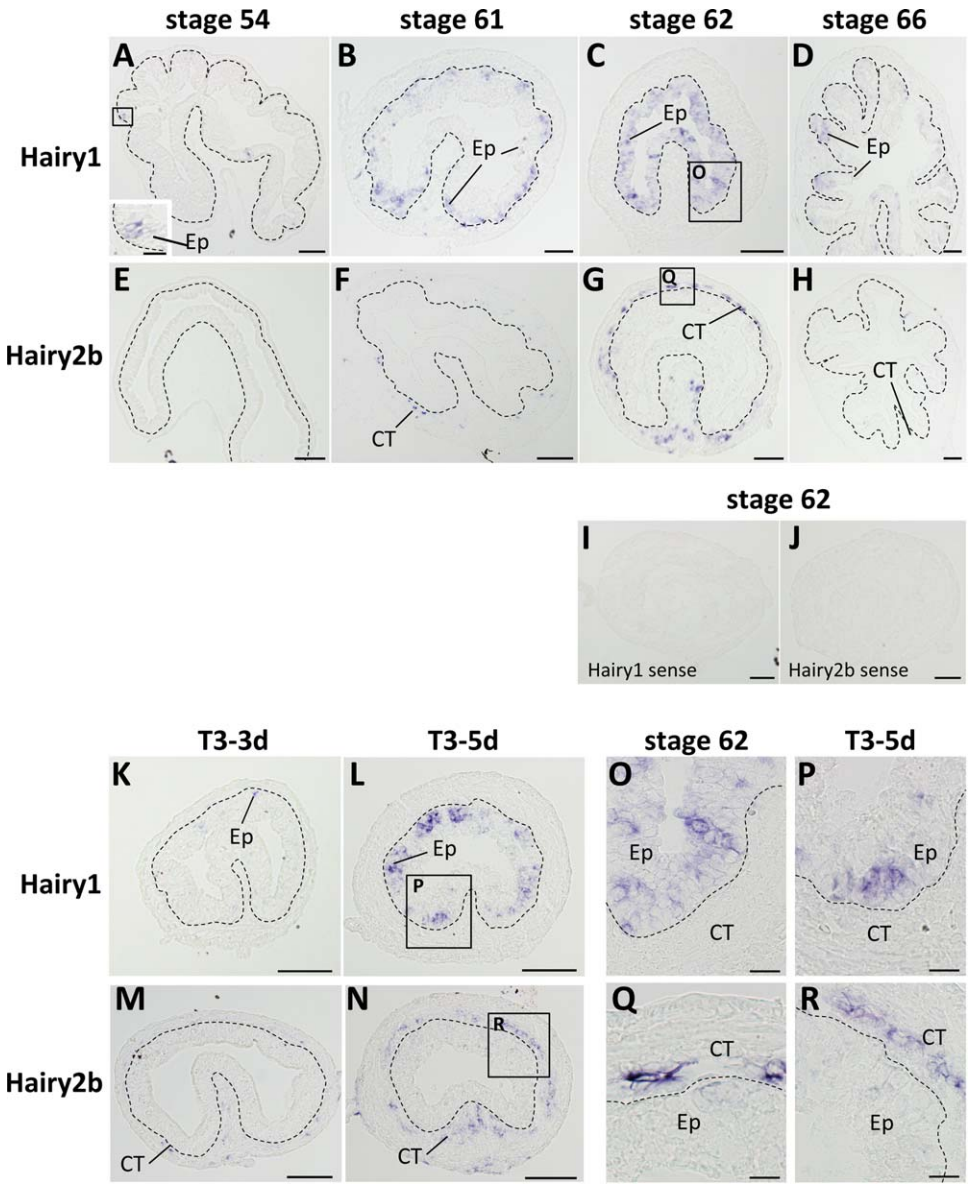

Figure 2. Localization of Hairy1 and Hairy2b mRNAs in the *X. laevis* intestine during natural and T3-induced metamorphosis. Cross-sections of the intestine from animals at premetamorphic stage 54 (**A, E**), metamorphic climax stages 61 (**B, F**) and 62 (**C, G, I, J, O, Q**), and the end of metamorphosis (stage 66; **D, H**), and stage-54 tadpoles treated with 10 nM T3 for 3 days (**K, M**) and 5 days (**N, L, P, R**) were hybridized with antisense Hairy1 (A-D, K, L, O, P), Hairy2b (E-H, M, N, Q, R), or their sense probes (I, J). Dark blue deposits indicate the sites of probe binding. Light or dark brown pigments in some pictures are melanin. Hairy1 is expressed in the epithelium (Ep), whereas Hairy2b is expressed in the connective tissue (CT). The boxed area in panel A is magnified to show the localization of Hairy1 expression in the larval Ep (panel A, inset). The boxed areas in panels C, G, L, N are magnified and shown in panels O, Q, P, R, respectively. The dashed-lines indicate the boundary of Ep and CT. Scale bars = 100 μm (A–L), 20 μm (inset of A, O–R). Abbreviation: CT, connective tissue, Ep, epithelium.

Localization of Hairy1 and Hairy2b mRNA in the Metamorphosing Intestine

We next examined by ISH the spatiotemporal expression of Hairy genes in the small intestine during natural and TH-induced metamorphosis (Fig. 2). At stage 54, the small intestine consists of a monolayer of the larval epithelial cells surrounded by thin layers of the outer longitudinal and inner circular muscles with intervening CT. The expression of Hairy genes was low or undetectable at this stage (Fig. 2A, 2E), though Hairy1 mRNA was occasionally detected in the larval epithelial cells (Fig. 2A inset). At the metamorphic climax (stages 61, 62), when the larval epithelial cells undergo apoptosis and AE/progenitor cells rapidly proliferate [46], the expression of these genes became higher. Hairy1 mRNA was specifically expressed in the Ep (Fig. 2B, 2C, 2O), whereas the expression of Hairy2b was exclusively detected in the CT (Fig. 2F, 2G, 2Q). Thereafter, both genes were down-regulated by stage 66 (Fig. 2D, 2H) when the adult epithelial cells are differentiated into the absorptive Ep and the Ep possesses a cell renewal system along the trough-crest axis of intestinal folds [47], similar to that along the crypt-villus axis of adult mammalian intestine [48, 49]. These expression patterns were also reproduced by T3 treatment of premetamorphic tadpoles at stage 54. Their mRNAs became detectable after 3 days of

