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Dkk2/Frzb in the dermal papillae regulates feather regeneration

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Abstract

Avian feathers have robust growth and regeneration capability. To evaluate the contribution of signaling molecules and pathways in these processes, we profiled gene expression in the feather follicle using an absolute quantification approach. We identified hundreds of genes that mark specific components of the feather follicle: the dermal papillae (DP) which controls feather regeneration and axis formation, the pulp mesenchyme (Pp) which is derived from DP cells and nourishes the feather follicle, and the ramogenic zone epithelium (Erz) where a feather starts to branch. The feather DP is enriched in BMP/TGF- β signaling molecules and inhibitors for Wnt signaling including *Dkk2/Frzb*. Wnt ligands are mainly expressed in the feather epithelium and pulp. We find that while Wnt signaling is required for the maintenance of DP marker gene expression and feather regeneration, excessive Wnt signaling delays regeneration and reduces pulp formation. Manipulating *Dkk2/Frzb* expression by lentiviral-mediated overexpression, shRNA-knockdown, or by antibody neutralization resulted in dual feather axes formation. Our results suggest that the Wnt signaling in the proximal feather follicle is fine-tuned to accommodate feather regeneration and axis formation.

Keywords

Feather follicle; Regeneration; Axis formation; Dermal papillae; Dkk2/Dkk3/Frzb

Introduction

Avian feathers serve as a useful model for developmental studies (reviewed in Lin et al., 2013, 2006; Yu et al., 2004). Major signaling pathways are involved in embryonic feather bud development, including Wnt/ β -catenin, BMP/Tgf- β , FGF, Shh, Notch/Delta, EGF, Eda/Edar etc. (reviewed in Lin et al., 2006). In particular, various Wnt ligands are involved, such as Wnt1, Wnt3a, Wnt5a, Wnt6, Wnt7a, Wnt11, and Wnt14 (Widelitz et al., 1999;

Chodankar et al., 2003; Chang et al., 2004). A similar set of signaling molecules and pathways are also involved in adult feather growth and regeneration (Yu et al., 2002; Yue et al., 2006, 2012). Previous studies have characterized the expression of Wnt ligands including Wnt3a, Wnt5a, Wnt6 and Wnt8c (Chodankar et al., 2003; Yue et al., 2006). Quantifying gene expression at the whole genome scale during these processes will help evaluate the contribution of each molecule at each developmental stage. Methods based on next generation sequencing technology provide such an opportunity, as compared with the more traditional hybridization-based microarray technology (Saha et al., 2002).

Similar to the mammalian hair follicle, the feather follicle is a “professional” regenerative organ that undergoes physiological renewal and regeneration after wounding or plucking (Lucas and Stettenheim, 1972; Lin et al., 2013; Yu et al., 2004). We have shown previously there are slow-cycling epithelial stem cells in the feather follicle that contribute to its episodic renewal (Yue et al., 2005). Furthermore, classical surgery experiments have established the critical role of the DP in feather regeneration (Lillie and Wang, 1941, 1944). It was shown that the DP controls feather shape, size, and axis determination (Lillie and Wang, 1941, 1943; Yue et al., 2006). However, little is known about the “molecular encoding” of the feather DP.

Molecular expression in the hair DP has been investigated in detail (Driskell et al., 2011, 2009; Rendl et al., 2005). Wnt3a, but not Shh signaling has been shown to maintain DP cell properties, i. e. hair reconstitution capability and marker gene expression (Kishimoto et al., 2000). DP-specific knockout of β -catenin disrupts hair regeneration (Enshell-Seijffers et al., 2010). In addition, BMP signaling plays an important role in maintaining DP cell properties (Rendl et al., 2008). In the DP niche, Sox2 may regulate the strength of BMP signaling and hair growth (Clavel et al., 2012). Moreover, TGF- β signaling may also modulate BMP signaling and contribute to hair regeneration (Oshimori and Fuchs, 2012).

Here we use the feather follicle as a model to analyze the regulatory logic of tissue regeneration. We started by using an unbiased transcriptional profiling of the feather DP. Interestingly, we noticed high levels of *Dkk2/Dkk3/Frzb*, which presumably encode Wnt signaling inhibitors. Functional analysis of the specific roles of these molecules revealed intriguing regulatory modes, i.e. both overexpression and knockdown of *Dkk2/Frzb* will lead to delayed feather regeneration and perturbation of feather axis formation. Our results have thus established novel concepts regarding the molecular mechanism of feather regeneration.

Results

A whole-genome survey of gene expression in the feather DP

The structure of the feather follicle has been described previously (Lin et al., 2013; Yu et al., 2004 and Fig. S1A). After plucking induced wounding, the follicle wall and DP (with the covering papillae ectoderm) still remain (Fig. S1B). This structure will always regenerate. However, if the DP is surgically removed, this “empty follicle” cannot regenerate, unless a DP is re-supplied (Lillie and Wang, 1944; Fig. S1 C). Feather regeneration is a rather quick process. After a short period of wound healing and remodeling (day 2), the follicle structure is re-established by day-4 (Fig. S1D–F).

To investigate the “molecular encoding” of the feather DP, we took an unbiased whole-genome profiling approach based on next generation sequencing technology. Compared to microarray, sequencing technology provides absolute quantification of gene expression and is more accurate (Saha et al., 2002). We isolated the DP, pulp mesenchyme (Pp) and ramosogenic zone feather epithelium (Erz) in growth phase follicles by microsurgery (Fig. 1A and B; and S2). The Pp is a distinct component in the feather follicle that is derived from DP cells and supports the actively growing feather epithelium (the hair follicle does not have a similar component) (Yue et al., 2012). Erz is included as a control because it is an epithelial component and should have very different molecular expression profile compared to the other mesenchymal components. Only high quality RNAs were used for analysis, which were monitored by an Agilent Bioanalyzer (Fig. S3).

