A Positive Regulatory Loop between foxi3a and foxi3b Is Essential for Specification and Differentiation of Zebrafish Epidermal Ionocytes

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Background. Epidermal ionocytes play essential roles in the transepithelial transportation of ions, water, and acid-base balance in fish embryos before their branchial counterparts are fully functional. However, the mechanism controlling epidermal ionocyte specification and differentiation remains unknown. Methodology/Principal Findings. In zebrafish, we demonstrated that Delta-Notch-mediated lateral inhibition plays a vital role in singling out epidermal ionocyte progenitors from epidermal stem cells. The entire epidermal ionocyte domain of genetic mutants and morphants, which failed to transmit the Delta-C-Notch1a/Notch3 signal from sending cells (epidermal ionocytes) to receiving cells (epidermal stem cells), differentiates into epidermal ionocytes. The low Notch activity in epidermal ionocyte progenitors is permissive for activating winged helix/forkhead box transcription factors of foxi3a and foxi3b. Through gain- and loss-of-function assays, we show that the foxi3a-foxi3b regulatory loop functions as a master regulator to mediate a dual role of specifying epidermal ionocyte progenitors as well as of subsequently promoting differentiation of Na+,K+-ATPase-rich cells and H+-ATPase-rich cells in a concentration-dependent manner. Conclusions/Significance. This study provides a framework to show the molecular mechanism controlling epidermal ionocyte specification and differentiation in a low vertebrate for the first time. We propose that the positive regulatory loop between foxi3a and foxi3b not only drives early ionocyte differentiation but also prevents the complete blockage of ionocyte differentiation when the master regulator of fox3 function is unilaterally compromised.

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INTRODUCTION

In terrestrial vertebrates, diverse types of ionocytes (ICs) that are distributed in the bladder, inner ear, and kidney have evolved to play essential roles in transepithelial ion, water, and acid-base transportation. For instance, ICs specializing in mediating salt and water absorption (principal cells), acid-base transport (intercalated cells), or both (inner medullary cells) have been characterized in collecting ducts of mouse kidneys [1,2]. In the mouse inner ear, forkhead-related (FORE) cells are also reported to play an essential role in maintaining osmotic pressure and the ionic composition [3]. For aquatic vertebrates like fish, the maintenance of osmotic and ionic gradients between body fluids and the external environment for internal homeostasis are more challenging. Fish gills have evolved as the most important extra-renal organ to mediate ion transport and acid-base regulation for compensating the passive leakage of ions [4,5]. At a cellular level, the functional units of ion and acid-base regulation in gills are carried out by branchial ICs (also known as chloride cells or mitochondrion-rich cells) which are primarily deposited on the junction regions between filaments and lamellae. At an organismic level, when the functional gill is not yet completely developed, epidermal ICs are precociously scattered on the skin to mediate ion and acid-base balance [6,7,8]. Based on morphology, vital dye binding and immunocytochemistry, distinct types of epidermal or branchial ICs have been reported in diverse fish species, and have been suggested to mediate an equivalent function as their counterparts in the kidneys of terrestrial vertebrates [9,10]. However, very little is known about whether distinct types of ICs are generated from common progenitors and what the molecular mechanisms controlling IC specification and differentiation are. We noted that the slow progress of studying IC development in aquatic animals is largely caused by insufficient molecular and genetic tools for non-model fish. Therefore, we sought to use zebrafish as a new model to study IC development by taking advantage of a mature embryonic manipulation technique as well as many available genetic mutants [11,12,13]. Recently, our team identified two types of epidermal ICs as Na+,K+-ATPase-rich cells (NaRCS) and H+-ATPase-rich cells (HRCs) in zebrafish embryonic skin [14]. In this study, we extend our knowledge to the molecular mechanisms determining IC identity and promoting the differentiation of NaRCS and HRCs in zebrafish.

The Delta-Notch (D-N) lateral inhibition mechanism is an evolutionarily conserved signal transduction cascade and plays essential roles in cell fate choices and boundary formation [15]. Taking Drosophila sensory organ formation as an example, the position of the sensory organ precursor (SOP) is determined by the basic helix-loop-helix (bHLH) transcription factors of the Achaete-Scute complex (AS-C) [16]. Within the SOP cluster, a nascent SOP expresses cell surface DSL (Delta, Serrate, Lag-2) ligands, NaRCs and HRCs in zebrafish.
and activates Notch in neighboring cells. Activation of the Notch pathway results in proteolytic cleavage to release the intracellular domain of Notch (NICD), which is subsequently translocated into the nucleus and is associated with CSL (CBF1/Stabilizing/Lag-1, also known as RBPj) proteins to activate target genes, such as the Hairy/Enhancer of Split (HES) family of bHLH transcriptional repressors (also known as bHLH-O). Activated bHLH-O inhibits the activity of AS-C genes in Notch signal-receiving cells (epidermal cells) and also prevents these cells from differentiating into SOPs [17,18]. Failure to transmit the Delta-Notch lateral inhibition signal results in overproduction of SOPs in <i>Drosophila</i> [19]. In zebrafish embryos, the epidermal ICs are surrounded by keratinocytes which are arranged in a mosaic pattern. This observation suggests that epidermal IC cell fate choice might also be mediated by the Delta-Notch lateral inhibition mechanism. We tested this hypothesis by examining the epidermal IC development in several genetic mutants or morphants which have defects in the Delta-Notch signaling pathway. Results demonstrated that Delta-Notch-mediated lateral inhibition indeed plays a role in selecting epidermal IC progenitors from the epidermal stem cell (SC) pool in zebrafish.

Winged helix/forkhead box transcription factors encode a large class of nuclear DNA-binding factors and function as cell fate determinants or cell differentiation regulators [20,21,22,23,24]. Recent studies have shown that IC differentiation in the mammalian inner ear and kidney is controlled by a strikingly conserved fox1-dependent mechanism. Gene knockout experiments demonstrated that in the absence of <i>fox1</i>, IC progenitors in the endolymphatic duct and collecting duct fail to differentiate into FORE cells and intercalated cells which respectively resulted in deafness [3] or distal renal tubular acidosis [25]. This <i>fox1</i>-mediated process of IC differentiation in the inner ear and urinary system of terrestrial vertebrates inspired us to postulate whether a similar mechanism is also utilized by aquatic vertebrates for controlling epidermal IC differentiation. In zebrafish, four members of <i>fox</i> genes have been identified as being expressed in the inner ear and pharyngeal arch (<i>fox1</i>) [26], in the chordamesoderm, retina, and pharyngeal arches (<i>fox2</i>) [27], and in mucous cells (<i>fox3a</i> and <i>fox3b</i>, we have renamed <i>fox3a</i>- and <i>fox3b</i>-expressing cells as epidermal ICs in this study) [27]. In this study, we manipulate the function of <i>fox3a</i> and <i>fox3b</i> by morpholino knockdown and mRNA misexpression to test whether they have a function in epidermal IC development. The data obtained from gain- and loss-of-function assays led us to discover a positive regulatory loop between <i>fox3a</i> and <i>fox3b</i> which is essential not only for acquiring IC identity but also for promoting IC differentiation.

RESULTS

<i>Na</i><sup>+</i>,<i>K</i><sup>+</i>-ATPase-rich Cells (NaRCs) Differentiate Earlier than <i>H</i><sup>+</i>-ATPase-rich Cells (HRCs)

We have previously characterized two distinct populations of epidermal ICs, NaRCs and HRCs, in 3-day post-fertilization (dpf) zebrafish embryos which respectively expressed <i>Na</i><sup>+</i>,<i>K</i><sup>+</i>-ATPase and <i>H</i><sup>+</i>-ATPase in a mutually exclusive manner [14]. However, we failed to detect the early ontogeny of NaRCs and HRCs due to the low concentration of <i>Na</i><sup>+</i>,<i>K</i><sup>+</i>-ATPase and <i>H</i><sup>+</i>-ATPase which accumulated in epidermal ICs of embryos younger than 36 hours post-fertilization (hpf) (data not shown), indicating the difficulty in certifying the lineage relationships between NaRCs and HRCs by immunocytochemistry. To overcome this constraint, we tested several NaRC markers: the <i>Na</i><sup>+</i>,<i>K</i><sup>+</i>-ATPase z1a.2 subunit (<i>atp1a1a.2</i>), <i>Na</i><sup>+</i>,<i>K</i><sup>+</i>-ATPase z1a.4 subunit (<i>atp1a1a.4</i>), <i>Na</i><sup>+</i>,<i>K</i><sup>+</</sup>-ATPase z1a.5 subunit (<i>atp1a1a.5</i>), Na,<i>K</i><sup>+</i>-ATPase β1b subunit (<i>atp1b1b</i>), N-myc downstream-regulated gene 1 (ndrg1), and potassium inwardly-rectifying channel, subfamily J, member 1 (<i>kcnj1</i>), as well as HRC markers: the <i>H</i><sup>+</i>-ATPase V<sub>0c</sub> subunit (<i>atp6veo</i>), type II arginase (arg2), transient receptor potential cation channel, subfamily M, member 7 (tprpm7), and carbonic anhydrase 2a (ca2a) by in situ hybridization. Among these epidermal IC markers, <i>atp1b1b</i> and ca2a were identified as being robust and early markers for labeling NaRCs and HRCs, respectively (<i>atp1b1b</i> is also weakly expressed in the differentiating HRC, see below for discussion). By performing fluorescent double in situ hybridization, we found that <i>atp1b1b</i> was expressed on epidermal ICs with a sporadic pattern as early as 14 hpf (data not shown). By the 1B- somite (1B-s) stage, ca2a was detected in about 10% of <i>atp1b1b</i>-positive cells (Figure 1A, highlighted by asterisks). By 24 hpf, the differentiating NaRCs and HRCs had gradually spread out and were scattered on the epidermal layer in a pattern similar to those detected by antibody staining in 3-dpf embryos [14]; differentiating HRCs were largely distributed on the yolk sac and extension regions, while differentiating NaRCs were more widely scattered on the epidermis (Figure 1B). Interestingly, neither NaRCs nor HRCs ever appeared on the cephalic epidermis (Figure 1A and 1B). For 24-hpf embryos, the differentiating HRCs expressed a balanced level of <i>atp1b1b</i> and ca2a (Figure 1B). However, as development proceeded, the <i>atp1b1b</i> level in differentiating HRCs was gradually downregulated and maintained at a basal expression level by 72 hpf (Figure 1C and 1D, labeled by asterisks). Differentiating NaRCs, on the contrary, strongly expressed <i>atp1b1b</i> but lacked ca2a expression. This observation suggests that both NaRCs (<i>atp1b1b</i>-positive cells) and HRCs (<i>ca2a</i>-positive cells) might come from common progenitors and then subsequently differentiate.