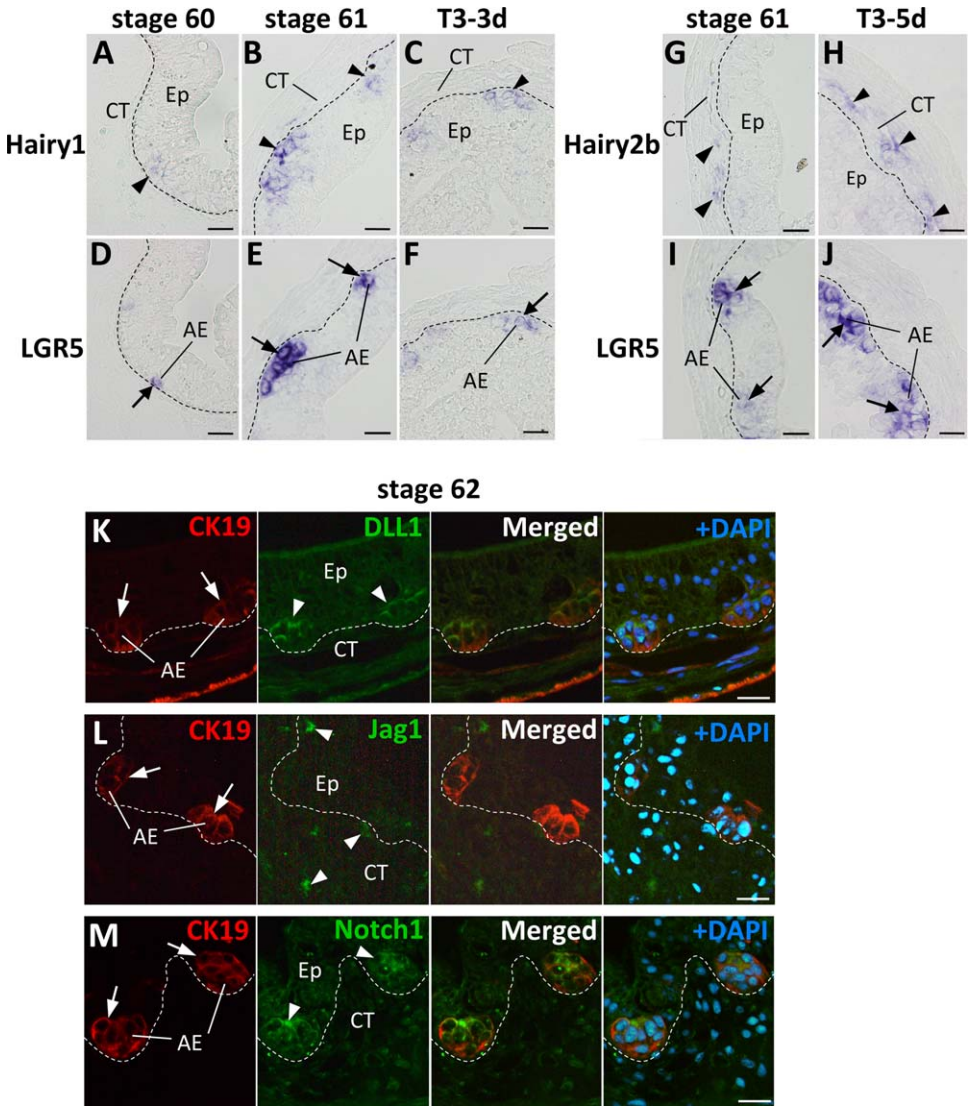

Figure 3. Correlation of the expression patterns of Notch pathway components with that of a stem/progenitor marker in the metamorphosing intestine. Cross-sections of the intestine from tadpoles at stages 60 (**A, D**) and 61 (**B, E, G, I**), and stage-54 tadpoles treated with 10 nM T3 for 3 (**C, F**) and 5 days (**H, J**) were hybridized with antisense Hairy1 (A-C) or Hairy2b (G, H) probes. The corresponding serial sections were hybridized with antisense LGR5 probe (D–F, I, J) for comparison. Hairy1 is expressed in the adult epithelial stem (AE)/progenitor cells (A, arrowhead) when they first appear as the small islets expressing LGR5 (D, arrow) at stage 60. These islets grew in size as metamorphosis proceeded and continued to co-express Hairy1 (B, arrowheads) and LGR5 (E, arrows). During T3-induced metamorphosis, Hairy1 (C, arrowhead) is also expressed in the AE/progenitor cells expressing LGR5 (F, arrow) after 3 days of T3 treatment. Some, although not all, cells expressing Hairy2b were in the connective tissue (CT) (G, arrowheads) underlying the AE/progenitor cells expressing LGR5 (I, arrows). Hairy2b is also expressed in the CT after 5 days of T3 treatment (H, arrowheads), again, with some underlying the AE/progenitor cells (J, arrows). Cross-sections of the intestine from tadpoles at stage 62 were double-immunostained with anti-CK19 to detect the islet of AE/progenitor cells (**K–M**, red) and anti-DLL1 (K, green), anti-Jag1 (L, green), or anti-Notch1 (M, green) followed by counterstaining with DAPI (K–M, blue). DLL1 (K, arrowheads) and Notch1 (M, arrowheads) are expressed in the islets (arrows). The cells expressing Jag1 are scattered in both the larval Ep and CT with some just beneath the islet, but not in the islets (L, arrowheads). The dashed-lines indicate the boundary of the Ep and the CT. Scale bars = 20 μm. Abbreviations: AE, adult epithelial stem/progenitor cells, CT, connective tissue, Ep, epithelium.

10 nM T3 treatment (Fig. 2K, 2M) and were strongly expressed after 5 days (Fig. 2L, 2N, 2P, 2R). These results agree well with those obtained by RT-PCR analysis shown in Figure 1.

To study their localization more precisely, we compared the expression patterns of Hairy1 and Hairy2b with that of LGR5, an adult intestinal stem cell marker gene, by using serial sections. At stage 60, when the AE/progenitor cells first appear as small roundish islets between the larval Ep and the CT, Hairy1 expression was detected in these islets (Fig. 3A). The same region in the serial section expressed LGR5 (Fig. 3D). As the islets rapidly grew in size at stage 61, the ISH signals for Hairy1 became stronger and showed a specific localization in the islets (Fig. 3B). These islets again expressed LGR5 at this stage (Fig. 3E). Their co-expression was also observed in the intestine of premetamorphic tadpoles treated with 10 nM T3 for 3 days (Fig. 3C, 3F). On the other hand, some, although not all, cells expressing Hairy2b in the CT were located adjacent to the islets expressing LGR5 (Fig. 3G-3J). In addition, we analyzed by IHC the localization of DLL1, Jag1, and

Notch1 at metamorphic climax (stage 62). To identify the islets of AE/progenitor cells, CK19 was immunostained as a marker (Fig. 3K-3M, arrows) [45]. DLL1 and Notch1 were predominantly detected in the islets (Fig. 3K, 3M, arrowheads). On the other hand, Jag1-expressing cells were scattered in both the larval Ep and CT but not in the islets. Some CT-cells expressing Jag1 were located just beneath the islets (Fig. 3L, arrowheads). These results indicate that Hairy1 is an AE/progenitor cell-specific gene and that Notch signaling is activated in both the Ep and CT during intestinal remodeling.

