Current Biology 23, 1559–1565, August 19, 2013 ©2013 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2013.06.035

Report

Wnt/Dkk Negative Feedback Regulates Sensory Organ Size in Zebrafish

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Summary

Correct organ size must involve a balance between promotion and inhibition of cell proliferation. A mathematical model has been proposed in which an organ is assumed to produce its own growth activator as well as a growth inhibitor [1], but there is as yet no molecular evidence to support this model [2]. The mechanosensory organs of the fish lateral line system (neuromasts) are composed of a core of sensory hair cells surrounded by nonsensory support cells. Sensory cells are constantly replaced and are regenerated from surrounding nonsensory cells [3], while each organ retains the same size throughout life. Moreover, neuromasts also bud off new neuromasts, which stop growing when they reach the same size [4, 5]. Here, we show that the size of neuromasts is controlled by a balance between growth-promoting Wnt signaling activity in proliferation-competent cells and Wnt-inhibiting Dkk activity produced by differentiated sensory cells. This negative feedback loop from Dkk (secreted by differentiated cells) on Wnt-dependent cell proliferation (in surrounding cells) also acts during regeneration to achieve size constancy. This study establishes Wnt/Dkk as a novel mechanism to determine the final size of an organ.

Results and Discussion

The mechanosensory organs of the fish lateral line system (neuromasts) are composed of a core of sensory hair cells surrounded by nonsensory support cells (Figure 1A). Sensory cells are constantly replaced and are regenerated from surrounding nonsensory cells in response to traumatic events [3]. Neuromasts retain the same size throughout fish life, but they can also bud off new neuromasts, which will grow until they reach the same size again [4, 5]. Embryonic neuromasts on the body and tail of zebrafish are deposited by a migrating primordium, where cell proliferation compensates for cell loss due to neuromast deposition. The Wnt signaling pathway is essential for cell proliferation in this system [6–8] (Figure 1B). The secreted inhibitor of Wnt signaling Dickkopf1b (Dkk1b; Figure 1B) restricts Wnt activity to the leading region of the primordium and thereby contributes to primordium patterning [9].

To address a possible role of Wnt in later steps of neuromast development, we assessed Wnt activity in neuromasts with a reporter line in which a destabilized form of GFP (dGFP) is expressed under the control of binding sites for an effector of Wnt signaling, LEF1 (*tcf/lef-minip:dgfp*, hereafter *lef:dgfp*) [10]. We focused our study on two sets of neuromasts on the head: O1 and O2, which are the first neuromasts to be deposited and to differentiate, around 36 hr postfertilization (hpf) [11], and OP1, which is the first neuromast to bud off a new neuromast (OP2), around 72 hpf [4]. We observed that dGFP is present at high levels in the budding cells that will form OP2, but not in O1, O2, or OP1 (Figure 1C). The presence of dGFP gradually subsides as the budding structure becomes converted into a fully differentiated OP2 neuromast (Figure 1D). The difference in levels of dGFP parallels a difference in the proportion of cells undergoing mitosis, which is high in OP2 and much lower in O1, O2, and OP1, as detected by anti-phosphohistone H3 (pH3) labeling (Figures 1E and 1F).

We examined whether Wnt signaling promotes cell proliferation in neuromasts by using the recessive mutation *apc*, which results in constitutive activation of the Wnt target β-*catenin* [12]. The number of dividing cells in neuromast O2 was increased significantly in *apc* relative to wild-type at 36 hpf (1.5 ± 1.1 pH3-positive cells per neuromast in *apc*; 0.4 ± 0.6 in wild-type; Figure 1F). This increase in mitotic rate might conceivably be not a direct consequence of the change in Wnt signaling but rather an indirect effect due to a change in the proportion of cell types within neuromasts. The expression of *atoh1a* was increased in *apc* mutant embryos, however (see below), suggesting that the number of hair cells was increased in proportion to neuromast size and that there was no substantial change in the proportion of cell types.

Because Dkk acts as an inhibitor of Wnt signaling in various systems, including the migrating primordium of the posterior lateral line [9], we examined whether Dkk is present in developing neuromasts. Among the five genes of the zebrafish *dkk* family, three are putative Wnt inhibitors as predicted by sequence similarity to the mouse orthologous genes: *dkk1a*, *dkk1b*, and *dkk2* (see Figure S1 available online) [13]. Neither *dkk1a* nor *dkk1b* is detectably expressed in neuromasts, but *dkk2* mRNA was detected at 36 hpf in the central cells of O1 and O2 (Figures 1G and 1H) and later in other neuromasts as well. We also observed that the gene coding for the Dkk receptor, *kremen* (*krm*) [14], is broadly expressed in neuromasts (Figure S1).