<i>fox3a</i> and <i>fox3b</i> are Early Markers for the Epidermal IC Progenitors

To test the common progenitor hypothesis, we needed a molecular marker to trace the early epidermal IC development before the onset of differentiation. <i>fox3a</i> and <i>fox3b</i> are duplicated winged helix/forkhead transcription factors and are sporadically expressed on the epidermis at the gastrula stage [27]. According to fluorescent double in situ hybridization, <i>fox3a</i> was expressed within a subdomain (hereafter, defined as the epidermal IC domain) of the ventral ectoderm at around the 90% epiboly to tail bud (tb) stages (Figure 2A). By the 5-s stage, <i>fox3b</i> was rapidly upregulated in the majority of <i>fox3a</i>-positive cells and resulted in a mixed population of cells with either <i>fox3b</i><sub>low</sub> (red), <i>fox3a</i><sub>low</sub>/<i>fox3b</i><sub>low</sub> (yellow), or <i>fox3a</i><sub>high</sub>/ <i>fox3b</i><sub>low</sub> (light green) expression (Figure 1E). By the 14-s stage, <i>fox3b</i> expression in <i>fox3a</i>-positive cells was sharply upregulated, resulting in more cells showing <i>fox3a</i><sub>low</sub>/ <i>fox3b</i><sub>high</sub> (green) expression (Figure 1F). It is worth noting that by the 14-s stage, <i>fox3a</i> expression in some <i>fox3a</i><sub>low</sub>/ <i>fox3b</i><sub>low</sub> cells was sharply downregulated to a very low level, suggesting that these cells were differentiating into different cell fates. From the 18-s stage onwards, the differentiation process may have been completed since the majority of cells expressed either <i>fox3b</i><sub>high</sub>/ <i>fox3b</i><sub>low</sub> (Figure 1G and 1H). By 24 hpf, we noted that the spatial distribution patterns of <i>fox3a</i> and <i>fox3b</i> were highly similar to those of HRCs and NaRCs (compare Figure 1B with 1H or [14]). The comparable distribution pattern persisted until at least 72 hpf (Figure 1I and J). <i>fox3a</i>-positive cells had a restricted distribution on the yolk and the yolk extension region (similar to the ca2a expression pattern); while <i>fox3b</i>-positive cells were widely scattered on the epidermis (similar to the <i>atp1b1b</i> expression pattern). In addition, we also noted that the colocalized
expressions of fox3a and fox3b (fox3a\textsuperscript{high}/fox3b\textsuperscript{low}) in the yolk extension region (Figure 1K) were highly similar to those of ca2a and atp1b1b (Figure 1C). To clarify the identity of fox3a-positive and fox3b-positive cells, we conducted triple-labeling with a fox3a or fox3b riboprobe together with NaRC- (anti-Na\textsuperscript{+},K\textsuperscript{+}-ATPase) and HRC-specific (anti-H\textsuperscript{+}-ATPase) antibodies on 72-hpf embryos. The results showed that fox3a was only expressed in HRCs (red arrow), while fox3b was expressed by both HRCs (red arrow) and NaRCs (green arrow). (N) Schematic diagram showing the major events of epidermal ionocyte development in zebrafish embryos at the progenitor stage (the 90% epiboly to 14-s stages), differentiation stage (the 14-s stage to 36 hpf), and maturation stage (from 36 hpf onwards). The developmental stage is indicated in the left lower corner of each panel.

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expression of fox3a and fox3b was co-expressed in a sub-population of P63-positive cells at the tb stage (Figure 2A, labeled with asterisks).

Lateral Inhibition Plays a Role in Restricting the Number of Epidermal IC Progenitors

In zebrafish embryos, fox3a-positive epidermal IC progenitors appear on the IC domain as early as the 90% epiboly stage. By examining the expressions of fox3a and epidermal IC progenitors (see Figure S1 for details), we categorized three major development events of epidermal ICs in zebrafish as the progenitor stage (from the 90% epiboly to the 14-s stage), differentiation stage (from the 14-s stage to 36 hpf), and maturation stage (from 36 hpf onwards). According to the successive expression of the progenitor markers (fox3a and fox3b) as well as differentiation markers (atp1b1b and ca2a), we proposed that NaRCs and HRCs may be generated from common progenitors by the differentially regulated expressions of fox3a and fox3b (summarized in Figure 1N).
by asterisks). However, from the tb stage onwards, nuclear P63 staining in foxi3a-positive IC progenitors was gradually down-regulated and eventually reached an undetectable level by 24 hpf (Figure 2B and 2C). This observation suggests that epidermal IC progenitors may be generated from the epidermal SC. We were interested in the molecular mechanism controlling the cell fate choice between epidermal ICs and epidermal SCs. Based on the mosaic pattern of epidermal ICs and epidermal SCs (Figure 2C), we speculated that a D-N-mediated lateral inhibition mechanism may be involved. To test this hypothesis, we initially treated embryos with the γ-secretase inhibitor, DAPT, to inhibit Notch proteolysis, nuclear translocation, and signaling [29]. The DAPT-treated or control embryos were then fixed at the tb stage, and the foxi3a expression area was quantified by imaging software (see Materials and Methods). By comparing the sporic pattern in wild-types (Figure 2D) or the DMSO sham control (data not shown), it was noted that foxi3a expression in DAPT-treated embryos had become about 2.2-fold denser and more homogeneous (Figure 2E; also summarized in Table 1). This result suggests that Notch signaling might play a role in regulating the IC progenitors in an antimorphic allele of mibta52b (mibta52b), which fails in lateral inhibition due to widely compromised Notch signaling and results in premature differentiation of neural progenitors and neuron mass-production [30,31]. We also detected about 2.3-fold denser foxi3a expression in mibta52b mutants (Figure 2G; Table 1) than siblings. In addition, in both DAPT-treated and mibta52b embryos, loss of Notch activity was confirmed by desynchronized deltaC expression in somite stripes (data not shown). Therefore, we confirmed that Notch signaling indeed plays a role in restricting the number of epidermal IC progenitors in zebrafish embryos.

Whether epidermal ICs behave as D-N signal-sending cells or receiving cells was unknown. We attempted to determine this by manipulating the Suppressor of Hairless [Su(H)] activity with mRNAs encoding either the dominant-active form of X-Su(H)/Ank or the dominant-negative form of X-Su(H)/Ank mRNA-injected embryos (F), foxi3a expression in the epidermal IC progenitor domain of mibta52b-positive epidermal IC progenitors was sharply reduced in X-Su(H)/Ank mRNA-injected embryos (F), while X-Su(H)/Ank mRNA-injected embryos (G) or X-Su(H)DBM mRNA-injected embryos (H), foxi3a expression in the epidermal IC progenitor domain is greatly reduced. The developmental stage is indicated in the left lower corner of each panel.

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Figure 2. Number of Epidermal Ionocyte Progenitors is Restricted by Delta-Notch-Mediated Lateral Inhibition. (A–C) Dynamic expression of the epidermal ionocyte progenitor marker of foxi3a (black, cytoplasmic staining) and the epidermal stem cell marker of P63 (green, nuclear staining) on the zebrafish epidermis. In the beginning, foxi3a was activated in some P63-positive epidermal stem cells at the tail bud (tb) stage (indicated by asterisks, A). However, as development precedes, P63 expression in epidermal ionocyte progenitors is sharply down-regulated (at the 14-somite (14-s) stage, B) ultimately to an undetectable level (24 hour post infection (hpf), C). (D–I) The mosaic distribution of epidermal ionocyte progenitors within the epidermal ionocyte domain is controlled by Delta-Notch-mediated lateral inhibition. In DAPT-treated embryos (F), and mibta52b, (G) or X-Su(H)DBM mRNA-injected embryos (H), the foxi3a expression in the epidermal ionocyte domain has become denser and more homogeneous. While in X-Su(H)/Ank mRNA-injected embryos (I), foxi3a expression in the epidermal ionocyte domain is greatly reduced. The developmental stage is indicated in the left lower corner of each panel.