Notch Signaling is Required for the Up-Regulation of Hairy Genes and the Development of Adult Intestinal Stem Cells

To investigate the role of Notch signaling in TH-dependent intestinal remodeling, we used DBZ, a well-known γ-secretase inhibitor (GSI) that blocks the proteolytic cleavage of Notch receptor, thereby inhibiting the expression of target genes of Notch signaling. When premetamorphic tadpoles at stage 54 were pre-treated with dimethyl sulfoxide (DMSO, control) or 5 μM DBZ for 3 days (Fig. 4A), the expression levels of Hairy genes significantly decreased in the intestine of tadpoles treated with DBZ (Fig. 4B), suggesting that Notch signaling is required for basal level expression of the Hairy genes in the premetamorphic intestine. When such pre-treated tadpoles were further treated with 10 nM T3 for 3 or 5 days in the presence of DMSO or 5 μM DBZ (Fig. 4A), we observed that the gross morphology of the tadpoles was similar between control and DBZ-treated tadpoles after 5 days (Fig. 4C-4D') except for internal hemorrhage around the nose and along the digits of hindlimb in the DBZ-treated tadpoles (Fig. 4D, 4D', arrowheads). ISH analysis showed that DBZ treatment strongly inhibited TH-induced up-regulation of Hairy1 and Hairy2b in the intestine (Fig. 4E-4H). More importantly, LGR5-positive cells were reduced by the DBZ treatment (Fig. 4I, 4J), suggesting that DBZ inhibited the formation and/or proliferation of adult intestinal stem cells. In contrast, T3-repression of IFABP, a differentiation marker of absorptive cells in the both larval and adult epithelia and a well-known T3-down-regulated gene [38], was delayed when DBZ was present (Fig. 4K-4N), suggesting that Notch pathway is also important for the T3-induced larval epithelial degeneration during metamorphosis.

To analyze Notch responsiveness of those genes quantitatively, we performed real-time RT-PCR. After 3 and 5 days of T3 administration, TH-induced up- or down-regulation of all of the genes examined were significantly inhibited by DBZ (Fig. 5). Specifically, the up-regulation of Hairy genes (Fig. 5A, 5B) was markedly reduced by DBZ treatment (Hairy1: 25%-30% of control, Hairy2b: 15% of control), while LGR5 (Fig. 5C) expression was modestly affected (60%-80% of control). The down-regulation of IFABP (Fig. 5D) was also inhibited by DBZ treatment (160%-190% of control). These results suggest that the expression of Hairy genes is dependent on Notch signaling [50] and that Notch singling affects AE cell development.

Notch Signaling Affects the Differentiation of Adult Epithelial Cells During Intestinal Metamorphosis

We first sought to examine the effects of DBZ on adult Ep development in vivo by treating tadpoles with DBZ for longer time. All tadpoles treated with T3 in the presence of DBZ for longer than 5 days died, before the formation of adult Ep. Thus, we performed organ culture experiments by using the intestine isolated from tadpoles at stage 56/57 when T3 levels were still low. The intestinal fragments were pre-cultured with DMSO or 10 μM DBZ in the absence of T3 for 3 days as done for in vivo experiments above (see Fig. 4A). Then, the intestinal fragments were cultured with 20 nM T3 in the presence of DMSO or 10 μM DBZ for 5 or 7 days. We observed up-regulation of Hairy genes by T3 after 5 days and more importantly, the inhibition of the T3-induced up-regulation by DBZ treatment, indicating that both T3 regulation and DBZ inhibition was organ-autonomous (Fig. 6A-6D). To investigate the effects of Notch inhibition on cell proliferation, proliferative activity in the DBZ-treated intestine was compared with that in the control intestine by immunohistochemical analysis of PCNA, a marker for cell proliferation. The percentage of the epithelial cells positive for PCNA was significantly reduced by DBZ treatment (Fig. 6E, 6F; control vs. DBZ = 23.5% ± 0.5% vs. 18.2%±1.2, $p < .01$), whereas that of the CT cells was not significantly different (control vs. DBZ = 21.3%±0.6 vs. 22.3%±0.7, $p = .31$). After 7 days of T3 treatment, when the adult Ep completely replaces the larval one in vitro [42], IFABP was expectedly detected in most of the epithelial cells of the intestine without DBZ treatment (Fig 6G, arrowheads), indicating differentiation to the adult absorptive cells. However, IFABP expression was much weaker in the DBZ treated intestine (Fig. 6H). To determine whether Notch inhibition led to the failure of adult stem cells to differentiate to any adult cell type due to diminished stem cell activity (Figs. 5C, 6F), we performed PAS staining to detect mucus-secreting goblet cells. In the control intestine treated with T3, goblet cells, whose supranuclear region can be stained with PAS [30], were expectedly detected (Fig. 6I), as were those in the intestine of the postmetamorphic froglets at stage 66 (Fig. 6K). In contrast, in the Ep of DBZ and T3-treated intestines, numerous clusters of PAS-positive secretory cells were observed (Fig. 6J). These results indicate that inhibiting Notch signaling prevented differentiation to the absorptive cells while promoting the differentiation to the secretory cells during *X. laevis* adult intestinal development.