In about 19,000 chicken genes in our database used for analysis, about 45% are expressed in the feather DP. A detailed description of the data processing and analysis procedure is in Supplemental data. Using the criteria of 2-fold difference and a false discovery rate (FDR) < 0.001, 794 genes are considered DP specific when compared with Erz and Pp (Fig. 1C). Similarly, 475 genes are Erz specific, and 904 genes are Pp specific. A full list of the differentially expressed genes, and the results of pathway enrichment/gene ontology (GO) analysis are shown in Supplemental Table 1. Potential “marker genes” for each compartment, together with the GO analysis results are also shown (Fig. 1D).

Previously there are very few molecules known to be expressed in the feather DP, mainly extracellular matrix proteins or cell adhesion molecules such as *Fibronectin*, *Tenascin*, *Laminin*, and *Ncam* (*neural cell adhesion molecules*) (Lin et al., 2006; Yue et al., 2012). Their expressions were confirmed by gene profiling analysis and by immunohistochemistry (Fig. 2A). A significant molecular feature of the feather DP is enrichment in muscle-related genes. These include *Actg2* (*smooth muscle actin, gamma 2*), *Acta2* (*smooth muscle actin, alpha 2*), *Desmin*, *Myh11* (*myosin heavy chain 11*), *Myl4* (*myosin light chain 4*), *Myl9* (*myosin light chain 9*), *Mylk* (*myosin light chain kinase*), etc. These genes are expressed at comparable or higher levels than β -actin. *Sox2* is not expressed, but *Ncam* is present in the DP. The developmental origin of the feather DP has not been clarified yet, however, gene expression profiling suggests a close relationship with muscle cells. GO analysis also revealed features such as “muscle tissue development”, “vascular smooth muscle contraction”. The specific expression patterns of some genes listed were confirmed by immunohistochemistry (Fig. 2A).

Due to the critical roles of the DP in feather formation, we paid special attention to molecules involved in major signaling pathways. A list of genes with their expression levels are shown in Table 1. *Dkk2/Dkk3/Frzb* and *Sfrp1/Sfrp2* are expressed at high levels in the DP, which presumably encode inhibitors of the Wnt signaling pathway. The expression levels of Wnt ligands, mainly *Wnt5a/Wnt5b/Wnt6*, are very low in the DP, but high in the feather epithelium and pulp. The receptors including *Fzd1/Fzd2/Fzd7/Fzd8* are expressed in the DP, suggesting active Wnt signaling in this tissue. We previously suggested a Wnt3a signaling gradient in the epithelium controls feather A–P axis orientation (Yue et al., 2006). Here we found *Wnt3a* is actually expressed at a low level in the feather Erz. A clear gradient distribution is found for *Wnt5a/Wnt6*, *Dkk2/Dkk3/Frzb* and a structural protein, feather

keratin A in the Erz region (Fig. 2B). We further cloned or synthesized the full ORFs of the chicken *Dkk2*, *Dkk3*, *Frzb* genes, expressed in a prokaryotic system and purified the encoded proteins. We made and affinity purified polyclonal antiserum for each of these proteins (Fig. S4). These antisera confirmed the specific expression patterns of these proteins by immunohistochemistry (Fig. 2A).

Previous work suggested BMP signaling is involved in feather formation (Yu et al., 2002). GO analysis revealed TGF- β signaling is a distinct feature of DP gene expression (Fig. 1D). Here we found *BMP4*, *BMP7*, *Tgfb2*, *Tgfb3*, *Noggin2*, *Chordin-like 1*, *Decorin*, but not *BMP2* or *Noggin* are expressed at high levels in the DP (Table 1). Other signaling molecules expressed include *FGFR1*, *PDGF*, *CTGF* and *Notch1/Jagged 1/Serrate2*. Interestingly, members of the Shh signaling pathway are absent, consistent with their later role in feather epithelial branching (Yu et al., 2002; Harris et al., 2002, 2005). We confirmed the expression of some molecules by RT-PCR (Fig. 2C) and in situ hybridization (Fig. 2D). The results also confirmed the specific expression patterns of several marker genes. For example, *Shh* gene is only expressed in the Erz but not DP or Pp, and *SMA/Desmin* is highly enriched in the DP but not Erz or Pp. In summary, we find many signaling molecules or regulators of major signaling pathways are expressed in the feather DP, consistent with its critical role in feather growth and regeneration.

cDkk2/cFrzb antagonizes Wnt signaling

The gene expression profile of the feather DP provides interesting insights into the functional role of this structure. In early embryonic development, the organizer (or the node) controls body axis formation and elongation, which expresses high levels of FGF/TGF- β signaling molecules and regulators, and inhibitors of Wnt signaling such as *Dkk1* (reviewed in Niehrs, 2001; De Robertis, 2006). In the hair follicle, the critical role of BMP signaling in DP function is well-documented (Rendl et al., 2008; Clavel et al., 2012). Wnt signaling is required for DP function (Kishimoto et al., 2000; Enshell-Seijffers et al., 2010), and inhibitors such as *Wif1* and *Sfrp1/Sfrp2* are also expressed in the hair DP (Rendl et al., 2005). *Dkk1* overexpression blocks hair follicle morphogenesis; however, its expression level in the hair DP is very low (Andl et al., 2002). Therefore, the function of these inhibitors in the hair follicle has not been investigated in detail.