In order to determine the dynamics of *dkk2* expression during neuromast development, we focused on the stereotyped sequence whereby the opercular neuromast OP1 buds off OP2 at 3–4 days postfertilization (dpf) [4]. We observed that *dkk2* is highly expressed in the central cells of OP1, but not at all in its budding process (Figures 1I and 1I′) or at the earliest stages of OP2 expansion (Figures 1J and 1J′). As proliferation proceeds and OP2 rounds up, expression of *dkk2* is initiated in a few central cells (Figures 1K and 1K′). When OP2 has attained the size of OP1, *dkk2* mRNA expression has become comparable in the two neuromasts (Figures 1L and 1L′). We conclude that *dkk2* expression is triggered when the neuromast reaches a certain, predetermined size.

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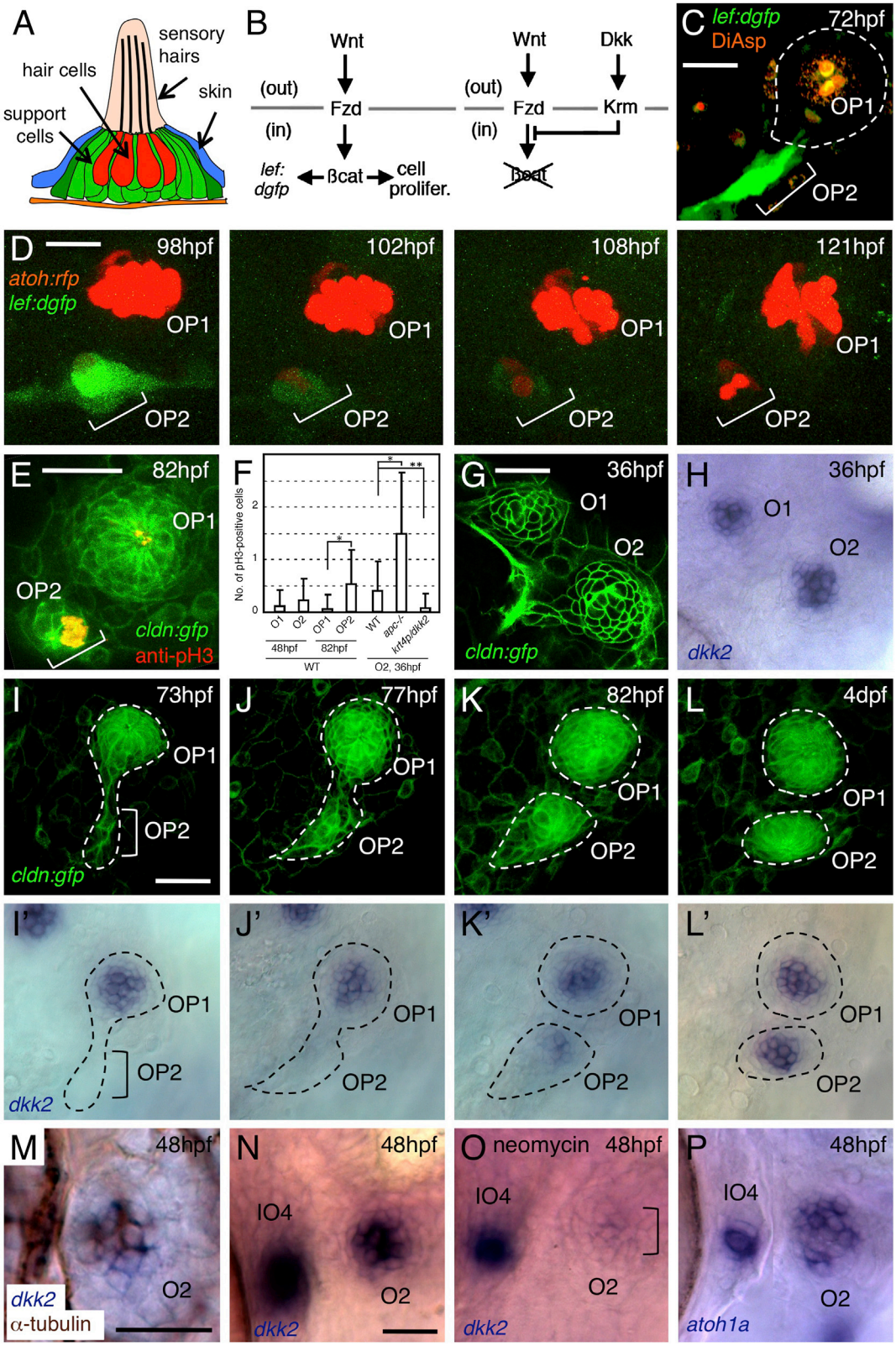

Figure 1. Wnt Signaling and Expression of *dkk2* Are Associated with Neuromast Proliferation

(A) Schematic drawing of a neuromast in transverse view.