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Table 1. Quantitative measurement of foxi3a or deltaC expression in ventral ectoderm of treated embryos aged at tail bud stage

| Treatment                  | foxi3a-area (μm²) | N  | deltaC-area (μm²) | N  |
|----------------------------|-------------------|----|-------------------|----|
| wild types                 | 48490±9499        | 17 | 18321±7866        | 17 |
| GFP RNA                    | 48733±10014       | 17 | NA                | NA |
| DAPT                       | 105347±11502 *    | 27 | NA                | NA |
| mibta52b                   | 113311±15815 *    | 10 | 105268±20847 *    | 10 |
| X-Su(H)DBM RNA             | 99793±17659 *     | 13 | 71457±19542 *     | 13 |
| X-Su(H)/Ank RNA            | 1942±2559 *       | 18 | 27±74 *           | 18 |
| bet3a2f4                     | 120642±23994 *    | 10 | 100661±28423 *    | 10 |
| bed3a12b                     | 113259±6829 *     | 10 | 103544±11996 *    | 10 |
| desth35b                    | 68249±8898 *      | 16 | 68046±20224 *     | 16 |
| notch1b MO                  | 44990±5257        | 21 | NA                | NA |
| notch1a MO+desth35b         | 63949±10181 *     | 10 | NA                | NA |
| notch1 MO+desmibta52b       | 106885±13361 *    | 17 | NA                | NA |
| notch1a ICD RNA            | 2174±2750 *       | 23 | 2008±1542 *       | 20 |
| notch1a ICD RNA            | 878±1974 *        | 11 | NA                | NA |

N, number of embryos scored. NA, not assayed. The values are shown as means±S.D. * significant difference (Student’s t-test, p<0.05) comparing with wild types.

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should be other mechanisms which delineate the foxi3a-positive epidermal IC domain first, and the D-N-mediated lateral inhibition subsequently functions within this domain to single out epidermal IC progenitors from epidermal SCs.

The Excessive Epidermal IC Phenotype in mibb\textsuperscript{a52b} Mutants is at the Expanse of Epidermal SCs

The excessive epidermal IC phenotype observed in either mibb\textsuperscript{a52b}, DAPT-treated, or X-Su(H)\textsubscript{DBM} mRNA-injected embryos might be generated by excessive cell division or by the cell fate choice. We examined cell division of foxi3a-stained embryos using phospho-histone 3 (pH3) antibody staining. Within the epidermal IC domain, we counted the number of foxi3a-positive epidermal ICs in mibb\textsuperscript{a52b} mutants and found that it was about 2-fold higher than that of its siblings (151 ± 6 vs. 75 ± 6, n = 7, p < 0.05). However, the proliferation activity in whole embryos (28 ± 4 vs. 33 ± 8, n = 7) or in epidermal IC progenitors (7 ± 0 vs. 5 ± 2, n = 7) showed no significant difference between mibb\textsuperscript{a52b} and sibling embryos (Figure 3A–3C). Therefore, it is unlikely that the excessive epidermal IC phenotype observed in mibb\textsuperscript{a52b} mutants is generated by an excess of cell proliferation. Is it due to an aberrant cell fate choice? By counting the number of epidermal ICs (including both NaRCs and HRCs) and epidermal SCs (by P63 antibody staining) at 24 hpf, we detected significantly higher epidermal ICs (NaRCs, 41 ± 5 vs. 28 ± 5, n = 7, p < 0.05; HRCs, 88 ± 8 vs. 21 ± 3, n = 7, p < 0.05) and lower epidermal SCs (135 ± 8 vs. 212 ± 11, n = 7, p < 0.05) in mibb\textsuperscript{a52b} mutants. When the total number of epidermal ICs and epidermal SCs was taken into account, there was no significant difference between mibb\textsuperscript{a52b} and sibling embryos (Figure 3D–3J). These results clearly demonstrate that excessive epidermal ICs occur at the expense of epidermal SC cell fate, and also suggest the default cell fate of the IC domain is set as epidermal ICs when the Notch signal is absent. However, when Notch activity is enhanced, the entire epidermal IC domain will adopt an epidermal SC cell fate. Therefore, we concluded that a balanced mixture of epidermal ICs and epidermal SCs within the IC domain is maintained by the D-N-mediated lateral inhibition mechanism.

Lateral Inhibition of Singling-out Epidermal IC Progenitors is Mediated by the DeltaC Ligand and Notch1a/Notch3 Receptors

D-N-mediated lateral inhibition plays essential roles in the cell fate choice and cell differentiation of diverse target tissues by utilizing...
different combinations of Delta/Jagged ligands and Notch receptors in a stepwise manner [33,34]. In zebrafish, at least eight Delta/Jagged genes: deltaA [35], deltaB [36], deltaC [37], deltaD [38], delta-like 4 (dll4) [39], jagged1a [40], jagged1b [40], and jagged2 [36,40], and four notch genes: notch1a [41], notch1b [42], notch2 [42], and notch3 [42] have been identified so far. To figure out which combination of ligand and receptor is used, we performed in situ hybridization with deltaA, deltaB, deltaC, deltaD, dll4, jagged1a, jagged1b, and jagged2 probes to look for possible Delta/Jagged components expressed on epidermal IC progenitors around the tb to 3-s stages. As a result, only deltaC was detected as being expressed on the ventral ectoderm in a salt-and-pepper pattern (Figure S2). This result suggests that deltaC may be the only ligand involved in mediating IC specification. In zebrafish, although the epidermal expression of deltaC has previously been described [37], the identity of deltaC-positive cells is unknown. To validate whether deltaC is expressed in the epidermal IC lineage, we performed fluorescent double in situ hybridization with deltaC and foxi3a probes on zebrafish embryos at the tb stage. Results demonstrated that both foxi3a and deltaC were largely co-expressed in epidermal IC progenitors (Figure 4A). However, we noted that the territory (expressed as the degree of the angle shown in Figure 4A) of deltaC-positive cells (69° ± 4°, n = 16) was greater than that of foxi3a-positive cells (49° ± 2°, n = 11). A high-magnification view confirmed that most deltaC-positive cells outside the IC domain were indeed foxi3a-negative (Figure 4B). We speculated that these deltaC-positive/foxi3a-negative cells might be the progenitors for other epidermal cell types. It was also noted that deltaC expression on the epidermal IC lineage was relatively transient compared to that of foxi3a (Figure 4C and 4D). By 24 hpf, when foxi3a was robustly being expressed, deltaC was sharply downregulated to an undetectable level in the epidermal ectoderm (Figure 4D).

Next, we searched for possible Notch receptors by performing in situ hybridization with notch1a, notch1b, notch2, and notch3 probes. Results showed that notch1a was diffusely and strongly expressed in the ventral ectoderm (Figure S3) and possibly functions as a receptor to receive DeltaC signals. Other notch genes, on the contrary, were only expressed in the ventral ectoderm at a basal level (Figure S3). To further validate the right components for transmitting lateral inhibition for selecting epidermal IC progeni-

![Figure 4. Lateral Speciation on Singling-out Epidermal Ionocyte Progenitors is Mediated by deltaC Ligand and notch1a/notch3 Receptors.](image)

(A) Fluorescence double in situ hybridization shows the overlapping expression between deltaC (green, left) and foxi3a (red, middle) on the epidermal ionocyte domain of the ventral ectoderm at the tail bud (tb) stage. The angles of the deltaC and foxi3a expression domain are presented as the mean ± S.D. (B) The area demarcated by the dotted line in (A) is viewed at high magnification. Basically, foxi3a-epidermal ionocyte progenitors (red) also co-express deltaC (green, asterisks). However, some deltaC+ cells outside the epidermal ionocyte domain are negative for foxi3a, suggesting that they are not epidermal ionocytes. (C–D) Fluorescent double in situ hybridization with deltaC (green) and foxi3a (red) probes to show that deltaC is transiently expressed in the epidermal ionocyte lineage. As development proceeds, deltaC is sharply downregulated in the epidermal ionocyte lineage. (E–L) Evaluation of foxi3a expression by genetic mutants or morphants with reduced or enhanced Notch activity at the tb stage. foxi3a expression in the epidermal ionocyte domain was more homogeneous in deltaC mutants of beat[deltaC] (G), and in a notch3 MO-injected des[1MO] mutant (H). The foxi3a expression in the epidermal ionocyte domain was severely reduced in notch1a intracellular domain (ICD) RNA- (J) or notch3 ICD RNA-injected embryos (K). (H) notch1a/des[1MO] mutants injected with the notch1b MO showed no significant difference with uninjected mutants. N1a, notch1a; N1b, notch1b; N3, notch3; ICD, intra-cellular domain; MO, morpholino.

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tors, we assayed the loss-of-function phenotypes of deltaC, notch1a, notch1b, notch2, and notch3 genes in genetic mutants or morphants by examining their foxi3a expression at the tb stage. Results confirmed that deltaC is indeed a crucial ligand and the only one which sends a lateral inhibition signal to single out epidermal IC progenitors, since the entire IC domain homogeneously expressed foxi3a in deltaC/

MO (Figure 4E; Table 1) and deltaC/beatw212b

mutants (Figure 4F; Table 1). In notch1a/des535

mutants, we also detected an increase in foxi3a expression within the IC domain (Figure 4G; Table 1). However, its foxi3a-positive area was much less dense than those of mibta52b

beatit446 or beatw212b

mutants (Table 1). These results suggest that there should be other notch receptor(s) which synergistically function with notch1a in mediating lateral inhibition. We explored this possibility by injecting notch1b morpholino (MO), notch2 MO, or notch3 MO into des535

mutants and assaying foxi3a expression at the tb stage. As a result, we failed to detect any significant increase in the foxi3a-positive area when notch1b

MO (Figure 4H; Table 1) or notch2

MO (data not shown) was injected into des535 mutants. However, when notch3

MO was injected into des535

mutants, the entire IC domain homogeneously expressed foxi3a (Figure 4I; Table 1). In addition to the loss-of-function assay, we also validated the functions of notch1a and notch3 through a gain-of-function approach. As we expected, the foxi3a-positive area was greatly reduced or even completely lost when we enhanced Notch activity by misexpressing intracellular domains (ICD) of either notch1a (Figure 4J; Table 1) or notch3 (Figure 4K; Table 1). Taken together, we concluded that notch1a plays a more-prominent role than notch3 in receiving deltaC signals from epidermal ICs. In addition, the cooperation between notch1a and notch3 carries out D-N-mediated lateral inhibition in a synergistic manner to maintain a balanced population of epidermal ICs and SCs.