To examine whether *Dkk2/Dkk3/Frzb* are truly inhibitors for Wnt signaling, we tested their functions both in vitro and in vivo. Previous work suggested that *Xenopus Dkk2* (*xDkk2*) could activate or inhibit Wnt signaling depending on the specific context, while *xDkk3* did not regulate Wnt signaling (Wu et al., 2000). In a Wnt responsive reporter assay in HEK 293 T cells, we found *cDkk2* is a potent inhibitor for Wnt signaling, even stronger than the positive control used here, *hDkk1* (Fig. 3A). *cFrzb* is a weaker but consistent inhibitor, whereas *cDkk3* does not significantly interfere with Wnt signal transduction. In *Xenopus* embryos, mRNAs of chicken *Dkk2*, *Dkk3* or *Frzb* were injected into all four blastomeres at 4-cell stage. *cDkk3* injected embryos were largely normal with very few (3/33) showing weak anteriorization, an indication of Wnt inhibition. In contrast, all *cFrzb* injected embryos (52/52) were modestly anteriorized. Even stronger anteriorization effect was observed after *cDkk2* injection, in which 7 out of 35 injected embryos had enlarged head and shortened

trunk/tail structures (Fig. 3B). A summary of the tests in *Xenopus* embryos is shown in Fig. 3C. These results suggest that *cDkk2* is a potent Wnt inhibitor, *cFrzb* is a mild but consistent Wnt inhibitor, whereas *cDkk3* shows almost no activity in this regard.

Overexpression of *Dkk2*/*Frzb* but not *Dkk3* disrupts feather regeneration

To directly examine the roles of *Dkk2*/*Dkk3*/*Frzb* in feather regeneration, we developed methods based on lentiviral delivery that could either overexpress or knockdown gene expression. Previously, gene overexpression in the feather follicle was achieved by RCAS-mediated gene delivery, which led to widespread and long-term expression (Yu et al., 2002; Yue et al., 2006). However, this method does not permit RNAi-mediated gene knockdown. Recently, methods based on siRNA – (Harpavat and Cepko, 2006) or microRNA – (Das et al., 2006; Smith et al., 2009) mediated gene knockdown were developed. Here we show that lentiviral-mediated gene delivery is efficient in the feather follicle. Moreover, while RCAS infection is usually limited to actively replicating cells, lentivirus has the advantage of infecting both dividing and non-dividing cells. This property is of particular significance, because DP cells usually replicate infrequently. Using a lentivirus carrying a GFP reporter, we found extensive viral gene expression at day 4 post-infection (Figs. 4M and N and S5). In our study, we also found extensive yet distinct phenotypes in feather formation by lentiviral-mediated *Dkk2*/*Frzb*/*Shh*/*Lfringe*/*Notch1* gene overexpression, further confirmed the effectiveness of this method (see below and data not shown).

We found overexpression of *Dkk2* or *Frzb* led to defective feather regeneration, whereas *Dkk3* overexpression did not have an obvious phenotype (Fig. 4A–H). Both *Dkk2* (13/20) and *Frzb* (8/15) delayed regeneration in about half the cases, whereas an empty pLVX virus (control) produced no abnormality (n = 15). Based on their growth rates, virus perturbation delayed feather regeneration for about 1–2 weeks. The impacts of virus-mediated gene perturbation on Wnt signaling were monitored by staining a phosphorylated form of β -catenin (Rhee et al., 2007; Song et al., 2009; Livnat et al., 2010). Compared to control or *Dkk3* over-expression, *Dkk2*/*Frzb* overexpression reduced pY489- β -catenin both in the collar epithelium and the DP (Fig. 4I–L). *Dkk2*/*Frzb*-induced regeneration defects were further characterized by reduced cell proliferation in the feather follicles, as shown by BrdU incorporation assay (Fig. 4O; Fig. S6), and increased cell apoptosis, as shown by TUNEL staining (Fig. 4P; Fig. S7).

We examined marker gene expression after perturbation. In normal regeneration, Desmin/Laminin/SMA marked the DP, while Tenascin showed widespread expression in the follicle (Fig. 5A–D). However, overexpression of *Dkk2* or *Frzb* led to reduced expression of DP markers including Desmin/Laminin/SMA, whereas Tenascin maintained its widespread pattern (Fig. 5E–L). In Masson staining, the dermal sheath appeared blue due to collagen fibers, but the DP appeared red due to its muscle property (Fig. 5M). *Dkk2*/*Frzb* overexpression reduced the DP property (Fig. 5N–O). This is not due to an oblique section plane, because we collected every other sections of the follicle and did not find a normally stained sample. Eventually, all feather follicles recovered and new feather growth resumed, possibly because not all cells were infected, and the virus might be silenced after certain

period of time. In addition, the virus was not spreading. In summary, *Dkk2* or *Frzb* overexpression delays feather regeneration, and reduces the DP properties (Fig. 5P).

RNAi-mediated knockdown of *Dkk2*/*Frzb* but not *Dkk3* disrupts feather regeneration

To specifically knockdown gene expression, we designed shRNA for *Dkk2*, *Dkk3* or *Frzb* and used lentiviral vectors to deliver these constructs into the regenerating feather follicle. The effectiveness of shRNA was confirmed in DF-1 cells in vitro. Compared with a random control construct, RNAi-*Dkk2* or RNAi-*Dkk3* reduced endogenous gene expression to less than 20%, while RNAi-*Frzb* had an efficiency of 50% knockdown (Fig. 6A). The in vivo impacts of RNAi knockdown were also confirmed (Fig. S8). Interestingly, RNAi knockdown of *Dkk2* or *Frzb* but not *Dkk3* led to delayed feather regeneration, which was characterized by significantly reduced epithelial and mesenchymal cell mass (Figs. 6B–E, and S8). In 15 feather follicles examined for each gene, about 70% (10/15) showed obvious defects when analyzed at day 4 post-infection. A control virus targeting a random sequence did not produce visible effect ($n = 10$). The impacts of RNAi-mediated gene knockdown were also monitored by pY489 β -catenin staining. Compared to control or *Dkk3* knockdown, *Dkk2*/*Frzb* knockdown significantly increased pY489 β -catenin (Fig. 6F–I), suggesting increased Wnt signaling. Virus expression was confirmed by GFP staining (Figs. 6J–K; S5). Again cell proliferation was decreased in the feather follicles after *Dkk2*/*Frzb* knockdown (Figs. 6L; S6), but no change in apoptosis was found (Figs. 6M; S7).