(B) Schematic representation of Wnt signaling through its receptor, Frizzled (Fzd), and of its inhibition by Dickkopf (Dkk) signaling through its receptor, Kremen (Krm). out, extracellular compartment; in, intracellular compartment.

(C) Wnt signaling is detectable only in budding cells (OP2), but not in mature neuromast (OP1), as revealed in a Wnt-responsive GFP reporter line.

(legend continued on next page)

The presence of *dkk2* mRNA in the central cells of neuromasts, and its appearance at or shortly before the onset of hair cell differentiation (Figures 1K and 1K′), led us to examine whether the origin of Dkk2 was the hair cells themselves. We observed that *dkk2* is expressed in and near the sensory cells (Figure 1M), consistent with this possibility. To settle this question, we ablated all differentiated hair cells by neomycin poisoning [15]. Hair cell ablation at 48 hpf severely reduced the expression of *dkk2* mRNA in mature neuromast O2. Neomycin poisoning had no effect on *dkk2* expression in immature neuromast IO4 (Figures 1N and 1O), at a stage when hair cell precursor cells [16] are already present (Figure 1P) but have not yet generated fully differentiated neomycin-sensitive hair cells [11]. We conclude that the expression of *dkk2* starts in hair cell precursor cells and is maintained in mature hair cells.

We assessed a putative role of Dkk signaling in neuromast size control by inactivating either *dkk2* or *krm* (Figure 1B) through antisense morpholino oligonucleotides (MOs) (Figure S2). Because MOs injected in zygotes only function over the first few days of life, we focused on neuromast O2, which is the first neuromast to develop and which has almost reached its final size at 3 dpf (Figure 2A). Inactivation of *dkk2* or *krm* led to a significant increase in the overall size of O2 at 2 dpf, as measured by the area occupied (Figures 2C–2E). The increase in size reached 50% after inactivation of *dkk2* and 80% for *krm* (control, 1,098 ± 107 μm^2; *dkk2*-MO, 1,362 ± 174 μm^2; *krm*-MO, 1,887 ± 306 μm^2). We counted the neuromast cells and found a significant increase in cell number of 22% after inactivation of *krm* (control, 49.3 ± 6.1; *krm*-MO, 60.3 ± 8.9; $p < 0.001$, t test). The number of hair cells was also increased by about 40% in either morphant embryo (Figure 2B). By contrast, injection of *dkk1a*-MO or *dkk1b*-MO did not affect the number of hair cells (Figure 2B), consistent with the lack of expression of *dkk1a* or *dkk1b* mRNA in neuromasts (Figure S1).

In order to confirm the importance of Dkk2 in the control of cell number and size, we performed a gain-of-function experiment. We constructed a *dkk2-rfp* fusion gene and put it under the control of UAS sequences. When *UAS:dkk2-rfp* was expressed using a ubiquitous *gal4* driver (SAGFF73A) [17], fin fold formation was altered (Figure S3A), as is also the case after overexpression of *dkk1a* (Figure S3A) or *dkk1b* [18, 19] or after depletion of Wnt target genes [20], indicating that the fusion protein was functional. The depletion of *krm* function attenuated the effect of overexpression of *dkk2-rfp* (Figures 2B and S3B), confirming the functionality of the fusion protein, the specificity of the *krm*-MO, and the role of Krm in mediating Dkk2 signaling. When expressed ectopically in the skin using the *keratin4p(krt4p):gal4* line (Figure S3C), *dkk2-rfp* caused a significant reduction in neuromast area (626 ± 101 μm^2 versus 1,098 ± 107 μm^2 in controls; Figure 2F) as well as in the total number of neuromast cells (18 ± 2.9 cells versus 49.3 ± 6.1 cells in controls). The number of hair cells was also decreased (by 60%; Figure 2B) and was further reduced to about 5% of its normal value when using the stronger *gal4* driver, SAGFF73A (Figure 2B). The observation that a stronger *gal4* driver leads to a more extreme phenotype indicates that ectopic expression of *dkk2* decreases neuromast size and hair cell number in a dose-dependent manner, suggesting that *dkk2* is involved not in an all-or-none decision, but in a graded process.