Discussion

In the present study, we have provided evidence for active Notch signaling during *X. laevis* intestinal remodeling by analyzing the expression of genes involved in Notch signaling pathway, including its target genes (Hairy1, Hairy2b), ligands (DLL1, DLL3, Jag1, Jag2) and receptor (Notch1). We have shown here that their expression levels peaked during metamorphic climax and that they were up-regulated in response to T3. In particular, Hairy1, but not the other genes analyzed, was significantly up-regulated even after 1 day of T3 treatment. However, Hairy1 may still be indirectly regulated by T3 as reported in the mouse intestine [51], because its up-regulation by T3 was impaired by Notch inhibition. Hairy2b is also considered to be a TH-indirect response gene, since its expression was not up-regulated until 4 days after T3 treatment. Since similar expression profiles were obtained for the ligands and receptor, they may be also TH-indirect response genes. These results suggest that T3 directly activates the expression of certain factor(s) that is responsible for up-regulation of

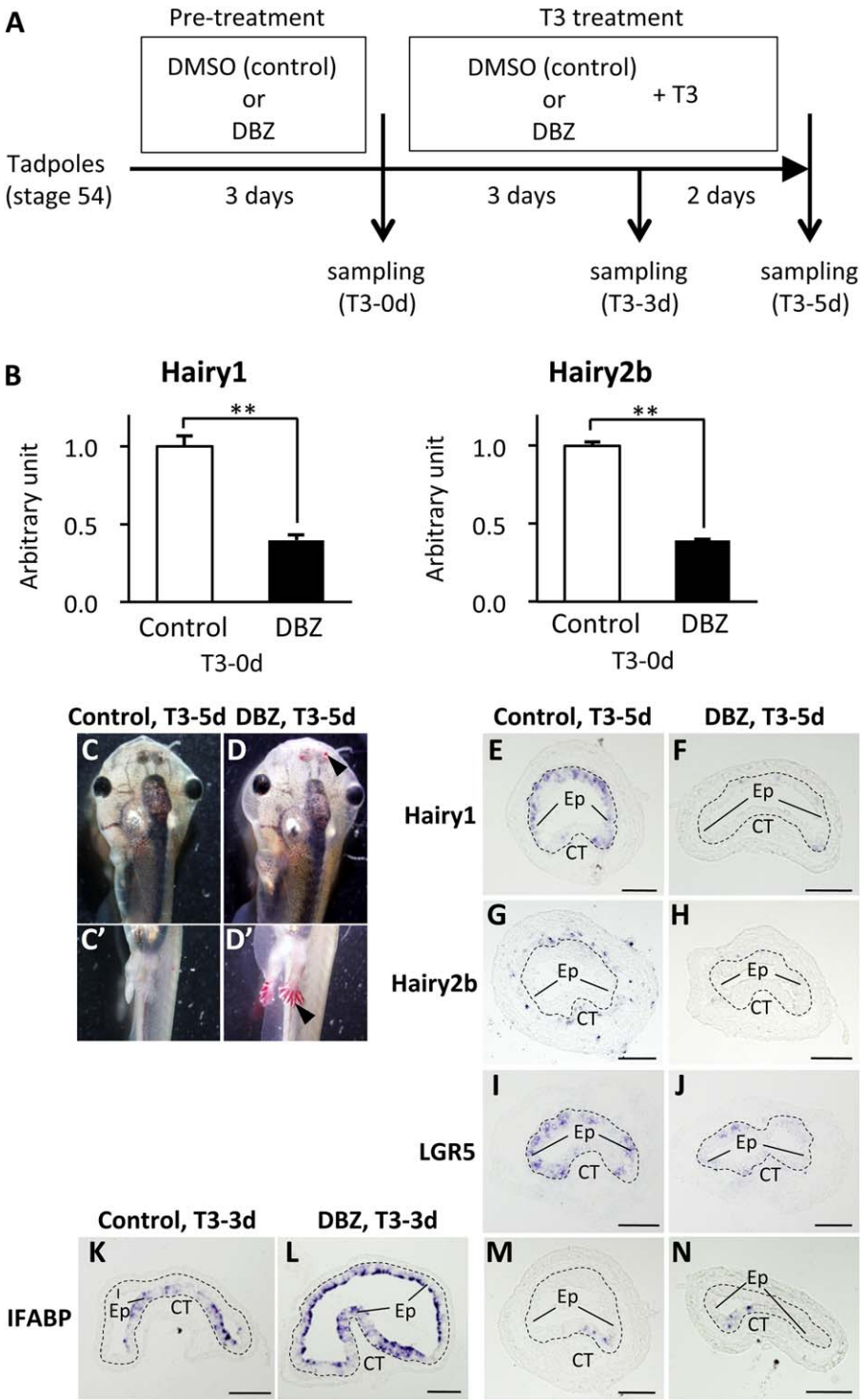

Figure 4. Inhibition of Notch signaling with dibenzazepine (DBZ) inhibits intestinal metamorphosis. (**A**): Schematic diagram of DBZ treatment study. Premetamorphic tadpoles at stage 54 were pretreated with DMSO (control vehicle) or 5 μM DBZ for 3 days. Then, the tadpoles were treated with 10 nM T3 in the presence of DMSO or DBZ. The intestine was isolated after 0, 3, or 5 days of T3 treatment and subjected to RNA extraction or cryosectioning. **(B)**: quantitative real-time RT-PCR revealed that pretreatment with DBZ significantly repressed the expression of Hairy1 and Hairy2b in the intestine of premetamorphic tadpoles. Error bars represent the SEM ($n = 3$). The results were analyzed by Student's *t*-test (**, $p < .01$). (**C-D'**): Gross morphology after 5 days of T3 treatment in the presence of DMSO (C, C') or DBZ (D, D'). Dorsal view of head–trunk region (C, D) and ventral view of hindlimbs (C', D') are shown. Internal hemorrhage around the nose and along the digits of hindlimb is indicated by arrowheads (D, D'). (**E–N**) DBZ treatment delays the down-regulation of IFABP and the formation of the adult intestinal stem cells. Cross-sections of the intestine from the tadpoles treated with T3 for 3 (K, L) or 5 (E–J, M, N) days in the presence of DMSO (E, G, I, K, M) or DBZ (F, H, J, L, N) were hybridized with antisense Hairy1 (E, F), Hairy2b (G, H), LGR5 (I, J), or IFABP (K–N) probes. The dashed-lines indicate the boundary of the epithelium and the connective tissue. Scale bars = 100 μm. Abbreviations: CT, connective tissue, DBZ, dibenzazepine, Ep, epithelium.