We further analyzed marker gene expression in the DP. In *Dkk2* or *Frzb* knockdown samples, similar expression levels of DP markers were found, including Desmin/Laminin/SMA and Tenascin (Fig. 7A–L). Masson staining confirmed the retained DP property, but no pulp was formed (Fig. 7M–O). These results differ significantly from *Dkk2*/*Frzb* overexpression. In summary, *Dkk2*/*Frzb* knockdown leads to delayed feather regeneration and reduced pulp formation (Fig. 7P).

Dkk2/*Frzb* regulates feather axis formation

Previous work suggested that a Wnt signaling gradient in the feather follicle helps set up the overall axis of the feather (Yue et al., 2006). However, here we found that Wnt ligands are absent or only weakly expressed in the feather DP. This raises the question of how the DP controls feather axis at the molecular level. In early embryonic development, *xDkk1* is the head-inducer and controls body axis formation (Glinka et al., 1998; Niehrs, 2001; De Robertis, 2006). We tested whether manipulation of *Dkk2*/*Dkk3*/*Frzb* expression in the feather follicle could also alter feather axis formation. To this end, the most dramatic phenotype obtained is by micro-bead coated antibody delivery into the regenerating feather follicle. In 20% of the cases (3/15), we observed the formation of feathers with two-axes from the same follicle (Fig. 8A). The final feather form is two-vanes joined together at the base (Fig. 8B–C), similar to those obtained by DP bisection (Lillie and Wang, 1943). Control serum or antibodies to each individual antigens produced morphologically normal feathers (Figs. 8D and S9). Viral infection mainly led to delayed feather regeneration. In some cases (20%; 3 out of 15), overexpression or RNAi knockdown of *Dkk2*/*Frzb* produced additional axis in the feather, confirming their roles in regulating feather axis formation (Fig. 8E–H). An empty control virus or LV-*Dkk3* transduced follicles remained normal ($n = 10$

for each). The incidence of phenotype by these manipulations was low (about 20%), and no bilateral to radial transition of feather morphology was observed, possibly because lentiviral infection is more homogenous, and only patches of infection would lead to a perturbation of the overall Wnt signaling gradient hence a clear phenotype.

Discussion

Comparison of molecular encodings in the feather and hair DP

Avian feather and mammalian hair are two prominent skin appendages in nature that undergo constant physiological renewal and have robust regeneration capability. They share many similarities, such as a follicular structure, slow-cycling epithelial stem cells, and an inductive DP (Paus and Cotsarelis, 1999; Lin et al., 2013; Yu et al., 2004). There are also distinctions between their growth cycle and regeneration. For example, after removal of the hair bulb together with the DP, the hair follicle still regenerates (Oliver, 1966a, 1966b; Jahoda et al., 1992, 1996; Waters et al., 2011). This is not the case for the feather follicle, as removal of the DP renders the follicle unable to regenerate (Lillie and Wang, 1941, 1944).

One notable molecular feature of the feather DP is the high levels of Wnt signaling inhibitors, including *Frzb*, *Dkk2*, *Dkk3*, *Sfrp1* and *Sfrp2*. Only *Wnt5a* is weakly expressed in the feather DP, otherwise there is an obvious lack of Wnt ligands. High levels of Wnt ligands are found in the epithelium and mesenchymal pulp, including *Wnt5a*, *Wnt5b*, *Wnt6* and *Wnt11*. This intriguing two-compartment distribution pattern may suggest a possible interactive mode (Fig. 9A). We also noticed many TGF- β super-family members and regulators are expressed at high levels in the feather DP, including *Tgf- β 2/3*, *Bmp4/7*, *Tgf- β R1*, *BmpR2*, *Activin-R1*, *Noggin2*, *chordin-like 1*, *decorin*, *Ltbp1*, etc. FGF ligands (*Fgf7*, *Fgf12*), receptors (*Fgfr1*, *Fgfr2*) and modulators (*Spry2*) are present. Consistently, our recent work suggested FGF signaling is important for feather DP maintenance and regulates the feather proximal–distal patterning (Yue et al., 2012). Interestingly, we noticed an absence of Shh signaling pathway members in the feather DP. We and others have shown previously *Shh* is expressed in the ramogenic zone epithelium and regulates feather branching morphogenesis (Yu et al., 2002; Harris et al., 2002, 2005). Given the critical role of Shh signaling in hair DP function (Woo et al., 2012), the absence of Shh signaling in the feather DP may suggest an important distinction. Together, our profiling data provide a rich resource for future investigations on feather growth, morphogenesis, and regeneration. They also offer the opportunity to compare with the hair follicle, an evolutionarily related skin appendage.