**deltaC Expression in Epidermal IC Progenitors is Positively and Negatively Regulated by ascl1a and notch, Respectively**

In Drosophila, the delta expression in signal-sending cells is positively enhanced by a proneural gene but negatively regulated by Notch activity [18,19]. This feedback regulatory loop can amplify the tiny difference in Delta expression between cells in a tissue at the beginning of the cell fate choice stage, and gradually specifies the cell identity between pure signal-sending and -receiving cells. In zebrafish, we were curious about whether the initial deltaC expression in epidermal IC progenitors is also positively activated by a proneural gene and negatively regulated by the Notch negative regulatory loop. To test this hypothesis, we first forced expression of the ascl1a (achaete-scute complex-like 1a) proneural gene (also expressed in epidermal IC progenitors, data not shown) by mRNA injection and assayed deltaC expression at the tb stage. Results showed that deltaC was upregulated by ascl1a mRNA misexpression; however, the penetrance was low (data not shown). To exaggerate the function of ascl1a, we fused a strong activation domain from a herpes simplex virus with full-length ascl1a cDNA to generate VP16:ascl1a chimera constructs [43]. Interestingly, when enhanced versions of VP16:ascl1a mRNA were forcibly expressed in zebrafish embryos, this was sufficient to induce ectopic deltaC expression in the cephalic epidermal ectoderm outside the IC domain (Figure 5A, green panel, indicated by arrows), but insufficient to induce foxi3a expression in this ectopic site (Figure 5A, red panel). These results indicated that, first, ascl1a can regulate deltaC but not foxi3a expression; and second, ectopic deltaC-expressing cells outside the IC domain are not epidermal ICs.

We also manipulated Notch activity to see whether deltaC expression is negatively regulated. Compared to wild-type embryos (Figure 5B), we found that the deltaC-positive area in the ventral ectoderm was 5.7-fold increased in mibta52b

(Figure 5C; Table 1), 5.5-fold increased in desth35b

(Figure 5D; Table 1) and 3.9-fold increased in X-Su(H)/Ank

mRNA-injected embryos (Figure 5E; Table 1), whose Notch activity was compromised due to a failure of deltaC ubiquitination, deltaC ligand expression, and transmission of the Notch signal through Su(H), respectively. On the contrary, in X-Su(H)/Ank

mRNA-injected embryos whose Notch signal was enhanced, deltaC expression in the ventral ectoderm was completely abolished (Figure 5F; Table 1). Therefore, we confirmed that deltaC expression by epidermal IC progenitors is positively and negatively regulated by ascl1a and notch, respectively.

**Reciprocal Regulation of foxi3a and foxi3b Expressions by a Positive Regulatory Loop**

foxi3a is activated earlier than foxi3b in epidermal IC progenitors (Figure 1N). We tested the hierarchical relationship between foxi3a and foxi3b by both loss- and gain-of-function approaches in 5-s embryos. Compared to wild types (foxi3a,

Figure 6A; foxi3b,
Figure 6D), we found that foxi3a expression was almost unaffected in foxi3b morphants (Figure 6C), while foxi3b expression was completely abolished in foxi3a morphants at the 5-s stage (Figure 6F).

However, as development proceeded, it was still possible to detect some foxi3a- or foxi3b-positive epidermal IC progenitors in foxi3a morphants, although their relative number was greatly reduced (Figure 6H) when compared to the wild type (Figure 6G). For the gain-of-function assay, foxi3b expression was 6.1-fold upregulated in foxi3a mRNA-injected embryos (Figure 6E). Surprisingly, foxi3a expression was 2-fold upregulated when foxi3b mRNA was forcibly expressed (Figure 6B). This result suggests that, first, foxi3a is genetically upstream of foxi3b and, second, foxi3a and foxi3b can be reciprocally regulated by a positive feedback loop.

The epidermal ectoderm of zebrafish gastrula can be divided into three subdomains along the A/P axis according to the differential expressions of gata2 and gata3 [44]. After completion of convergent extension, these three subdomains give rise to the epidermis covering the cephalic (zone I), trunk (zone II), and tail (zone III), respectively. For wild-type 5-s embryos, we found that foxi3a (Figure 6A) and foxi3b (Figure 6D) were restrictedly expressed within the epidermal IC domain (corresponding to zone II). However, either foxi3a (50%, n = 255, Figure 6E) or foxi3b (35%, n = 223, Figure 6B) misexpression was sufficient to generate extra epidermal IC progenitors at ectopic sites of zones I and III at the 5-s stage. Moreover, the generating potential of ectopic epidermal ICs gradually declined from zones I to III. This result demonstrated that both foxi3a and foxi3b are sufficient to specify the IC identity and that the entire epidermal ectoderm is permissive for epidermal IC formation.

In addition to functioning as epidermal IC determinants, it is not clear whether the unusually high level of exogenous foxi3a or foxi3b is related to the precocious differentiation of epidermal IC progenitors. Therefore, we addressed this event by performing fluorescent double in situ hybridization with atp1b1b and ca2a probes in either foxi3a mRNA- or foxi3b mRNA-injected embryos at the 5-s and 24-hpf stages. In a normal condition, NaRCs and HRCs did not differentiate until the 14- and 18-s stages, respectively. However, when foxi3a mRNA was misexpressed, we found that the elevated expression level of foxi3a was sufficient to promote precocious differentiation of both NaRCs (73%, n = 171) and HRCs (4%, n = 171) not only within the epidermal IC domain but also in the ectopic sites of the cephalic epidermis at the 5-s stage (Figure 7B). Only NaRCs differentiated precociously in foxi3b mRNA-misexpressed embryos at the 5-s stage (16%, n = 190, Figure 7C). But at 24 hpf, ectopic HRCs could be found in the cephalic epidermis in foxi3b mRNA-misexpressing embryos (52%, n = 60, Figure 7F). In addition, we also noted that ectopic epidermal ICs occurred at the expense of the epidermal SC fate, since the epidermal SC marker of P63 was completely abolished in some regions where high levels of atp1b1b and ca2a were expressed (Figure 7E and 7F, highlighted by arrows). Taken together, we concluded that foxi3a is more potent than foxi3b in promoting IC differentiation in wild-type embryos, and that the timing of NaRC and HRC differentiation is strongly dependent on the relative concentrations of foxi3a and foxi3b accumulated in epidermal IC progenitors.

Gain-of-Function Assay of foxi3a and foxi3b in X-Su(H)/Ank mRNA-Injected Embryos or bmp7 Morphants

Based on the differential expressions of foxi3a and foxi3b in differentiating NaRC (foxi3b\textsuperscript{low} and HRC (foxi3a\textsuperscript{low}/foxi3b\textsuperscript{low}) lineages, we speculated that they might have distinct functions in promoting the differentiation of NaRCs and HRCs. However, according to the misexpression data performed in wild-type embryos, it was difficult to distinguish the distinct functions between foxi3a and foxi3b in promoting NaRC and HRC differentiation. Actually, the misexpression results obtained in the wild-type background should be carefully interpreted when both Foxi3a and Foxi3b activities are present, because the positive regulatory loop will amplify downstream foxi3b when only foxi3a is misexpressed. In the same manner, the positive regulatory loop can also amplify upstream foxi3a when only foxi3b is misexpressed. Therefore, in the wild-type background, regardless of misexpression foxi3a or foxi3b, it will turn out to be a combinatorial effect derived from both foxi3a and foxi3b in promoting NaRC and HRC differentiation. In order to differentiate the functions of foxi3a and foxi3b more precisely, we sought to create a genetic background which contains no endogenous foxi3a or foxi3b expression by misexpressing X-Su(H)/Ank mRNA or bmp7 MO. When a high level of X-Su(H)/Ank mRNA (500 pg per embryo) was misexpressed, about 45% (n = 210) of the injected embryos showed a reduced body axis phenotype, and all the epidermal IC progenitors adopted the epidermal SC fate in such a high Notch activity background (Notch-ON embryos). We selected Notch-ON embryos aged at 24 hpf to perform an atp1b1b and ca2a in situ study, and found that only 3% of NaRCs and 1%
of HRCs (n = 104, Figure 8C) differentiating. However, when we forced foxi3a expression in Notch-ON embryos, it was sufficient to restore both NaRC (100%, n = 74) and HRC (100%, n = 74) differentiation with high penetrance (Figure 8D). foxi3b, on the contrary, was sufficient to restore full NaRC (100%, n = 86) and partial HRC (24%, n = 86) differentiation in Notch-ON embryos (Figure 8E).

In parallel, we also dissected the functions of foxi3a and foxi3b in bmp7 morphants. Bmp functions as upstream signals, which are essential for ventral cell fate and epidermal ectoderm development [45]. Because epidermal ICs are generated from epidermal SCs, we hypothesized that epidermal IC specification is also regulated by Bmp activity. Indeed, we found that foxi3a expression was very sensitive to Bmp activity. In 24-hpf bmp7 morphants, although we still could detect P63 expression, both NaRC (0%, n = 88) and HRC (0%, n = 88) differentiations were completely undetectable (Figure 8F) due to the loss of epidermal IC identity (detected by a foxi3a in situ study, data not shown). However, when we forced foxi3a expression in bmp7 morphants, it was sufficient to restore both NaRC (100%, n = 62) and HRC (100%, n = 62) differentiation with high penetrance (Figure 8G). foxi3b misexpression was sufficient to restore NaRC (100%, n = 103) and partially HRC (21%, n = 103) differentiation in bmp7 morphants (Figure 8H). Taken together, we concluded that first, either Foxi3a or Foxi3b activity may be sufficient to promote the default fate of NaRC differentiation; second, the alternative fate of HRC differentiation requires a high level of Foxi3a; and third, the partial restoration of HRCs in embryos injected with X-Su(H)/Ank mRNA+foxi3b mRNA or bmp7 MO+foxi3b mRNA may be mediated by Foxi3a activity. And this Foxi3a activity is probably boosted by the Foxi3b feedback regulatory loop.