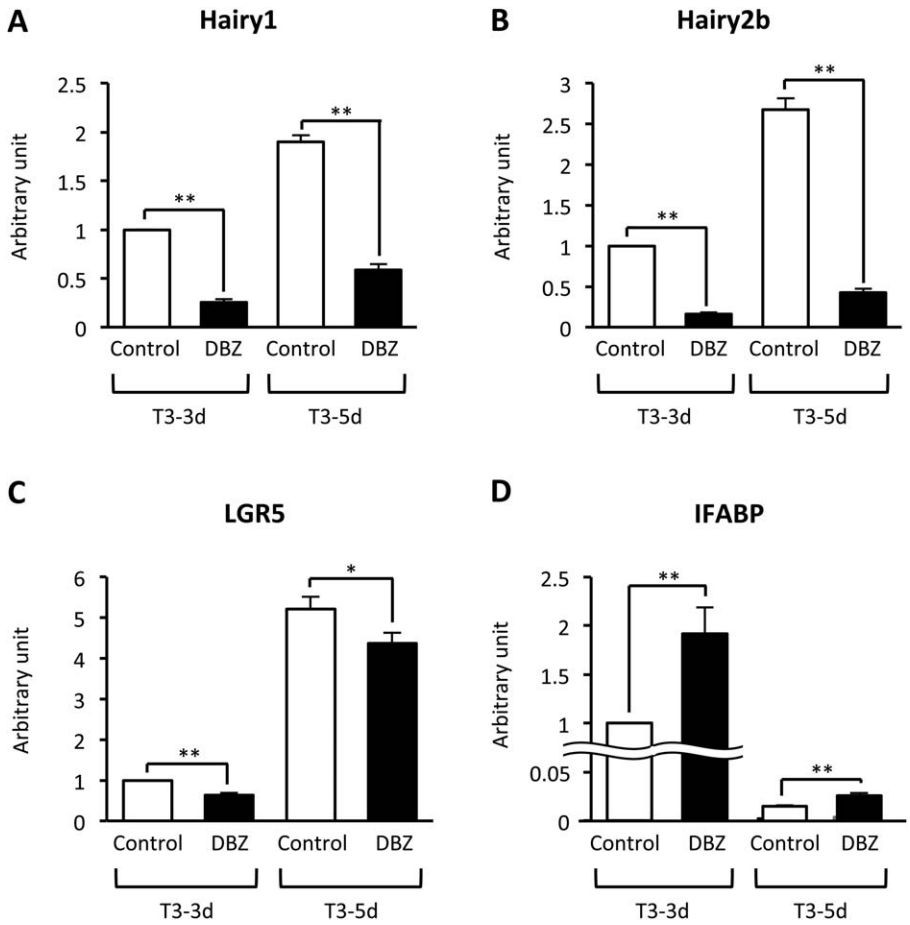

Figure 5. Inhibition of Notch signaling by dibenzazepine (DBZ) blocks the regulation of gene expression by T3. Total RNA was extracted from the intestine of tadpoles treated with T3 for 3 or 5 days in the presence of DMSO or DBZ as shown in Fig. 4A. quantitative real-time RT-PCR was carried out for Hairy1 (**A**), Hairy2b (**B**), LGR5 (**C**), and IFABP (**D**). Error bars represent the SEM ($n = 3$). The results were analyzed by Student's *t*-test (*, $p < .05$; **, $p < .01$). Abbreviation: DBZ, dibenzazepine.

Hairy genes. One such candidate could be the Notch ligand Jag1, which has been shown to be directly regulated by T3 in the mouse intestine [51], although Jag1 is unlikely a direct target of T3 in the *X. laevis* intestine (Fig. 1J). Several signaling pathways may be involved in modulation of the expression levels of Hairy genes [52]. Currently, it still remains unknown which factors other than those involved in Notch signaling directly up-regulate Hairy genes during intestinal remodeling. In addition, the factors that directly control the expression of Notch ligands and receptor in the metamorphosing intestine need to be elucidated.

Inhibition of Notch signaling by DBZ dramatically suppressed TH-induced up-regulation of Hairy genes, confirming their Notch dependency [18–21, 53]. Internal hemorrhage around the nose and along the digits of hindlimb in the DBZ-treated tadpoles may also be suggestive of Notch inhibition in those organs/tissues since it has been shown that loss of Notch1 causes vascular tumors and lethal hemorrhage in mouse [54]. In addition, DBZ treatment of premetamorphic tadpoles led to the repression of both Hairy1 and Hairy2b, suggesting that Notch signaling is already active during premetamorphosis. It is of interest to elucidate the role of this signaling pathway in the premetamorphic intestine in which the cell-renewal system has not yet been acquired. More importantly, TH-induced up-regulation of LGR5 and TH-induced down-regulation of IFABP were also inhibited by DBZ treatment. Furthermore, inhibition of Notch signaling resulted in decreased cell proliferation in the Ep during adult epithelial development. Similar results have also been reported for homeostasis of the adult mouse intestine, where the number of the $Lgr5^+$ intestinal stem cells and their proliferative activity decreased by Notch inhibition [55]. The effects of DBZ on the transcript levels of LGR5 were modest both in mouse [55] and *X. laevis* intestines (Fig. 5C) as compared to the marked repression of Hairy genes. This could be due to the active Wnt signaling that also up-regulates LGR5 expression [32]. Indeed, we have previously shown that TH activates both canonical and noncanonical Wnt signaling pathways that are involved in intestinal remodeling [43, 44]. Our results indicate for the first time that Notch signaling plays pivotal roles in development of adult stem cells during vertebrate intestinal development.

In the adult mouse intestine, Notch inhibition has been shown to affect the cell fate determination in the Ep [23, 30]. Interestingly, during the formation of the adult intestine during *X. laevis* metamorphosis, DBZ treatment led to hyperplasia of the secretory cells and reduction of the absorptive cells, resembling those observed in the adult mouse intestine. These results suggest that the role of Notch signaling in cell fate determination first takes place during the initial formation of the adult intestine and that this effect is likely evolutionarily conserved. It