The complex role of Wnt signaling in feather regeneration

Wnt signaling is required at various stages during tissue regeneration, such as maturation of the wound epidermis, formation of the blastema, and regenerative outgrowth (Stoick-Cooper et al., 2007a, 2007b). Here we find that Wnt signaling is important for feather regeneration. Overexpressing inhibitors of Wnt signaling such as *Dkk2* or *Frzb* delays feather regeneration. By reducing Wnt signaling, the expression levels of many DP marker genes are reduced, such as *Desmin/Laminin/SMA*. Therefore, Wnt signaling is important for the maintenance of the molecular properties of the feather DP. On the other hand, if *Dkk2/Frzb*

is knockdown, feather DP properties are maintained but pulp formation is delayed. Again feather regeneration is delayed. It seems proliferation and/or initial differentiation of the DP cells, hence the formation of the pulp, requires a reduced Wnt signaling. These results suggest that Wnt signaling controls many aspects of feather regeneration, and an appropriately fine-tuned Wnt signaling both in time and space is required for successful regeneration (Fig. 9B).

Wnt signaling triggers complex downstream events, often categorized into canonical and non-canonical pathways. Among the Wnt ligands expressed at high levels in the feather follicles, some are involved in non-canonical pathways such as *Wnt5a/Wnt11*. The details of these downstream signaling events will need further clarification. In this study, we manipulated the expression levels of *Dkk2/Frzb* by various methods, including overexpression and knockdown. The impacts on canonical Wnt signaling were monitored by activation of β -catenin. Overexpression of *Dkk2/Frzb* reduced pY489 β -catenin, whereas knockdown of *Dkk2/Frzb* increased this particular active form. These results are consistent with their expected roles in Wnt signal transduction.

Epithelial–mesenchymal interactions are important for embryonic development, and similarly for regeneration. Our model thus provides a mechanism for such interactions in feather regeneration: Wnt ligands in the epithelium/pulp maintain the DP property, and *Dkk2/Frzb* in the DP controls appropriate level of Wnt signaling. After wound plucking, the expression levels of Wnt ligands are reduced. The re-acquisition and accumulation of Wnt ligands seem to require the wound healing process and FGF signaling. Our previous work suggested FGF signaling expands, while spry diminishes the feather epithelium (Yue et al., 2012). FGF ligands and *Fgfr1/Fgfr2* are present in the feather DP and therefore may contribute to the re-acquisition of Wnt ligands.

Dkk2/Frzb in feather axis formation

In previous work, we showed that a Wnt signaling gradient controls feather axis and topological arrangement of the branching feather epithelium (Yue et al., 2006). By RCAS-mediated overexpression of *Wnt3a* or *Dkk1*, the feather phenotypes mostly changed from bilateral symmetry to radial symmetry. The more dramatic phenotypes such as two feather axes/vanes were not observed. Here we show that by manipulating *Dkk2/Frzb* expression, either through lentiviral-mediated overexpression/RNAi knockdown, or antibody neutralization, two axes/vanes can be produced. These results suggest that manipulating *Dkk2/Frzb* expression is a more efficient way to control the formation of feather axis. Alternatively, the different methods used to manipulate gene expression in the feather follicles may cause a difference. However, due to the inherited technical difficulties (an early analysis during development would be destructive), and the biological complexity in the system (apparently BMP/noggin signaling is also involved in feather axis formation, among other possible factors) (Yu et al., 2002), a clear description of the axis formation process at the molecular and cellular level is not achieved at this moment.

In summary, we show that the feather DP expresses high levels of *Dkk2/Frzb* that are inhibitors for Wnt signaling, whereas Wnt ligands are mainly expressed in the feather epithelium and pulp. This two-compartment distribution pattern suggests a feedback

interaction. Other signaling pathways may also involve in various stages of feather growth and regeneration, including FGF/BMP/Shh (Fig. 9A). These results provide new insights into the regulatory logic of tissue regeneration.

Materials and methods

Experimental animals

Three to 6 months old chickens were bought from a local farm, and housed in Fuzhou University Animal Facility Center. Adult *Xenopus laevis* frogs were obtained from Nasco. All operations and procedures were according to the Institutional Guidelines of Fuzhou University Ethics Board.

Microdissection

For whole-genome expression analysis, only wing flight feathers in their growth phase were used, which was determined by the overall feather length (usually feathers grow to their half-length were used). The chickens were sacrificed before sample collection. For dissection, the DPs were excised from the follicles directly after plucking the feathers or dissected in vitro (Fig. S2). Erz and Pp were separated using the open-prep procedure described previously (Yue et al., 2006). To collect the required 5 µg total RNAs for analysis, 10 DPs were collected from the wing flight feather follicles from two birds. Pp and Erz samples were from two follicles in their growth phase.

Whole-genome expression profiling and data analysis

Detailed descriptions of the process and data analysis were in Supplemental data. Briefly, samples were collected, and total RNAs were isolated using Trizol reagent (Invitrogen). Total RNAs were quality monitored by Agilent 2100 analysis and sequenced using the Illumina Genome Analyzer at the Beijing Genome Institute (BGI), Shenzhen, China. The sequencing results were annotated according to a reference chicken gene database provided by BGI. Raw data and processed data were deposited in the NCBI database under accession #GSE 42017.

Antibody production, purification and in vivo perturbation

We cloned the full ORF for *cDkk2*, and synthesized the full ORF for *cDkk3* and *cFrzb* (Sangon Biotech, Shanghai). To reduce the high GC content in *cDkk3/cFrzb*, we modified the sequences but kept the amino acid coding. These genes were subcloned into pET-32a, a bacterial expression vector, and His-tag fusion proteins were produced. We purified the proteins by a Ni column (Genescript) following the manufacturer's instruction, and produced antibodies in mice. We further affinity purified the antibodies using an antigen-coupled column (Genescript). These antibodies were used for immunohistochemistry. For feather follicle perturbation, 50 µl antiserum for each antigen, or their mix, or a control antiserum immunized with BSA, were mixed with equal volume of DEAE-Sepharose beads (BBI) in PBS and injected into a plucked wing flight feather follicle. The regenerated feathers were photographed when growing, and collected after finished the growth cycle.