We wondered whether cell death could contribute to the decrease in neuromast size observed after *dkk2* overexpression. Acridine orange staining did not reveal apoptotic cells in neuromasts, however (Figure S3D). Moreover, the number of hair cells did not change significantly over time in *dkk2*-overexpressing embryos (Figure 2A), suggesting that *dkk2* overexpression does not lead to substantial cell death in neuromasts. By contrast, we observed that overexpression of *dkk2* leads to a decrease in the number of dividing cells (Figure 2G; see also Figure 1F). Reduced cell proliferation, neuromast size, and hair cell number were also observed after overexpression of *dkk1a* (Figure 2B), suggesting that *dkk*-family genes can substitute each other for inhibiting Wnt signaling.

If the phenotypes associated with *dkk2* overexpression result from reduced cell proliferation, they should be mimicked when cell proliferation is blocked pharmacologically by aphidicolin [21]. Treatment with aphidicolin from 24 to 48 hpf led to a reduction of neuromast size by 40%, and the number of hair cells was reduced by 50% (Figures 2B and 2H). These phenotypes were identical to those we observed following *dkk2* overexpression, consistent with the idea that *dkk2* reduces neuromast size and hair cell number by suppressing cell proliferation, without affecting cell differentiation.

So far, we assumed that Wnt signaling, through its effect on cell proliferation, promotes the formation of hair cell precursor cells. In order to verify this assumption, we manipulated Wnt signaling directly. We found that inactivation of the major target of Wnt signaling, Lef1, through *lef1*-MO [20] injection, reduced hair cell number (Figure 2B). By contrast, overexpression of a constitutively active form of β-*catenin* (*ca-βcat*) [22] (Figure S3E) increased hair cell number (Figure 2B). Both results suggest that the number of hair cells is directly correlated to neuromast size and is therefore largely determined by cell proliferation activity within the neuromast.

Since *dkk2* is expressed in hair cells (Figures 1M–1P), the amount of *dkk2* expressed in the neuromast should be correlated to the number of hair cells present. On the other hand, both gain-of-function and loss-of-function experiments have shown that *dkk2* negatively regulates Wnt signaling and therefore reduces overall neuromast size, neuromast cell number, and hair cell number. It follows that *dkk2* should have a negative effect on its own expression. We observed that the expression domain of *dkk2* is indeed expanded in *apc* mutant embryos (Figures 3A and 3B), where the number

(D) The expression of dGFP gradually subsides as hair cells (labeled with *atoh:rfp*) are formed in O2.
(E) Anti-phosphohistone H3 (pH3) labeling indicates dividing cells in budding neuromast (OP2), but not in mature neuromast (OP1).
(F) Number of pH3-positive cells per neuromast. Mean ± SEM is indicated. *$p < 0.001$, **$p < 0.01$ (t test).
(G and H) *dkk2* mRNA is expressed by cells in the center of neuromasts O1 and O2.
(I–L′) Expression profile of *dkk2* during neuromast budding. *dkk2* mRNA expression coincides with neuromast maturation. The budding structures are outlined.
(M) *dkk2* mRNA (blue) is present in hair cells (brown).
(N and O) *dkk2* expression is reduced in O2 after ablation of hair cells but is not affected in immature neuromast IO4.
(P) *atoh1a* mRNA is present in O2 and IO4.
Scale bars represent 20 μm. See also Figure S1.