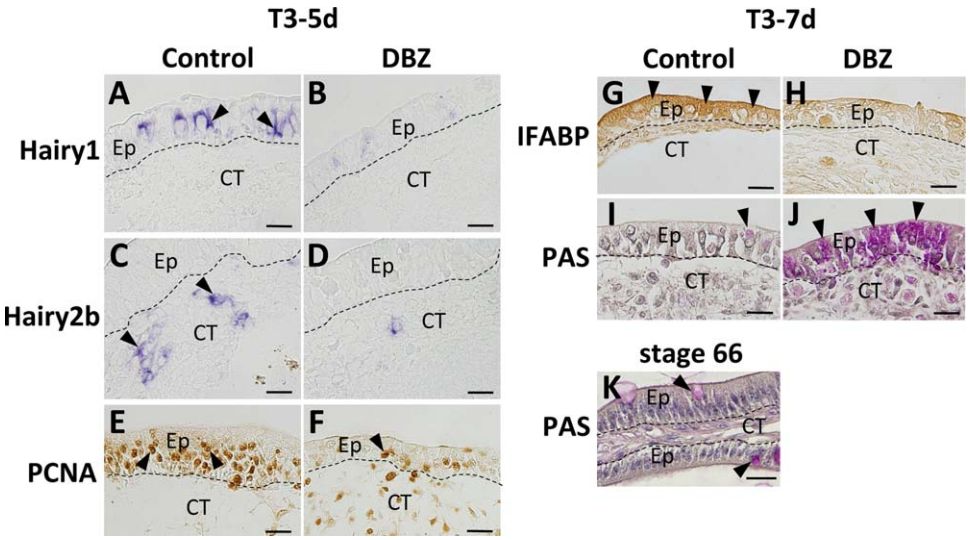

Figure 6. Inhibition of Notch signaling by dibenzazepine (DBZ) leads to decreased cell proliferation and secretory cell hyperplasia. The intestine isolated from stage-56/57 tadpoles was cultured with 20 nM T3 for 5 (**A-F**) or 7 (**G-J**) days in the presence of DMSO (**A, C, E, G, I**) or 10 μM DBZ (**B, D, F, H, J**) after precultivation of the isolated intestinal fragments with DMSO or 10 μM DBZ for 3 days (see Fig. 4A). Cryosections of the intestinal fragments were hybridized with antisense Hairy1 (**A, B**) or Hairy2b (**C, D**). Paraffin sections were incubated with anti-PCNA (**E, F**) or anti-IFABP (**G, H**) antibodies or subjected to Periodic acid-Schiff (PAS) staining followed by counterstaining with hematoxylin (**I, J**). As a control, paraffin sections of the intestine from wild type froglets at stage 66 were also stained with PAS to identify goblet cells (**K**, arrowheads). Note that after 5 days, TH-induced up-regulation of Hairy genes (**A, C**, arrowheads) was impaired by DBZ treatment in vitro (**B, D**). PCNA-positive epithelial cells also decreased by DBZ treatment (**E, F**, arrowheads). After 7 days, IFABP was detected in most of the newly formed adult epithelial cells of the control intestine (**G**, arrowheads), but not the DBZ-treated intestine (**H**). A small number of goblet cell was detected in the control intestine (**I**, arrowhead), whereas PAS-positive secretory cells increased by DBZ treatment (**J**, arrowheads). The dashed-lines indicate the boundary of the epithelium and the connective tissue. Scale bars = 20 μm. Abbreviations: CT, connective tissue, DBZ, dibenzazepine, Ep, epithelium, PAS, Periodic acid-Schiff.

would be interesting to examine the effect of Notch signaling during the maturation of the mouse intestine during postembryonic development, that is, the first 3 weeks after birth when T3 levels peak in mouse.

The ISH analysis of spatiotemporal expression of Hairy genes in the metamorphosing intestine revealed that Hairy1 is an AE/progenitor cell-specific gene, whereas Hairy2b is a CT-specific one, indicating that Notch signaling is activated in both tissues and regulates the expression of downstream genes in a cell/tissue-specific manner during intestinal remodeling. This is also supported by the expression patterns of Notch ligands and receptor. DLL1 and Notch1 were prominently expressed in the AE/progenitor cells, suggesting that DLL1–Notch1 pathway induces Hairy1 expression. It is also suggested that these cells in the islet express both DLL1 and Notch1 to signal each other at metamorphic climax, then become the signal-sending or signal-receiving cells via lateral inhibition to limit the number of stem cells at later stages as proposed in the mammalian intestine [31]. The cells expressing Jag1 were detected in both the larval Ep and CT, but not in the islet, suggesting that at least one of the roles of Jag1 is to induce Hairy2b expression in the CT. In addition, some Jag1-expressing cells were detected just beneath the islet, suggesting that Jag1 may be involved in the AE cell formation and/or proliferation. It is also interesting to study the role of Jag1 in the larval epithelial cells that are destined to undergo apoptosis [46]. It has been reported that Notch1 is weakly expressed in the villus mesenchyme in the mouse intestine at postnatal day 25 and that Notch3 and Notch4 are also expressed in this tissue [56]. Thus, it is possible that the antibody we used could not detect Notch1 expressed at low levels in the CT and/or other Notch receptors are predominantly expressed. It will be important to clarify which type of Notch receptor is expressed in the CT.

The expression pattern of Hairy1 coincides well with that of Hes1 (Hairy1 ortholog) in the adult mouse intestine, in which Hes1 is predominantly expressed in mid to lower crypt cells including the stem cells [56, 57]. More importantly, the lineage-tracing experiments have shown that Hes1 labels intestinal stem cells in the adult mouse [58]. Thus, Hairy1 expression in stem cells appears to be conserved in the vertebrate intestine. Interestingly, we also found that Hairy1 is expressed in a limited number of the larval epithelial cells in the intestine at the onset of metamorphosis (stage 54). Thus, it is tempting to speculate that these cells activate Hairy1 expression at such early stages when T3 levels are low and they in turn are destined to develop into the AE cells expressing high levels of Hairy1 at later stages.

Unlike Hairy1, there is little information available about expression patterns of Hes4 (Hairy2 ortholog) in the mammalian intestine. In other organs/tissues, several reports have shown that Hes4 is expressed in the stem/progenitor cells and is involved in their differentiation, migration, maintenance of undifferentiated state, and so on. [53, 59–62]. Our study here revealed that some cells expressing Hairy2b were located just beneath the adult stem/progenitor cells in the metamorphosing intestine. Thus, it is possible that these cells are involved in the stem cell formation and/or proliferation by functioning and facilitating the formation of stem cell niche. Hairy2b may also have some other roles since not all cells

expressing Hairy2b were located close to the Ep. However, it still remains unclear which type of cells in the CT expresses this gene, because the expression pattern of Hairy2b does not spatiotemporally coincides with that of any other CT-specific genes previously examined [34, 39, 40, 63, 64]. Clearly, it will be important to elucidate the mechanisms of differential regulation of these Hairy genes and more importantly, their functions during TH-dependent adult stem cell development and subsequent epithelial cell fate determination by making use of cell labeling with the Cre/lox system [65], overexpression system [66, 67] and gene knockout technologies such as the CRISPR/Cas9 system [68].

Acknowledgments

This work was supported in part by the Grants-in-Aid for Scientific Research (C) from the Ministry of Education, Science and Culture of Japan (Grant No. 25440160 to T.H. and No. 15K07136 to A.I.-O.), and in part by the intramural Research Program of NICHD, NIH.

Author Contributions

'T.H.: Conception and design, Financial support, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; K.F.: Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; M.K.: Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; L.F.: Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; Y.-B.S.: Conception and design, Financial support, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; A.I.-O.: Conception and design, Financial support, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflict of interest.