Loss-of-Function Assay Suggests that Foxi3a Functions as a Master Regulator to Maintain Epidermal IC Identity and Activate Epidermal IC Differentiation

In addition to the gain-of-function assay, we also interfered with foxi3a and/or foxi3b function by an MO injection. NaRC and HRC differentiation in 24-hpf morphants was then evaluated by fluorescent double in situ hybridization with atp1b1b and ca2a probes. For negative controls, both NaRC and HRC differentiations were largely undisturbed in either foxi3a MO<sup>−</sup> (Figure 9B) or foxi3b MO<sup>−</sup> (data not shown). For the weaker foxi3a MO1 injection (see Figure S4 for the specificity and efficacy tests), we found that the number of NaRCs was severely reduced to 14±8, while HRCs were completely lost (Figure 9C). With the stronger foxi3a MO2 injection, both NaRCs and HRCs were completely abolished (Figure 9D). It is worth noting that the effect of foxi3a MO is highly specific to epidermal IC lineages, since the expression domains of ca2a in the pronephric duct and spinal cord neurons, as well as the P63 expression in epidermal SCs were undisturbed in foxi3a morphants (Figure 9D). Furthermore, the loss of NaRCs and HRCs in foxi3a morphants could be restored by supplying foxi3a mRNA (the rescue rate for NaRCs was 95%, and that for HRCs was 56%, n = 95; Figure 9H and 9I), indicating that the observed IC phenotype is indeed due to a lack of Foxi3a activity. For foxi3b morphants, we found that HRCs were largely undisturbed while NaRCs were slightly reduced when embryos were injected with either foxi3b MO1 (Figure 9E), foxi3b MO2 (Figure 9F), or a mixture of both foxi3b MO1 and foxi3b MO2 (data not shown). Why are embryos insensitive to foxi3b MO? We propose that the foxi3a-foxi3b positive feedback loop can continuously supply foxi3b and eventually dilute out the foxi3b MO.
Interestingly, we found that NaRCs with weaker foxi3a MO1 could be completely eliminated when co-injected with the weaker foxi3b MO1 (Figure 9G). This result suggests that foxi3a and foxi3b also function in a synergistic manner to promote epidermal IC differentiation.

There are two possibilities to explain the completely abolished expression of atp1b1b and ca2u in 24-hpf foxi3a morphants: first, epidermal IC identity may have been completely lost; and second, epidermal IC progenitors are still alive but fail to differentiate into NaRCs and HRCs. By examining the progenitor markers of foxi3a and foxi3b, we learned that epidermal IC identity is reduced but not completely lost in foxi3a morphants (Figure 6H). However, surviving epidermal IC progenitors in foxi3a morphants are blocked in an undifferentiated status and lose the expression of the differentiating markers of ndrg1, kcnj1, atp1a1a.2, arg2, trpm7, and atp6a1k (Figure S5). In addition to loss of marker expression, we also provide additional evidence to show that surviving epidermal ICs in foxi3a morphants lose their biochemical properties and function. In zebrafish, functionally mature NaRCs and HRCs were able to absorb vital dyes of MitoTracker (Figure 9J, red) and Con-A (Figure 9J, green), respectively, through their unique apical openings [14]. However, we found that 3-dpf foxi3a morphants were unable to absorb MitoTracker or Con-A (Figure 9K). On the contrary, foxi3b morphants could still mediate MitoTracker or Con-A uptake through mature NaRCs and HRCs (Figure 9L). By a scanning electron microscopic examination, mature NaRCs and HRCs in wild-type embryos were characterized by their distinct types of apical openings in deep-hole (diameter = 2 μm, Figure 9M, green asterisks) or mesh-like shapes (5~8 μm, Figure 9M, red asterisks), respectively. In foxi3b morphants, the apical openings of both NaRCs and HRCs were reduced (Figure 9O), while they were no longer visible in foxi3a morphants (Figure 9N).

The specific disappearance of epidermal ICs in foxi3a morphants provided an excellent opportunity to evaluate their physiological function in vivo. By incubating foxi3a morphants in E3 embryonic medium, they suffered severe cardiac and yolk sac edema from 5 dpf onwards (Figure S6C) and eventually died by 10 dpf. If we raised the external osmolarity with isotonic Ringer's buffer, the edema phenotype was greatly reduced (Figure S6D). On the contrary, a more-severe edema phenotype was observed when foxi3b morphants were raised in double-distilled water (D2W) (Figure S6E). Therefore, we learned that the edema phenotype in foxi3a morphants is primarily caused by unbalanced osmoregulation due to the complete loss of epidermal IC differentiation. By measuring the Ca\textsuperscript{2+} content in morphants, we also provide direct evidence to show that epidermal ICs play an essential role in active Ca\textsuperscript{2+} uptake, since foxi3a morphants were unable to maintain Ca\textsuperscript{2+} homeostasis even if high-Ca\textsuperscript{2+} Ringer's solution was provided (Figure S6E). Therefore, our data strengthen the idea that epidermal ICs in fish embryos indeed play a role in maintaining water and ion homeostasis before their branchial counterparts are fully matured.

**Summary of the Epidermal IC Specification and Differentiation Program in Zebrafish**

In this study, we successfully characterized three developmental programs of epidermal ICs in zebrafish as specification (from the 90%E8 stage to the 14-s stages), differentiation (from the 14-s stage to 36 hpf), and maturation (from 36 hpf onwards) (summarized in Figure 1). The underlying mechanisms controlling epidermal IC specification and differentiation are summarized in Figure 10. For the specification phase, we found that Bmp signals are essential for epidermal IC formation. Once Bmp activity is reduced, the

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Figure 8. Dissection of the Function of foxi3a and foxi3b in X-Su(H)/Ank mRNA-Injected Embryos or bmp7 Morphants. (A) Schematic diagram showing the role of Bmp and Delta-Notch signaling in specifying the epidermal ectoderm (gray) and singling-out epidermal ionocytes (yellow) from the epidermal stem cell pool (blue). (B) Normal Na\textsuperscript{+} and H\textsuperscript{+} ATPase rich cell (NaRC) and H\textsuperscript{+} ATPase rich cell (HRC) differentiation in wild-type embryos aged at 24 hours post-fertilization (hpf). In wild-type embryos, a few IC progenitors were selected from the epidermal stem cell pool within the epidermal ionocyte domain by Delta-Notch-mediated lateral inhibition, and then differentiated into NaRCs (green, detected by atp1b1b in situ) or HRCs (red, detected by ca2u in situ) which are scattered on the epidermal layer. (C) For X-Su(H)/Ank mRNA-injected embryos (500 pg/embryo), the foxi3a and foxi3b expressions were inhibited due to the elevated Notch activity in the epidermal ionocyte domain. In such a condition, all epidermal ionocyte progenitors adopted the epidermal stem cell fate; therefore, subsequent NaRC and HRC differentiation was completely abolished. (D) When foxi3a mRNA (50 pg/embryo) and X-Su(H)/Ank mRNA (500 pg/embryo) were co-injected, high levels of exogenous foxi3a expression could compensate for the elevated Notch activity and restore both NaRC and HRC differentiation. (E) When foxi3b mRNA (50 pg/embryo) and X-Su(H)/Ank mRNA (500 pg/embryo) were co-injected, a high level of exogenous foxi3b expression was also sufficient to compensate for the elevated Notch activity to restore NaRC and partially restore HRC differentiation. (F) In bmp7 morphants (0.1 mM/embryo), although the low level of Bmp signals was still sufficient to promote P63 expression, it was insufficient to drive either foxi3a or foxi3b expression and finally the epidermal ionocytes lost their identity. (G) When foxi3a mRNA (50 pg/embryo) and bmp7 MO (0.1 mM/embryo) were co-injected, a high level of exogenous foxi3a expression could compensate for the low Bmp activity and restore both NaRC and HRC differentiation. (H) When foxi3b mRNA (50 pg/embryo) and bmp7 MO (0.1 mM/embryo) were co-injected, a high level of exogenous foxi3b expression could compensate for the low Bmp activity to restore NaRC and partial HRC differentiation. All embryos were scored at 24 hpf. doi:10.1371/journal.pone.0000302.g008
The epidermal IC identity is completely lost, and foxi3 expression becomes undetectable. In the wild-type, foxi3a-positive epidermal IC progenitors are activated on the IC domain of the ventral ectoderm as early as the 90% epiboly stage. D-N-mediated lateral inhibition plays a role in singling-out epidermal IC progenitors, while keeping most cells as epidermal SCs within the IC domain. The epidermal ICs themselves function as signal-sending cells and express high levels of the deltaC ligand. The lateral inhibition signal is then transmitted from epidermal ICs to the surrounding epidermal SCs and received by notch1a and notch3 receptors. When notch1a and notch3 are activated in epidermal SCs, the Notch signal is then mediated by Su(H) to activate the unknown member of bHLH-O in inhibiting expression of the pro-differentiating factors of ascl1a and foxi3a(3b) in the epidermal SC side. The low level of ascl1a in epidermal SCs renders deltaC unable to transmit the lateral inhibition signal back to epidermal IC progenitors ultimately creating a balanced population of epidermal ICs and epidermal SCs on the ventral ectoderm by such a negative feedback regulatory loop.