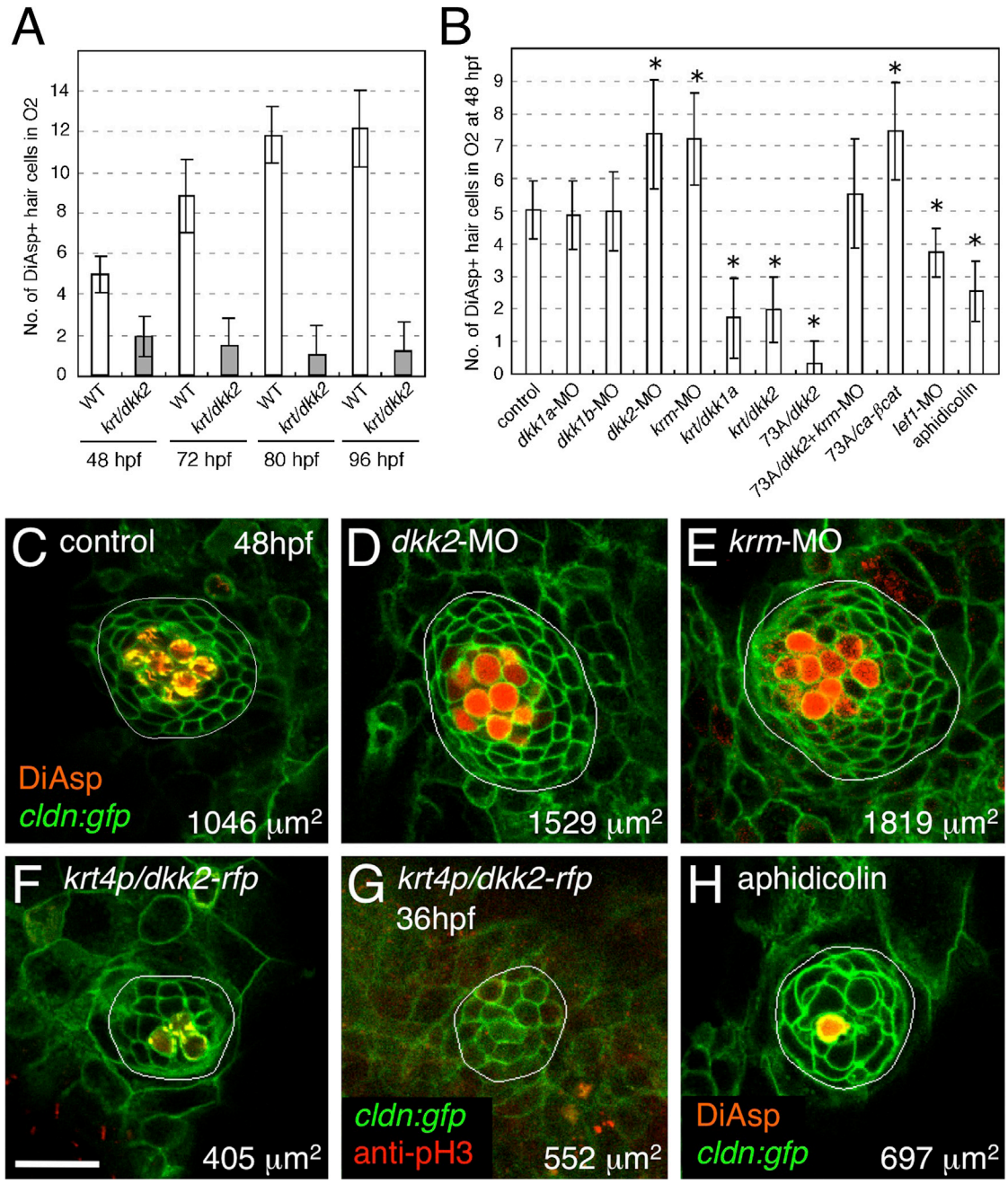

Figure 2. Wnt Signaling Positively Regulates Neuromast Size, Whereas Dkk2/Krm Signaling Negatively Regulates It

(A) Neuromast O2 reaches its final size at 80 hpf in wild-type embryos. By contrast, O2 reaches its final size before 48 hpf, and the number of hair cells does not change over time in *dkk2*-overexpressing embryos.

(B) Manipulations of Wnt signaling affect hair cell numbers. Mean ± SEM is indicated. *$p < 0.001$ (t test).

(C–E) Knockdown of *dkk2* (D) or *krm* (E) gene function increases neuromast size and hair cell number, as revealed by DiAsp labeling.

(F and G) Wnt inhibition by overexpression of *dkk2* reduces neuromast size, number of hair cells (F), and number of dividing cells (G). Scale bar represents 20 μm.

(H) Pharmacological blockade of cell division phenocopies inhibition of Wnt signaling.