References

1 Shi Y-B. Amphibian Metamorphosis: From Morphology to Molecular Biology. New York: John Wiley & Sons, Inc., 1999.
2 Ishizuya-Oka A. Regeneration of the amphibian intestinal epithelium under the control of stem cell niche. Dev Growth Differ 2007;49:99–107.
3 Shi Y-B, Ishizuya-Oka A. Biphasic intestinal development in amphibians: Embryogenesis and remodeling during metamorphosis. Curr Top Dev Biol 1996;32:205–235.
4 Ishizuya-Oka A, Hasebe T, Buchholz DR et al. Origin of the adult intestinal stem cells induced by thyroid hormone in *Xenopus laevis*. FASEB J 2009;23:2568–2575.
5 Tata JR. Gene expression during metamorphosis: An ideal model for post-embryonic development. Bioessays 1993;15:239–248.
6 Shi Y-B. Thyroid hormone-regulated early and late genes during amphibian metamorphosis. In: Gilbert LI, Tata JR, Atkinson BG, eds. Metamorphosis: Postembryonic Reprogamming of Gene Expression in Amphibian and Insect Cells. New York: Academic Press, 1996:505–538.
7 Kress E, Samarut J, Plateroti M. Thyroid hormones and the control of cell proliferation or cell differentiation: Paradox or duality? Mol Cell Endocrinol 2009;313:36–49.
8 Ishizuya-Oka A, Shi Y-B. Thyroid hormone regulation of stem cell development during intestinal remodeling. Mol Cell Endocrinol 2008;288:71–78.
9 Buchholz DR, Paul BD, Fu L et al. Molecular and developmental analyses of thyroid hormone receptor function in *Xenopus laevis*, the African clawed frog. Gen Comp Endocrinol 2006;145:1–19.
10 Lazar MA. Thyroid hormone receptors: Multiple forms, multiple possibilities. Endocr Rev 1993;14:184–193.
11 Shi Y-B, Matsuura K, Fujimoto K et al. Thyroid hormone receptor actions on transcription in amphibia: The roles of histone modification and chromatin disruption. Cell Biosci 2012;2:42.
12 Sun G, Fu L, Shi Y-B. Epigenetic regulation of thyroid hormone-induced adult intestinal stem cell development during anuran metamorphosis. Cell Biosci 2014;4:73.
13 Tsai MJ, O'malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994;63:451–486.
14 Yen PM. Physiological and molecular basis of thyroid hormone action. Physiol Rev 2001;81:1097–1142.
15 Buchholz DR, Heimeier RA, Das B et al. Pairing morphology with gene expression in thyroid hormone-induced intestinal remodeling and identification of a core set of TH-induced genes across tadpole tissues. Dev Biol 2007;303:576–590.
16 Shi Y-B, Brown DD. The earliest changes in gene expression in tadpole intestine induced by thyroid hormone. J Biol Chem 1993;268:20312–20317.
17 Sun G, Heimeier RA, Fu L et al. Expression profiling of intestinal tissues implicates tissue-specific genes and pathways essential for thyroid hormone-induced adult stem cell development. Endocrinology 2013;154:4396–4407.
18 Kopan R, Ilagan MX. The canonical Notch signaling pathway: Unfolding the activation mechanism. Cell 2009;137:216–233.
19 Castel D, Mourikis P, Bartels SJ et al. Dynamic binding of RBPJ is determined by Notch signaling status. Genes Dev 2013;27:1059–1071.
20 van Tetering G, Vooijs M. Proteolytic cleavage of Notch: "HIT and RUN". Curr Mol Med 2011;11:255–269.
21 Li Y, Hibbs MA, Gard AL et al. Genome-wide analysis of N1ICD/RBPJ targets in vivo reveals direct transcriptional regulation of Wnt, SHH, and hippo pathway effectors by Notch1. Stem Cells 2012;30:741–752.
22 van Es JH, van Gijn ME, Riccio O et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 2005;435:959–963.
23 Fre S, Huyghe M, Mourikis P et al. Notch signals control the fate of immature progenitor cells in the intestine. Nature 2005;435:964–968.
24 Jensen J, Pedersen EE, Galante P et al. Control of endodermal endocrine development by Hes-1. Nat Genet 2000;24:36–44.
25 Schwanbeck R, Just U. The Notch signaling pathway in hematopoiesis and hematologic malignancies. Haematologica 2011;96:1735–1737.
26 Louvi A, Artavanis-Tsakonas S. Notch signalling in vertebrate neural development. Nat Rev Neurosci 2006;7:93–102.
27 Wahi K, Bochter MS, Cole SE. The many roles of Notch signaling during vertebrate somitogenesis. Semin Cell Dev Biol 2016;49:68–75.
28 Chuang JH, Tung LC, Lin Y. Neural differentiation from embryonic stem cells in vitro: An overview of the signaling pathways. World J Stem Cells 2015;7:437–447.
29 Milano J, McKay J, Dagenais C et al. Modulation of Notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. Toxicol Sci 2004;82:341–358.
30 van Es JH, de Geest N, van de Born M et al. Intestinal stem cells lacking the Math1 tumour suppressor are refractory to Notch inhibitors. Nat Commun 2010;1:18.
31 Stamataki D, Holder M, Hodgetts C et al. Delta1 expression, cell cycle exit, and commitment to a specific secretory fate coincide within a few hours in the mouse intestinal stem cell system. PLoS One 2011;6:e24484.
32 Barker N, van Es JH, Kuipers J et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 2007;449:1003–1007.
33 Sun G, Hasebe T, Fujimoto K et al. Spatio-temporal expression profile of stem cell-associated gene LGR5 in the intestine during thyroid hormone-dependent metamorphosis in *Xenopus laevis*. PLoS One 2010;5:e13605.
34 Hasebe T, Kajita M, Fujimoto K et al. Expression profiles of the duplicated matrix metalloproteinase-9 genes suggest their