Histology, immunostaining and in situ hybridization

H&E staining, immunostaining and in situ hybridizations were processed as described (Yue et al., 2006). For immunostaining, the feather follicles were collected at day 4 post-infection. Eight μm cryosections were used. We used antibodies for LCAM, NCAM, Laminin, Desmin, SMA, Tenascin C, pY-489 P-catenin, BrdU (Developmental Study Hybridoma Bank), GFP (Santa Cruz). For a comparison of staining intensity in each group, same exposure time was used. For Masson staining, a kit was purchased from ZSBio Co (Beijing), and instructions were followed. RNA probes used in this study: Wnt5a (nt 382–1208; NM_204887.1), Wnt6 (nt 191–507; NM_001007594.2), Dkk2 (nt 961–1260; XM_420494.2), Dkk3 (nt 821–1050; NM_205125.1), Frzb (nt 521–771; NM_204772.2), Fzd8 (nt 800–1138; XM_418566.2), FGFR1 (nt 511–760; NM_205510.1), Sfrp2 (nt 651–925; NM_204773.1), TGF- β 2 (nt 1146–1375; XM_003640970.2), BMP4 (nt 486–755; NM_205237.3), BMP7 (nt 1212–1480; XM_417496.2).

BrdU staining

For BrdU staining, animals were i.p. injected with 50 mg/kg BrdU (Sigma) and samples collected 1 h later. Samples were fixed in 4% PFA in PBS at 4 °C overnight. Eight μm paraffin sections were collected, proceeded for BrdU staining and developed using an AEC substrate. Sections were counterstained with hematoxylin and photographed. Quantification of BrdU staining results were performed by counting the positive cells in each follicle in three sections of the corresponding samples, and normalized to that of a control pLVX virus-infected follicle.

TUNEL staining

For TUNEL staining, a commercial kit from Beyotimes was used and instructions followed. Briefly, paraffin sections were hydrolyzed and digested with 20 $\mu\text{g}/\text{ml}$ proteinase K at 37 °C for 15 min. After 3 \times PBS wash, TdT enzyme and FITC-dUTP reaction buffer was applied to the slide and incubated for 60 min at 37 °C. After 3 \times additional wash with PBS, slides were counterstained with DAPI, mounted and photographed under a Leica fluorescence microscope. Quantification of the staining results was performed by counting positive cells per follicle by three independent investigators.

RT-PCR and qRT-PCR analysis

Total RNAs were isolated using Trizol reagent (Invitrogen) and reverse transcribed using RevertAid first strand cDNA synthesis kit (Fermentas). PCR was performed using a pre-mix from CWBIO, Beijing. The conditions used were: 5 min at 95 °C, 29–33 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 7 min. qPCR was performed in triplicate using SYBR green pre-mix (CWBIO) and a LightCycler480 real-time PCR machine (Roche). Data were quantified using the delta-delta CT method. Each pair of primers was independently tested, with the correct size and single band in electrophoresis. Primer sequences available upon request.

Virus production and infection

Lentivirus were produced and harvested in 293 T cells using the standard protocol. The vector used for overexpression is pLVX-ZsGreen (a gift from Dr. Jun Xu, Tongji University, Shanghai, China), and for RNAi knockdown is pLL3.7. Sequences targeted in shRNA: Dkk2 (ggatgaactccatcaagtc), Dkk3 (gccacttcaagaggagaaa), Frzb (gctaccagaagacctatc), and a random control sequence (agatacagacagaggacact). These sequences were designed according to the Broad Institute website instruction <http://www.broadinstitute.org/rnai/public/>, and blasted to ensure they do not have significant sequence homology with other genes. To test the efficiency of shRNA knockdown, these constructs were transfected into DF-1 cells (ATCC). Cells were collected 48 h later, and total RNAs extracted. Endogenous gene expression levels were quantified and compared with the control. Virus transfection of regenerative feathers and sample processing were performed as described (Yu et al., 2002). Briefly, plucked feather follicles were washed with PBS, and virus supernatant injected immediately. Total injection volume is 80–120 μ l per follicle. To reduce variation in the experiment and avoid bleeding after plucking, only flight feathers in their resting phase were used.

Wnt reporter assay

Wnt responsive Super-TOPFLASH luciferase reporter assays in HEK293T cells were performed in 96-well plates in triplicate as described previously (Wang et al., 2010). Briefly, HEK293T cells reached 50–60% confluence at the time of transfection. Total DNA transfected per each well was 150 ng with VigoFect reagent (Vigorous), using pCS2+ to adjust the DNA amount. Wnt1 was used at 15 ng/well; 10 ng TOPFLASH and 1 ng Renilla luciferase plasmids were co-transfected. Forty-eight hours later, the cells were lysed and luciferase activity determined using Dual-luciferase assay kit (Promega). TOPflash luciferase activity was normalized to that of Renilla.

Xenopus embryo injection

cDkk2, *cDkk3* and *cFrzb* were subcloned into pCS2+ vector using PCR and verified by sequencing. The plasmids were linearized with NotI and transcribed with SP6 RNA polymerase according to the manufacturer's instructions (MEGAscript kit, Ambion). In vitro synthesized mRNAs were injected into 4-cell stage embryos at the equatorial region. For control animals, mRNA of *preprolactin* gene was injected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2014.01.010>.