See also Figures S1–S3.

of hair cells is increased (Figures 3D and 3E). By contrast, the expression domain of *dkk2* is significantly reduced in *dkk1a*-overexpressing embryos (Figure 3C), where the number of hair cells is decreased (Figure 3F). We conclude that *dkk2* exerts a negative feedback on itself (through its action on Wnt signaling and cell proliferation) and therefore belongs to a negative regulatory loop (Figure 4I). The presence of a negative loop is a necessary component of all homeostatic systems. In the case of neuromasts, mutual regulation of Wnt and Dkk signaling by each other would eventually lead to a homeostatic constant value in both signaling, where Wnt-dependent cell proliferation determines the final number of hair cells (i.e.,

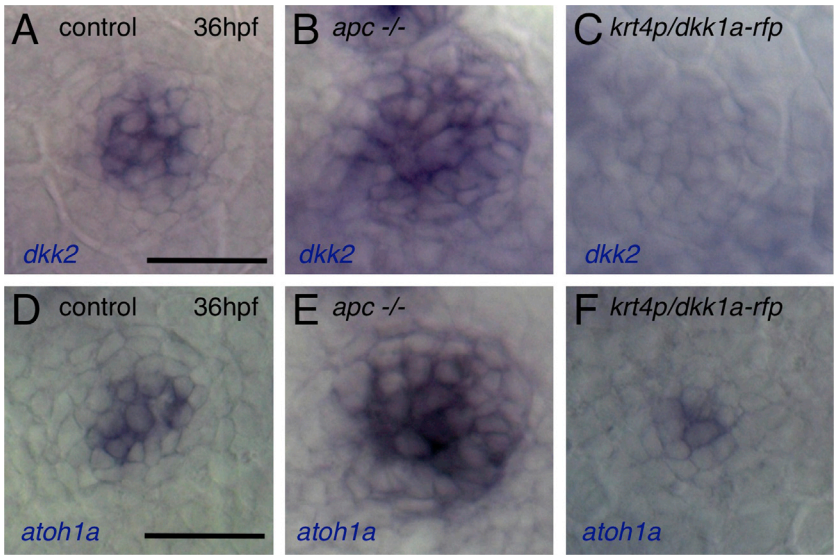

Figure 3. Manipulations of Wnt Signaling Affect the Expression of *dkk2* and *atoh1a*

dkk2 and *atoh1a* expression are enhanced in *apc* mutant embryos (B and E) and reduced in *dkk1a*-overexpressing embryos (C and F) as compared to wild-type (A and D). Scale bars represent 20 μm. See also Figure S3.

the number that prevents further growth of the organ size through Dkk signaling).

Hair cell regeneration is accompanied by cell proliferation after exposure to copper ions [23] or neomycin [24]. According to our scheme, the disappearance of hair cells should result in the disappearance of Dkk2 (Figures 1N and 1O), leading to disinhibition of Wnt signaling. After proliferation has allowed the formation of new hair cell precursor cells, the amount of Dkk2 secreted by these cells would progressively reestablish homeostatic control of neuromast size (Figures 4A–4D; 3.9 ± 1.6 hair cells were regenerated after 24 hr). We did observe some transient expression of the *lef:dgfp* construct after hair cell ablation (Figure S4). The result is not totally convincing, however, as expression was limited to a few neuromast cells, and nearby mesenchymal cells also exhibit Wnt signaling at this time. In order to confirm the implication of Dkk2 during hair cell regeneration, we examined whether *dkk2* overexpression would prevent hair cell regeneration. The result shows that this expectation is fulfilled, as hair cells were not regenerated in *dkk2*-overexpressing embryos (Figures 4E–4H; 0.04 ± 0.21 hair cells were regenerated). These results provide further support for a major role of Wnt/Dkk negative feedback in the achievement of neuromast size constancy (Figures 4I and 4J).

Our results strongly suggest the existence of a simple and novel mechanism for organ size control. This mechanism is based on a negative regulatory loop whereby Wnt signaling induces cell proliferation, which leads to the formation of hair cells, which secrete Dkk, which inhibits Wnt signaling, thus reaching a stable, homeostatic regulation of organ size (Figures 4I and 4J). Under normal conditions, replication takes place only in peripheral cells [3], where the levels of hair-cell-secreted Dkk are expected to be lower. After removal of the hair cells, on the other hand, Dkk levels would decrease all over, and replication should be observed throughout the neuromast, as was indeed observed [25, 26]. Thus, the reported distributions of BrdU-incorporating cells are entirely consistent with our proposal. Because replicating cells may either remain at the periphery or move to the center of the neuromast before dividing [3], the position of pH3-positive mitotic cells is not directly related to the position where these cells entered the S phase of the cell cycle, however.