The apical openings of NaRCs and HRCs in wild-types are shaped as deep holes (green box) or a mesh (red box), respectively. The apical openings were totally undetected in foxi3a morphants due to blockage of the entire differentiation program. For foxi3b morphants, the apical openings for both NaRCs and HRCs were reduced. Embryos in (A–I) were scored at 24 hpf, while in (J–O), they were scored at 72 hpf. In I, embryos are orientated in a dorsal-up and anterior-top position.

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DISCUSSION
Epidermal ICs are a New Target Site for D-N Lateral Inhibition
Our studies provide evidence to show that epidermal ICs are a novel target for D-N lateral inhibition, and also highlight a strikingly conserved mechanism of D-N in binary cell fate choice from invertebrates to vertebrates. In all cases, blockage of Notch signaling leads to a failure in lateral inhibition and to a great excess of one cell type at the expanse of another. The supernumerary cell types are neural precursors in the neuronal ectoderm [30,46,47], ciliated cells in the Xenopus epidermis and zebrafish pronephric duct [48,49], hair cells in the mouse and zebrafish ear [36,50,51], secretory cells in the zebrafish gut [52], luminal cells in the chicken stomach [53], alpha cells in the zebrafish pancreas [34], and ICs in the zebrafish epidermis (this study).

The epidermal layer in aquatic vertebrates is relatively simpler than that of their terrestrial vertebrate counterparts. The epidermal layer in fish embryos, for example, is covered by two layers of cells as the enveloping layer (EVL) and deeper layer (DEL). When development proceeds, the EVL is gradually shed
and replaced by a true epidermis from the DEL [54]. Actually, epidermal cells in fish and frog embryos are not as simple as we expected. In addition to epidermal cells, other cells like solitary chemosensory cells [55], mucous cell [56], taste buds [57], ciliated cells [48], and epidermal ICs [14,58] have also been reported in either fish or frogs. Interestingly, all of them are scattered on the epidermis in a salt-and-pepper pattern. According to the data obtained from studies of epidermal ICs (this study) and ciliated cells [48], such a salt-and-pepper distribution pattern is the outcome of D-N lateral inhibition. Therefore, it will be intriguing to test whether Notch signaling plays a role in the specification and differentiation of solitary chemosensory cells, mucous cells, as well as taste buds in the future.

The **foxi3a** Expression Pattern on Ventral Ectoderm Supports the Concept of the Ectomere

In vertebrates, the neuremocyte is patterned by the combination of different signal molecules like *wnt, fgf*, and *bmp* along the A/P axis [59,60]. Compared to the neural ectoderm, the mechanism of patterning of the epidermal ectoderm has been addressed less fully. Couly and Douarin (1990) transplanted the anterior neural crest and epidermal ectoderm between quail and chicken neurula embryos and found that the presumptive facial and hypobranchial ectoderm is also divided into units along the A/P axis [61]. This observation led them to propose a concept of an “ectomere” to highlight the idea that the epidermal ectoderm is also compartmentalized as its neuromere counterpart in neural anlagen [61]. Afterwards, the prospective epidermal ectoderm in the gastrula stage in both *Xenopus* and zebrafish was identified as being patterned into three subdomains along the A/P axis with different levels of *gata2* and *gata3* expressions [44]. Recently, Matsuo-Takasaki and colleagues (2005) discovered that *Xenopus foxi1a* is expressed in the anterior ventral ectoderm (corresponding to zone I) of the neurula and plays an essential role in ventral specification of the later cephalic ectoderm [62]. In this study, we provide more evidence to strengthen the concept of the ectomere by examining the expression and function of zebrafish *foxi3a*. Endogenous *foxi3a* is activated in the middle ventral ectoderm (corresponding to zone II) during gastrulation and functions to specify epidermal IC identity. However, when exogenous *foxi3a* is pan-embryonically misexpressed, we learned that other zones of the ectomere are also permissive to generating ectopic epidermal ICs but with different potentials. In general, such permissive potential to generate ectopic epidermal ICs gradually declines from the anterior toward the posterior. Currently, the molecular mechanism of the patterning of *foxi3a* expression within the IC domain is unclear. Further studies addressing *fgf, bmp*, and *wnt* signal gradients may provide an answer.

**Foxy3a and Foxy3b Function as Highly Potent Epidermal IC Determinants**

Winged helix/forkhead genes encode a subset of the helix-turn-helix class of transcription factors and are involved in a wide range of cell-type specifications and differentiation during organogenesis across diverse animal phyla [20,21,22,23,24]. In mice, *foxi1* has been reported to function as a master regulator in regulating differentiation of FORE cells in the inner ear [3] and intercalated cells in the urinary system [25]. This observation led Al-Awqati and Schwartz to propose a “kidney-inner ear axis” model to highlight the strikingly conserved mechanism of *foxi1* for kidney and inner ear differentiation [2]. In addition to promoting cell differentiation, *foxi1* has also been reported to be a strong cell fate determinant on shaping and patterning the inner ear in zebrafish [26,63]. Zebrafish *foxi1* is the earliest marker identified as being expressed in the otic placode. Misexpression of *foxi1* is sufficient to activate the expressions of downstream target genes of *pax8, dlx3b, dlx4b, and dlx5a* in several ectopic sites [26]. In this study, we also demonstrated that *foxi3a* is the earliest marker expressed in epidermal IC progenitors. *foxi3a* and *foxi3b* are both sufficient to determine the epidermal IC identity by a novel positive regulatory loop. Even in the condition of low *Bmp* or high Notch activities which does not favor epidermal IC formation, overexpression of either *foxi3a* or *foxi3b* is still sufficient to rebuild the epidermal IC identity as well as to promote a later differentiation program. Why do *foxi* genes function so potently? A recent study revealed that *foxi1* is capable of remodeling chromatin’s higher-order structure and can stably create site-specific changes in chromatin to either create or remove DNase I hypersensitive sites [64]. This creates a stable transcriptional “ground state” for cells to rapidly and appropriately respond to external developmental cues. Further studies on the structure and function of Foxi3a by domain analysis will benefit our understanding of its transactivating function.
Do Foxi3a and Foxi3b Play Distinct Functions in Promoting NaRC and HRC Differentiations?

According to the differential expressions of foxi3a and foxi3b in differentiating epidermal ICs, foxi3a and foxi3b may play different roles in HRC and NaRC differentiation. Although the loss-of-function assay suggested that only foxi3a but not foxi3b is required for NaRC and HRC differentiation, the gain-of-function assay showed that both exogenous foxi3a and foxi3b are sufficient to promote ectopic NaRC and HRC differentiation in a wild-type background. The results obtained from the gain- and loss-of-function studies do not totally contradict each other when taking the positive feedback regulatory loop between foxi3a and foxi3b into account. This novel regulatory loop can amplify the downstream foxi3b concentration when upstream foxi3a is misexpressed, and vice versa. Therefore, regardless of misexpression of foxi3a or foxi3b in wild-type individuals, the final readout will be the combinatorial results derived from both foxi3a and foxi3b. By blocking the upstream expression of foxi3a in either foxi3a morphants, X-Sia(H)/Ank mRNA-injected embryos, or bmp7 morphants, the foxi3a:foxi3b positive regulatory loop will break down and the entire epidermal IC differentiation program will be abolished. However, when exogenous foxi3b is supplied, it is sufficient to restore the primary setting of NaRC differentiation. The secondary setting of HRCs, on the contrary, requires a higher concentration of foxi3a for its restoration. This result clearly demonstrates that both foxi3a and foxi3b are sufficient to promote the primary setting of NaRC differentiation, while the secondary setting of HRC differentiation strongly relies on a higher concentration of foxi3a. What is the molecular mechanism which breaks the balanced expression between foxi3a and foxi3b in common IC progenitors and then drives the lineage separation between NaRCs and HRCs? Our current data are insufficient to answer this basic question. In Danophila SOP development, four daughter cells, socket, hair, sheath and neuronal cells, can be generated from a single SOP by a stepwise asymmetric cell division which is mediated by Notch and Numb interactions [15,65]. In zebrafish, by systematically analyzing the D-N components, we concluded that deltaC-notch1a/3-mediated lateral inhibition plays a role in singling out epidermal IC progenitors from the epidermal SC pool at around the 90% epiboly to tb stages. However, D-N-mediated lateral inhibition might also play a role in later NaRC and HRC lineage separation, since increases in NaRCs and HRCs are not proportional in mibta52b but are skewed toward the HRC lineage [Figure 3]. Further studies of NaRC and HRC differentiation on other D-N mutants or morphants will provide insights into this possibility.

In addition, we also noted that foxi3a expression is earlier and stronger than that of foxi3b in epidermal IC progenitors. If HRC differentiation really relies on a higher concentration of foxi3a, then, why don’t epidermal IC progenitors differentiate into HRCs as the default differentiation setting? The most reasonable interpretation is that the HRC differentiation program is not directly executed by foxi3a. We suggest that foxi3a might activate other downstream gene(s) to initiate the HRC differentiation program later than that for NaRCs. Indeed, forkhead genes often collaborate with other transcription factors to control the timing of differentiation, exerting both positive and negative effects on these processes [2]. Therefore, searching for genes which are downstream of or associated with foxi3 genes by in situ screening, mutant screening, microarrays, or a yeast-two-hybrid analysis may provide insights into this topic in the future.