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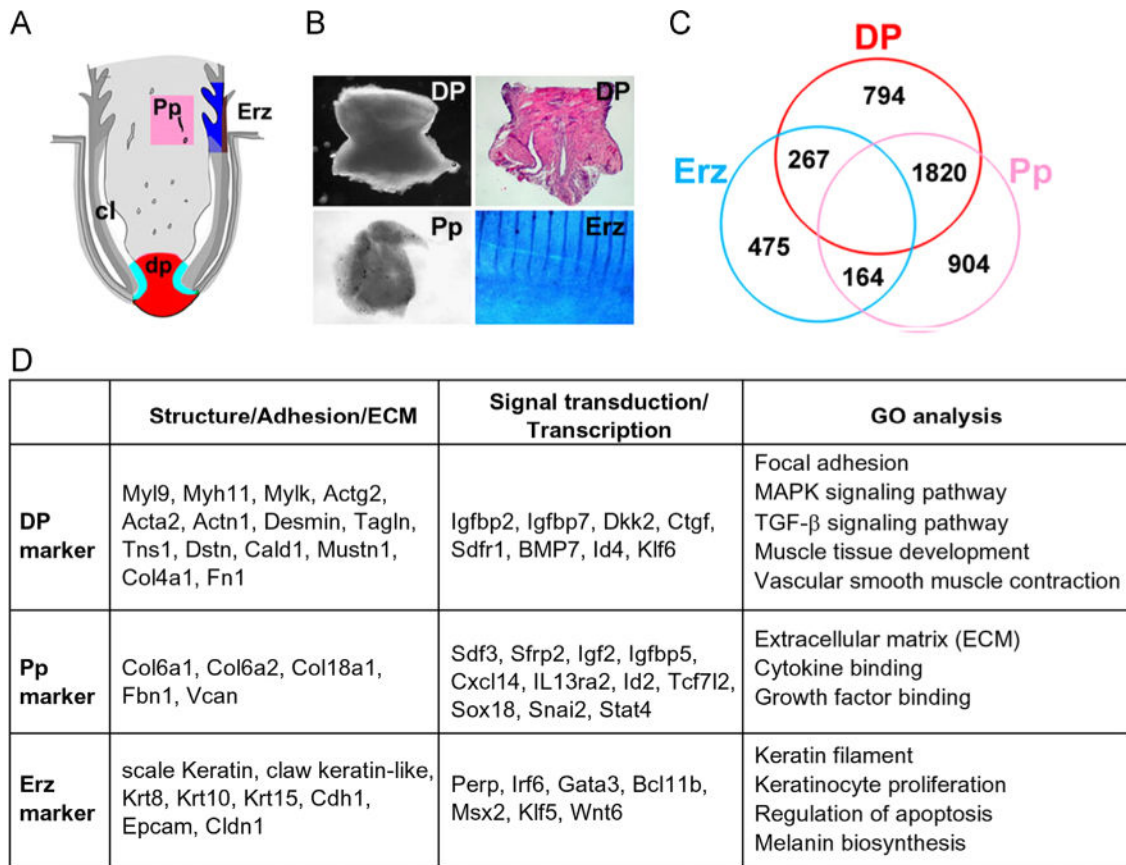


Fig.1.

Gene expression profiling in the feather follicle. (A) Diagram and (B) examples showing the feather structure and the dissection process. H&E showing the structure of the DP. Erz was illustrated based upon DAPI staining. The stripes in the Erz sample are feather branches. dp, dermal papilla; Pp, pulp; Erz, ramogenic zone feather epithelium. (C) Venn diagram showing differentially expressed genes among DP, Pp and Erz. (D) Lists of highly expressed genes in each compartment that could serve as “markers”. The gene abbreviations are according to the NCBI listings. Gene ontology (GO) analysis results are also shown.