To achieve the feedback loop, hair cells must be generated at a constant rate in proportion to neuromast size. Notch-Delta signaling is essential for hair cell formation through a mechanism of lateral inhibition, making it a good candidate to determine the ratio of hair cells to other neuromast cell types [27, 28]. Another potential factor is *atoh1a*, which is also required for hair cell development [29, 30]. The precise mechanism that ensures the formation of hair cells in numbers proportional to the size of the neuromasts (i.e., to the number of support cells) remains to be elucidated.

Experimental Procedures

Fish Stocks

Zebrafish *cldnb(cldn):gfp* [31], *atoh1a:dtomato(atoh:rfp)* [4], *apc* mutant [12], *UAS:egfp* [32], *tcf/lef-minip(lef):dgfp* [10], and a *Gal4* enhancer trap line (SAGFF73A) [17] were used. The transgenic lines *keratin4p(krt4p):gal4*, *UAS:dkk1a-rfp*, *UAS:dkk2-rfp*, and *UAS:ca-βcat-rfp* were generated in this study (see below). All experimental animal care was in accordance with institutional and national guidelines and regulations.

Isolation of Genes and Generation of Transgenic Lines

A 2.2 kb genomic region of the *keratin4* (*krt4*) gene [33] was amplified by PCR to generate the *krt4p:gal4* line. A partial cDNA fragment of *ctnnb1* (β-*catenin*) corresponding to a constitutively active form (*ca*-β*cat*) [22] was isolated by RT-PCR. Full-length cDNA fragments of *dkk1a*, *dkk1b* [18, 19], *dkk2*, and *kremen* (*krm*) were isolated by RT-PCR. The monomeric *rfp* gene [34] was introduced into the C terminus of each gene. See Supplemental Experimental Procedures for details.

Gene Knockdown by Antisense Morpholino Oligonucleotides

Partial genomic sequences of *dkk1a*, *dkk1b*, *dkk2*, and *krm* were cloned and sequenced, and antisense MOs that block splicing of these genes were generated by Gene Tools. See Supplemental Experimental Procedures for details.

Pharmacological Treatments

To ablate neuromast hair cells, we incubated embryos in 400 μM neomycin sulfate (Sigma) in embryonic medium for 1 hr [15]. To suppress proliferation of cells, we incubated embryos in a mixture of 75 μM aphidicolin/7.5 μM hydroxyurea (Sigma) in embryonic medium containing 1% DMSO from 24 to 48 hpf [9].

Immunohistochemistry, In Situ Hybridization, and Cell Labeling

Immunohistochemistry was performed according to standard protocols [35], using anti-acetylated α-tubulin antibody (Sigma) or anti-pH3 (Upstate Biotechnology). In situ hybridization using RNA probes was performed as described previously [35]. To count number of neuromast hair cells, we labeled 48 hpf embryos with 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (DiAsp, Sigma). Unpaired Student's t tests were performed to determine p values.

Accession Numbers

The DNA Data Bank of Japan (DDBJ) accession numbers for the zebrafish *dkk1a*, *dkk2*, and *krm* genes reported in this paper are AB753453, AB753454, and AB753455, respectively.