Epidermal ICs Provide a Simple Model to Study Branchial IC Differentiation

The gills are a high energy-demanding multiple-function organ which contains at least pavement cells (equivalent to keratinocytes), neuroepithelial cells, mucous cells, and branchial ionocytes to mediate the diverse functions of air exchange, oxygen sensing, protection, acid-base balance, and ion transport [5]. Branchial ionocytes in most fish species are concentrated in the interlamellar regions. However, when fish were challenged with lower temperature [MY Chou et al., unpublished observation], hypoxia [66], lower ionic water [67,68] or a high concentration of a heavy metal [69], branchial ionocytes extend to the more-distal region of the lamella in a short time. However, due to constraints of the complex organization of the gill epithelium and a lack of convincing gene markers to label undifferentiated ionocyte progenitors, whether these ectopic branchial ionocytes are generated from cell migration, cell proliferation, or in situ differentiation still remains unknown. In this study, we provide direct evidence to show that foxi3a and foxi3b are novel markers for epidermal ionocyte progenitors and play essential roles in specifying epidermal IC identity and promoting epidermal IC differentiation in a concentration-dependent manner. This observation raises the possibility that the renewal and differentiation of branchial ionocytes in adult gills might be also mediated by a foxi3-dependent pathway. Preliminary data suggest that this answer might be true since we found that foxi3a expression and the number of branchial ICs in the secondary lamella were strongly positively correlated (MY Chou et al., unpublished observation). This intriguing finding suggests that ectopic branchial ICs in the secondary lamella might be differentiated from preexisting progenitors. Further studies by manipulating foxi3a expression levels in zebrafish gill using transgenic approaches may provide more-direct evidence to support this working hypothesis.

What was the evolutionary driving force to evolve such an elegant, positive regulatory loop between foxi3a and foxi3b to regulate epidermal IC differentiation? We showed in zebrafish that although the epidermal IC progenitors appear very early at the gastrula stage, they are not functionally mature until 36 hpf based on a Ca2+ ion-flux assay [58], an electro-physiological assay [14], vital dye staining, and apical opening observations (this study). Actually, the maturation of epidermal ICs is a very complicated process that involves sequential activation of many genes involved in cell differentiation, ion transport, acid-base regulation, as well as cell shape remodeling [4]. We suggest that the coupled reciprocal regulation between foxi3a and foxi3b has at least two benefits for zebrafish embryos, as follows: first, it promotes epidermal IC progenitors entering the differentiation program by rapidly producing high amounts of foxi3a and foxi3b in a short time frame, and secondarily prevents the complete breakdown of epidermal IC differentiation when the master regulator of fox3 function is unilaterally compromised. Therefore, the two duplicate copies of fox3 genes may have been preserved during vertebrate evolution by a sub-functionalization mechanism.

MATERIALS AND METHODS

Animals

The wild-type AB strain and mutant lines of zebrafish (Danio rerio) were maintained as described previously [70]. Homozygous mutants were obtained by in-crossing between heterozygous carriers for the mibta52b, desb59b, bed44b, and beat52b alleles. All animal procedures were approved by the Animal Use and Care Committee of Academia Sinica and A-Star.
Whole-Mount in Situ Hybridization
For single-color in situ hybridization, digoxigenin-labeled antisense riboprobes for *foxi3a* [27], *foxi3b* [27], *deltaA* [35], *deltaC* [37], *deltaB* [38], *dll4* [39], *jadgeda* [40], *jadgedb* [40], *jadged2* [36,40], *notchla* [41], *notchlb* [42], *notchc* [42], *nrx* [71], * deleted [72], *atp1nl* [73], *asg* [74], *typm7* [75], and *atp6v0c* [76] were generated by in vitro transcription, and whole-mount in situ hybridization was performed as previously described [77]. Images were taken with a DIC microscope (BX51, Olympus) equipped with a digital camera (DP70, Olympus). For fluorescent double in situ hybridization, *foxi3a* and *ca2a* [72] riboprobes were labeled with digoxigenin, while *foxi3b*, *deltaC*, and *atp1lb* [73] riboprobes were labeled with dinutritol. We followed the protocol originally described in [78] and improved by [49]. Images were taken with a confocal laser scanning microscope (FluoView FV1000, Olympus). Since epidermal ICs are located on the superficial epidermis, we found that it was unnecessary to perform proteinase K digestion. To optimize the clarity of the presented images, the anterior is to the left and dorsal is up in all panels unless otherwise indicated, and scale bars are provided only in the absence of standard anatomical landmarks.

Whole-Mount Antibody Staining
Whole-mount antibody staining was performed as described previously [14]. Antibodies and their dilutions were used as follows: monoclonal anti-chicken Na⁺,K⁺-ATPase, 1: 200 (clone z5, Developmental Studies Hybridoma Bank); polyclonal anti-kifilin H⁺-ATPase, 1: 200 [79]; polyclonal anti-phospho-histone 3 (Ser10), 1: 200 (Santa Cruz); monoclonal anti-human P63, 1: 200 (clone TP63, R&D Systems); and goat anti-mouse IgG-Alexa Flour 568, 1: 200; and goat anti-rabbit IgG-Alexa Flour 488, 1: 200; goat anti-rabbit IgG-Alexa Fluor 568, 1: 200; and goat anti-mouse IgG-Alexa Fluor 647, 1: 200.

Vital Dye Staining
MitoTracker and Con-A have been utilized as vital dyes to label mitochondria in NaRCS and glycoproteins in the apical openings of HRCs in zebrafish embryos [14]. To evaluate the viability of NaRCS and HRCs in wild-type and morphants, we incubated 3-dpf embryos in 50 ppm of MitoTracker Orange CM-H2TMRos (Molecular Probes, Eugene, OR) or Alexa Fluor 488-conjugated Con-A (Molecular Probes) for 10 minutes in the dark. After extensive washing with E3 embryonic medium (0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, and 0.1% methylene blue), the stained embryos were observed under a fluorescence microscope (Axioplan2, Zeiss, Germany). The maximal dosage that caused no obvious toxic effect on embryogenesis was used as follows: *foxi3a* MO: +72 tor/96, against ATG); *5’-AGACGTGTGAAGCTATGTTGAT-3’* at 0.5 mM/embryo; *foxi3a* MO2 (+44 tor/68, against 5’UTR); *5’-TCTTTCTGTTTCTCTTGTTAGG-3’* at 0.5 mM/embryo; *foxi3a* MO1 (**+72 tor/96, against ATG**); *5’-AGAGTGTTGCAAGAATACTGTGATG-3’* at 0.5 mM/embryo; *foxi3b* MO1 (+89 tor/113, against ATG); *5’-CTTTGAGCTGTAGGATGTCATTG-3’* at 0.5 mM/embryo; *foxi3b* MO2 (+64 tor/88, against 5’UTR); *5’-CTGATCCGAGGTGGTTCGCAGTT-3’* at 0.5 mM/embryo; *foxi3b* MO1 (**+89 tor/113, against ATG**); *5’-TCTTTCACTCCTGCTAGCAGTGTGATT-3’* at 0.5 mM/embryo; *notchla* MO: 5’-CTCTCCCCATTCATCGTGGTTAGG-3’ at 0.5 mM/embryo [82]; *notchlb* MO: 5’-CCCTTTCACTCCTGCTAGCAGTGTGATT-3’ at 0.5 mM/embryo [82]; *notchc* MO: 5’-CCAATCCAGGAACTCATCGATTAGTT-3’ at 0.1 mM/embryo [83]. To validate the specificity and efficacy of the MOs, we designed two independent MOs against either the translational start site (MO1) or the 5’ untranslated region (UTR) (MO2) and co-injected them with mRNA Injection
All constructs cloned in the pCS2+ and pCS2+GFP XL vectors were linearized by NotI, and the capped RNA was transcribed using an SP6 message RNA polymerase kit (Ambion). The pCS2+VP16ascl1a construct was linearized by SacII, and the capped RNA was transcribed by SP6 RNA polymerase. We injected capped mRNA into yolks at the 1–2-cell stage at the following concentrations: *foxi3a*, 50 pg/embryo; *foxi3b*, 50 pg/embryo; *foxi3aUTR:GFP*, 250 pg/embryo; *foxi3bUTR:GFP*, 250 pg/embryo; *notchla* ICD [81], 125 pg/embryo; *notch3* ICD [31], 125 pg/embryo; X-Su(H)/Ank [32], 500 pg/embryo; X-Su(H)/pCS2 [32], 500 pg/embryo; and VP16ascl1a [43], 125 pg/embryo.

Morpholino (MO) Injection and Control
To archive the maximal knock-down effect, 1 nl of serially-diluted MOs (purchased from Gene Tools) at the concentrations of 1, 0.5, 0.25 and 0.1 mM were injected into yolks at the 1–2-cell stage. The maximal dosage that caused no obvious toxic effect on embryogenesis was used as follows: *foxi3a* MO: +72 tor/96, against ATG); *5’-AGACGTGTGAAGCTATGTTGAT-3’* at 0.5 mM/embryo; *foxi3a* MO2 (+44 tor/68, against 5’UTR); *5’-TCTTTCTGTTTCTCTTGTTAGG-3’* at 0.5 mM/embryo; *foxi3a* MO1 (**+72 tor/96, against ATG**); *5’-AGAGTGTTGCAAGAATACTGTGATG-3’* at 0.5 mM/embryo; *foxi3b* MO1 (+89 tor/113, against ATG); *5’-CTTTGAGCTGTAGGATGTCATTG-3’* at 0.5 mM/embryo; *foxi3b* MO2 (+64 tor/88, against 5’UTR); *5’-CTGATCCGAGGTGGTTCGCAGTT-3’* at 0.5 mM/embryo; *foxi3b* MO1 (**+89 tor/113, against ATG**); *5’-TCTTTCACTCCTGCTAGCAGTGTGATT-3’* at 0.5 mM/embryo; *notchla* MO: 5’-CTCTCCCCATTCATCGTGGTTAGG-3’ at 0.5 mM/embryo [82]; *notchlb* MO: 5’-CCCTTTCACTCCTGCTAGCAGTGTGATT-3’ at 0.5 mM/embryo [82]; *notchc* MO: 5’-CCAATCCAGGAACTCATCGATTAGTT-3’ at 0.1 mM/embryo [83]. To validate the specificity and efficacy of the MOs, we designed two independent MOs against either the translational start site (MO1) or the 5’ untranslated region (UTR) (MO2) and co-injected them with...
UTR:GFP mRNA into embryos as fluorescent reporters. The UTR:GFP constructs containing the 5’UTR, partial exon 1 of foxi3a (or foxi3b) was then in-frame-fused with the GFP reporter. Therefore, MO1 and MO2 were expected to simultaneously inhibit the expression of both endogenous foxi3a (foxi3b) and exogenous GFP in injected embryos. Results showed that both MO1 and MO2 reduced the expression of exogenous foxi3a (foxi3b) UTR:GFP, suggesting that they are also able to knock-down the expression of endogenous foxi3a or foxi3b. The five mismatched control MO1 or MO2 were unable to target exogenous foxi3a (foxi3b) UTR:GFP, suggesting that the mis-targeting problem can be ignored. The efficacy of MO2 was superior to that of MO1 at inhibiting the translation of foxi3a or foxi3b (Figure S4).