different roles in apoptosis of larval intestinal epithelial cells during *Xenopus laevis* metamorphosis. Dev Dyn 2007;236:2338–2345.
35 Nieuwkoop PD, Faber J. Normal Table of Xenopus Laevis (Daudin). New York: Garland Publishing Inc, 1994.
36 Karpinka JB, Fortriede JD, Burns KA et al. Xenbase, the *Xenopus* model organism database; new virtualized system, data types and genomes. Nucleic Acids Res 2015;43: D756–D763.
37 Shi Y-B, Liang VC. Cloning and characterization of the ribosomal protein L8 gene from *Xenopus laevis*. Biochim Biophys Acta 1994;1217:227–228.
38 Shi Y-B, Hayes WP. Thyroid hormone-dependent regulation of the intestinal fatty acid-binding protein gene during amphibian metamorphosis. Dev Biol 1994;161:48–58.
39 Hasebe T, Hartman R, Matsuda H et al. Spatial and temporal expression profiles suggest the involvement of gelatinase A and membrane type 1 matrix metalloproteinase in amphibian metamorphosis. Cell Tissue Res 2006;324:105–116.
40 Hasebe T, Kajita M, Fu L et al. Thyroid hormone-induced sonic hedgehog signal upregulates its own pathway in a paracrine manner in the *Xenopus laevis* intestine during metamorphosis. Dev Dyn 2012;241:403–414.
41 Ishizuya-Oka A, Shimozawa A. Induction of metamorphosis by thyroid hormone in anuran small intestine cultured organotypically in vitro. In Vitro Cell Dev Biol 1991;27A: 853–857.
42 Ishizuya-Oka A, Ueda S, Damjanovski S et al. Anteroposterior gradient of epithelial transformation during amphibian intestinal remodeling: Immunohistochemical detection of intestinal fatty acid-binding protein. Dev Biol 1997;192:149–161.
43 Ishizuya-Oka A, Kajita M, Hasebe T. Thyroid hormone-regulated Wnt5a/Ror2 signaling is essential for dedifferentiation of larval epithelial cells into adult stem cells in the *Xenopus laevis* intestine. PLoS One 2014;9: e107611.
44 Hasebe T, Fujimoto K, Kajita M et al. Thyroid hormone activates Wnt/beta-catenin signaling involved in adult epithelial development during intestinal remodeling in *Xenopus laevis*. Cell Tissue Res 2016;365:309–318.
45 Hasebe T, Buchholz DR, Shi Y-B et al. Epithelial-connective tissue interactions induced by thyroid hormone receptor are essential for adult stem cell development in the *Xenopus laevis* intestine. Stem Cells 2011; 29:154–161.
46 Ishizuya-Oka A, Ueda S. Apoptosis and cell proliferation in the *Xenopus* small intestine during metamorphosis. Cell Tissue Res 1996;286:467–476.
47 McAvoy JW, Dixon KE. Cell proliferation and renewal in the small intestinal epithelium of metamorphosing and adult *Xenopus laevis*. J Exp Zool 1977;202:129–138.
48 Bjerknes M, Cheng H. The stem-cell zone of the small intestinal epithelium. III. Evidence from columnar, enteroendocrine, and mucous cells in the adult mouse. Am J Anat 1981;160:77–91.
49 Cheng H, Bjerknes M. Whole population cell kinetics and postnatal development of the mouse intestinal epithelium. Anat Rec 1985;211:420–426.
50 Davis RL, Turner DL. Vertebrate hairy and Enhancer of split related proteins: Transcriptional repressors regulating cellular differentiation and embryonic patterning. Oncogene 2001;20:8342–8357.
51 Sirakov M, Boussouar A, Kress E et al. The thyroid hormone nuclear receptor TRa1 controls the Notch signaling pathway and cell fate in murine intestine. Development 2015; 142:2764–2774.
52 Liu ZH, Dai XM, Du B. Hes1: A key role in stemness, metastasis and multidrug resistance. Cancer Biol Ther 2015;16:353–359.
53 Vega-Lopez GA, Bonano M, Tribulo C et al. Functional analysis of Hairy genes in *Xenopus* neural crest initial specification and cell migration. Dev Dyn 2015;244:988–1013.
54 Liu Z, Turkoz A, Jackson EN et al. Notch1 loss of heterozygosity causes vascular tumors and lethal hemorrhage in mice. J Clin Invest 2011;121:800–808.
55 VanDussen KL, Carulli AJ, Keeley TM et al. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development 2012;139: 488–497.
56 Schröder N, Gossler A. Expression of Notch pathway components in fetal and adult mouse small intestine. Gene Expr Patterns 2002;2:247–250.
57 Kayahara T, Sawada M, Takaishi S et al. Candidate markers for stem and early progenitor cells, Musashi-1 and Hes1, are expressed in crypt base columnar cells of mouse small intestine. FEBS Lett 2003;535: 131–135.
58 Kopinke D, Brailsford M, Shea JE et al. Lineage tracing reveals the dynamic contribution of Hes1+ cells to the developing and adult pancreas. Development 2011;138:431–441.
59 Cakouros D, Isenmann S, Hemming SE et al. Novel basic helix-loop-helix transcription factor hes4 antagonizes the function of twist-1 to regulate lineage commitment of bone marrow stromal/stem cells. Stem Cells Dev 2015;24:1297–1308.
60 El Yakoubi W, Borday C, Hamdache J et al. Hes4 controls proliferative properties of neural stem cells during retinal ontogenesis. Stem Cells 2012;30:2784–2795.
61 Nagatomo K, Hashimoto C. *Xenopus* hairy2 functions in neural crest formation by maintaining cells in a mitotic and undifferentiated state. Dev Dyn 2007;236:1475–1483.
62 Nichane M, Ren X, Souopgui J et al. Hairy2 functions through both DNA-binding and non DNA-binding mechanisms at the neural plate border in *Xenopus*. Dev Biol 2008;322:368–380.
63 Hasebe T, Kajita M, Shi Y-B et al. Thyroid hormone-up-regulated hedgehog interacting protein is involved in larval-to-adult intestinal remodeling by regulating sonic hedgehog signaling pathway in *Xenopus laevis*. Dev Dyn 2008;237:3006–3015.
64 Ishizuya-Oka A, Ueda S, Shi Y-B. Transient expression of stromelysin-3 mRNA in the amphibian small intestine during metamorphosis. Cell Tissue Res 1996;283:325–329.
65 Rankin SA, Hasebe T, Zorn AM et al. Improved cre reporter transgenic *Xenopus*. Dev Dyn 2009;238:2401–2408.
66 Fu L, Buchholz D, Shi Y-B. Novel double promoter approach for identification of transgenic animals: A tool for in vivo analysis of gene function and development of gene-based therapies. Mol Reprod Dev 2002;62: 470–476.
67 Rankin SA, Zorn AM, Buchholz DR. New doxycycline-inducible transgenic lines in *Xenopus*. Dev Dyn 2011;240:1467–1474.
68 Wang F, Shi Z, Cui Y et al. Targeted gene disruption in *Xenopus laevis* using CRISPR/Cas9. Cell Biosci 2015;5:15.