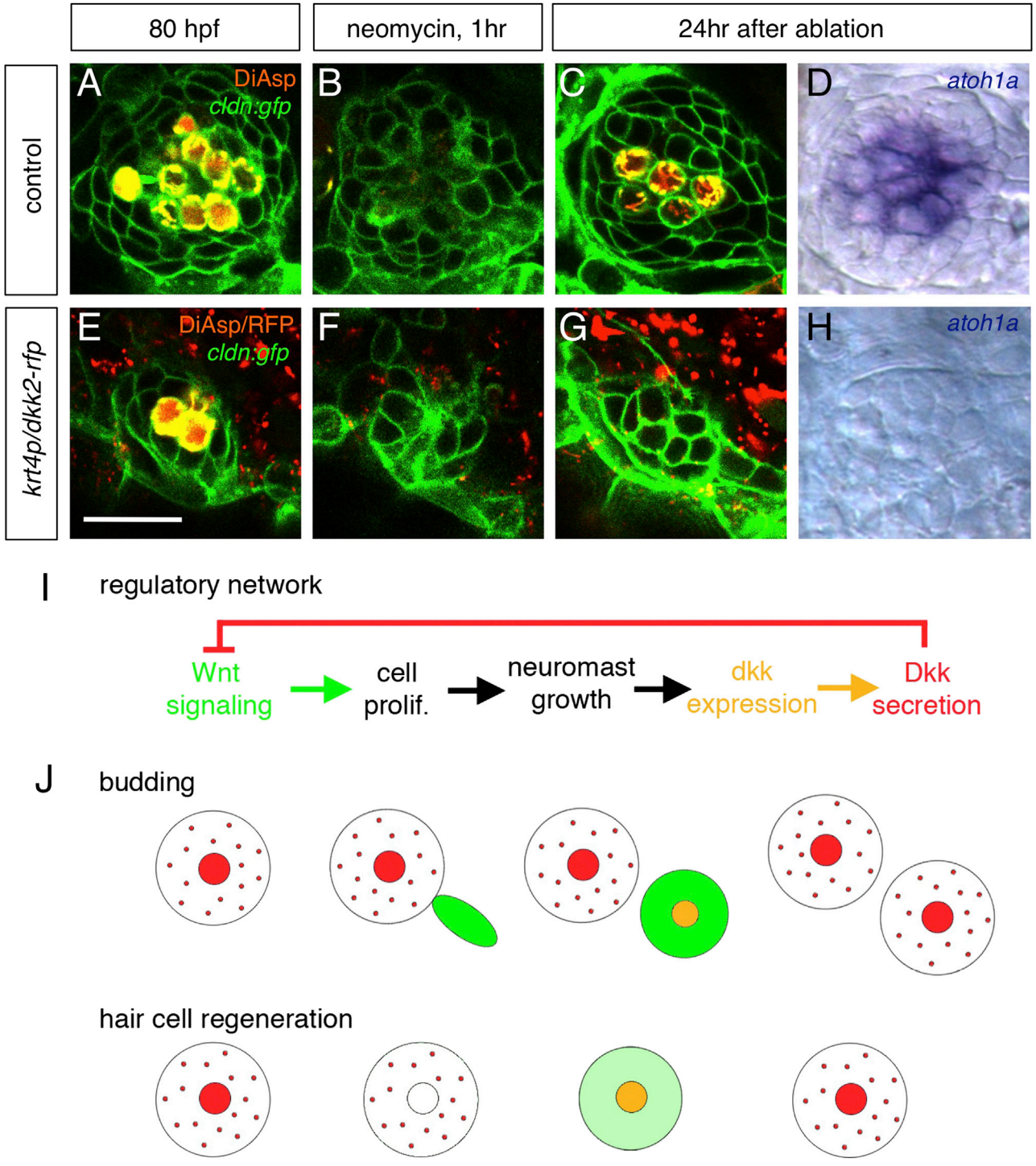

Figure 4. Inhibition of Wnt Signaling Prevents Regeneration of Hair Cells

(A–D) Regeneration of hair cells in normal embryos. New hair cells are regenerated within 24 hr after ablation.

(E–H) In *dkk2*-overexpressing embryos, where Wnt signaling is inhibited, new hair cells never form (G and H). Scale bar in (E) represents 20 μm.

(I) Proposed mechanism for neuromast size control, based on a regulatory network comprising a negative feedback step.

(J) Sequence of events leading to homeostasis in the case of budding of a new neuromast and in the case of hair cell regeneration. Large circles indicate neuromasts; smaller circles indicate hair cells. Wnt signaling (green) is active whenever Dkk (red) is absent. *dkk2* mRNA (orange) is expressed in differentiating and mature hair cells. Dkk2 protein (red) secreted by the hair cells reaches the peripheral cells and inhibits Wnt signaling (i.e., cell proliferation). The size of neuromasts is maintained during both budding and regeneration.

See also Figure S4.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.035.

Acknowledgments

We thank C. Dambly-Chaudière for critical reading of the manuscript. We also thank the anonymous reviewers for many valuable suggestions. The help of M. Iwasaki is gratefully acknowledged. We thank D. Gilmour, M. Itoh, S. Higashijima, H. Okamoto, H. Clevers, T. Piotrowski, and members of the Kawakami lab for fish strains and reagents. This work was supported by the National BioResource Project and funded by the Precursory Research for Embryonic Science and Technology (PRESTO) program of the Japan Science and Technology Agency (to H.W.).

Received: February 1, 2013
Revised: May 16, 2013
Accepted: June 13, 2013
Published: July 25, 2013

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Note Added in Proof

It has recently been demonstrated, using a pharmacological approach, that Wnt signaling is involved in cell proliferation and regeneration in mature neuromasts: Head, J.R., Gacioch, L., Pennisi, M., and Meyers, J.R. (2013). Activation of canonical Wnt/β-catenin signaling stimulates proliferation in neuromasts in the zebrafish posterior lateral line. Dev. Dyn. *242*, 832–846.