Scanning Electron Microscopy (SEM)
Embryos were pre-fixed with 4% paraformaldehyde in PBS at 4°C overnight and then post-fixed with 2.5% glutaraldehyde and 0.1 M sodium cacodylate again overnight at 4°C under gentle agitation. After rinsing with 0.1 M sodium cacodylate and fixation using 1% osmium tetroxide, samples were washed again in 0.1 M sodium cacodylate and then dehydrated stepwise by successive immersion in 50%, 70%, 80%, 90%, 95%, and 100% ethanol. Traces of ethanol in the samples were evaporated with a critical point dryer (HCP-2, Hitachi) and then coated with gold–palladium particles using a sputter coater (Cressington 108). The surface structure of the apical openings was evaluated using an environmental scanning electron microscope (ESEM, FEI Quanta 200).

Physiology Assay
Initially, we raised morphants and wild-types in E3 embryonic medium from days 0 to 3. On day 3, 100 morphants and wild-type embryos were selected and transferred to Ringer’s solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, and 5 mM Heps; pH 7.2), E3 or double-distilled water in three to five duplications (procedures are highlighted in Figure S6A). On day 7, we scored the survival rate (number surviving at 7 dpf/normally developing embryos at 3 dpf) x 100%) and edema rate (edematous embryos at 7 dpf/number surviving at 7 dpf) x 100%). To measure the whole-body Ca2+ content, 30 morphants or wild-type embryos at 7 dpf were selected from double-distilled water-, E3-, and Ringer’s solution-acclimated groups and then dehydrated at 65°C overnight. The dry embryos were digested with 1000 mM NaOH overnight and then subjected to atomic absorption spectrophotometry (Z-8000, Hitachi) following our previously described protocol [58].

Ionocyte Quantitation and Statistical Analyses
The embryos probed with foxi3a, foxi3b, or deltaC were oriented in a ventral-up position for the photographs. The gene expression area was quantified using ImageJ software (NIH). Values are presented as the mean (SD) and were compared using Student’s t-test.

SUPPORTING INFORMATION
Figure S1 Detection of the apical opening of epidermal ionocytes in zebrafish embryos. (A-F) The epidermal layer covering the yolk ball of wild-type embryos was scanned by a scanning electron microscope at different developmental stages (indicated in the lower left-hand corner). The first apical opening of the epidermal ionocyte appeared at 36 hours post-fertilization (hpf) (asterisks).

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Figure S2 Screening possible delta/jagged ligand expression in the epidermal ionocyte domain. Embryos aged at the tail bud (b) to 3-somite (3-s) stages were evaluated in situ with either (A) deltaA, (B) deltaB, (C) deltaC, (D) deltaD, (E) delta-like 4, (F) jagged1a, (G) jagged1b, or (H) jagged2 probes. Among the eight delta/jagged genes tested, only deltaC was detected as being expressed on the epidermal ionocyte domain (highlighted by dotted lines) of the ventral ectoderm.

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Figure S3 Screening for possible notch receptor expression in the epidermal ionocyte domain. Embryos aged at the tail bud (b) to the 3-somite (3-s) stages were evaluated in situ with either (A) notch1a, (B) notch1b, (C) notch2, or (D) notch3 probes. Results show that notch1a was strongly and ubiquitously expressed in the epidermal ionocyte domain. Other notch genes, on the contrary, were expressed in the ventral ectoderm at a low level.

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Figure S4 Control experiments to validate the specificity and efficacy of the morpholino. Schematic diagrams show the relative positions of the designed MOs and reporter constructs for either foxi3a (A) or foxi3b (B). The 5’UTR and partial exon 1 sequences of foxi3a or foxi3b were PCR-amplified from zebrafish cDNA and in-frame-fused with the green fluorescent protein (GFP) reporter gene. The resulting chimeric vectors of pCS2+3aUTR:GFP or pCS2+3bUTR:GFP contained the complementary sequences for testing the specificity and efficacy of both MO1 and MO2. (C) When five mismatched controls of 3a MO1miss (0.5 mM) were co-injected with 3aUTR-GFP mRNA (250 pg), all embryos (100%, n = 94) showed strong GFP expression. This result suggests that the 3a MO1mis is unable to target endogenous foxi3a mRNA. (D) When the 3a MO1 (0.5 mM) was co-injected with 3aUTR-GFP mRNA (250 pg), GFP expression was completely abolished in 78% of the injected embryos (n = 83). (E) When 3a MO2 (0.5 mM) was co-injected with 3aUTR-GFP mRNA (250 pg), GFP expression was completely abolished in all injected embryos (n = 119). This result suggests that both 3a MO1 and 3a MO2 can target endogenous foxi3a mRNA. However, the efficacy of 3a MO2 was superior to 3a MO1. (F) When five mismatched controls of 3b MO1miss (0.5 mM) were co-injected with 3bUTR-GFP mRNA (250 pg), all embryos (100%, n = 94) showed strong GFP expression. This result suggests that the 3b MO1mis is unable to target endogenous foxi3b mRNA. (G) When the 3b MO1 (0.5 mM) was co-injected with 3bUTR-GFP mRNA (250 pg), it was only sufficient to abolish GFP expression in 58% of injected embryos (n = 71). (E) When the 3b MO2 (0.5 mM) was co-injected with 3bUTR-GFP mRNA (250 pg), it was sufficient to abolish GFP expression in 75% of injected embryos (n = 145). This result suggests that both 3b MO1 and 3b MO2 can target endogenous foxi3b mRNA. However, the efficacy of 3b MO2 was superior to 3b MO1. The number and percentage on the right bottom corner of C-H refer to the ratios of embryos that express GFP. ORF, open reading frame; UTR, untranslated region.

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Figure S5 Global down-regulation of epidermal ionocyte markers in foxi3a morphants. (A-F) Comparison of the epidermal ionocyte marker expression between wild-type (left panel) and foxi3a morphants (right panel). The expression of markers is completely abolished in the epidermal ionocyte lineage in foxi3a morphants. Note that the pronephric duct expressions in mhyd1, kray1, atp1a1a.2 and tcpn7 were largely undisturbed, which shows that the foxi3a morphant phenotype specifically targets epidermal
ionocytes. Na+,K+-ATPase-rich cell (NaRC) markers were nrg1 (A), knly (B), and atp1a1a2 (C). H+-ATPase-rich cell (HRC) markers were mfr (D), htrn7 (E), and atp6oxc (F). All embryos were scored at 24 hours post-fertilization (hpf).

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12. Ackermann GE, Paw BH (2003) Zebrafish: a genetic model for vertebrate ion homeostasis in zebrafish embryos. (A) Procedure to assay the physiological functions of epidermal ionocytes in zebrafish embryos. After injecting them with fox3a MO, morphants were initially raised in E3 up to 3 days post-fertilization (dpf) and then challenged with either Ringer’s solution, E3, or double-distilled water (D2W). The survival rate, edema rate, and whole-body Ca2+ content between wild-types (wt) and morphants were measured at 7 dpf, and results are summarized in (E). The wild-types had a strong water balance ability and showed no edema phenotype in either E3 (B), Ringer’s solution, or D2W (not shown). The morphants displayed a severely edematous phenotype in hypotonic E3 (C) or D2W (not shown), while the abnormality was greatly rescued in isotonic Ringer’s solution (D). The values are shown as the mean ± SD (n = 10). Asterisks (*) indicate a significant difference from the wild-type (Student's t-test, p < 0.05). Found at: doi:10.1371/journal.pone.0000302.s006 (7.09 MB TIF)

Figure S6 Role of epidermal ionocytes in water and ion homeostasis in zebrafish embryos. (A) Procedure to assay the physiological functions of epidermal ionocytes in zebrafish embryos. After injecting them with fox3a MO, morphants were initially raised in E3 up to 3 days post-fertilization (dpf) and then challenged with either Ringer’s solution, E3, or double-distilled water (D2W). The survival rate, edema rate, and whole-body Ca2+ content between wild-types (wt) and morphants were measured at 7 dpf, and results are summarized in (E). The wild-types had a strong water balance ability and showed no edema phenotype in either E3 (B), Ringer’s solution, or D2W (not shown). The morphants displayed a severely edematous phenotype in hypotonic E3 (C) or D2W (not shown), while the abnormality was greatly rescued in isotonic Ringer’s solution (D). The values are shown as the mean ± SD (n = 10). Asterisks (*) indicate a significant difference from the wild-type (Student’s t-test, p < 0.05). Found at: doi:10.1371/journal.pone.0000302.s005 (9.54 MB TIF)

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Author Contributions

Conceived and designed the experiments: YJ CH PH. Performed the experiments: CH YG. Analyzed the data: YJ CH PH. Contributed reagents/materials/analysis tools: YJ MM MY. Wrote the paper: YJ CH PH.
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