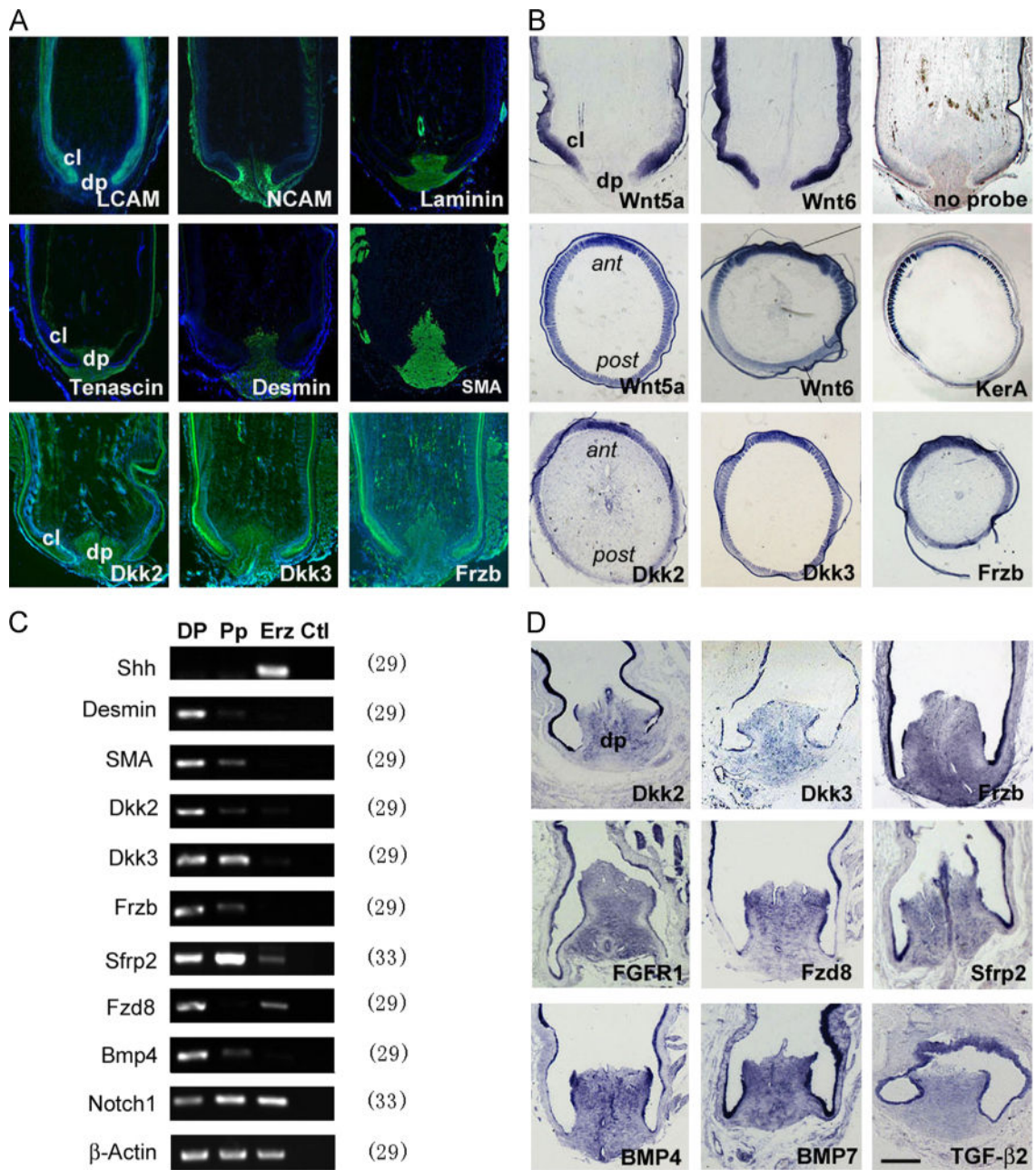


Fig. 2. Gene expression analysis in the feather follicle. (A) Marker gene expression shown by immunofluorescence (green). LCAM marks the feather epithelium. NCAM marks the DP/dermal sheath and weakly the feather branching epithelium. Laminin marks the DP and vessel walls. Tenascin marks the DP/dermal sheath. Desmin is more DP specific, and SMA marks the DP and vessel walls. Dkk2/Dkk3/Frzb is enriched in the DP, presents in the pulp but less in the epithelium. Some unspecific staining is found in the keratinized feather sheath. (B) Expression of Wnt ligands and inhibitors in the feather follicles shown by in situ hybridization. Notice *Wnt5a* and *Wnt6* appear primarily in the epithelium. A gradient distribution pattern is detected for *Wnt5a/Wnt6*, *Dkk2/Dkk3/Frzb* and feather keratin A in the

Erz region. A control staining with no probe is also shown. (C) Semi-quantitative RT-PCR and (D) in situ hybridization analysis of gene expression in the feather follicles. No template reactions are used as control for PCR analysis, and equal amount of RNAs are monitored by β -Actin gene expression. The number after each gene indicates PCR cycles. dp, dermal papilla; cl, collar; ant, anterior where the rachis locates; post, posterior as opposite to the rachis position. Bar = 1 mm in A and B, and 0.5 mm in D (shown in D).

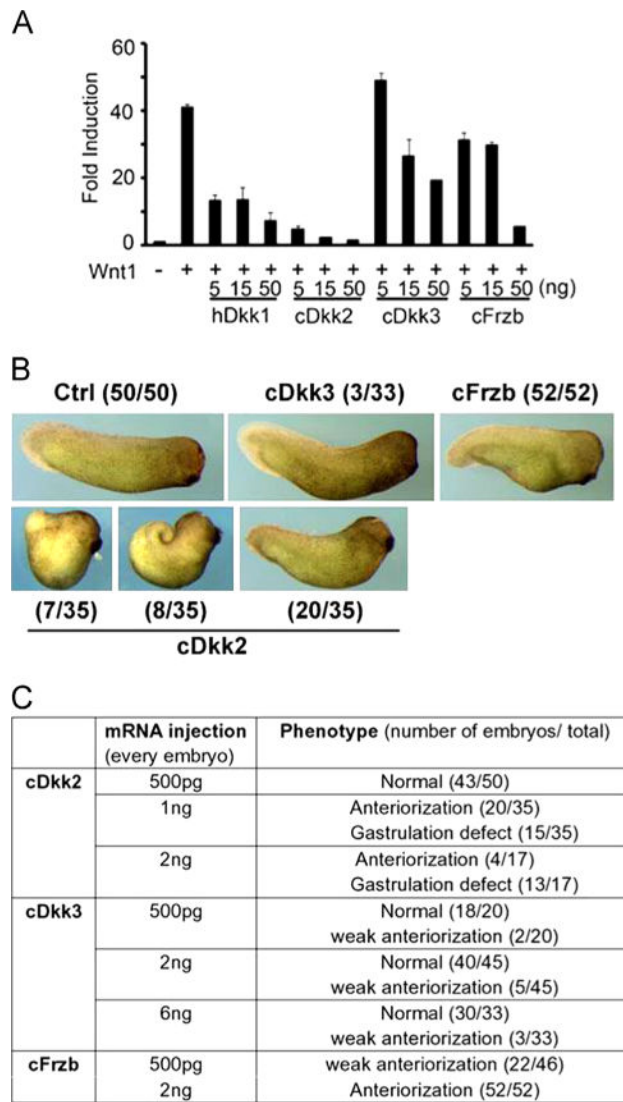


Fig. 3. *Dkk2/Frzb* antagonizes Wnt signaling. (A) Wnt reporter assay in HEK 293T cells. SuperTOPFLASH, a Wnt responsive reporter was co-transfected into HEK293T cells together with Wnt1 and other plasmids as indicated. hDkk1 (human Dkk1) was used as a positive control. Fold induction of Wnt reporter activity is shown. The numbers for each gene indicates the amount of DNA transfected; total amount of DNA transfected in each well was 150 ng, adjusted with pCS2+ plasmid. (B) Anteriorization of *Xenopus* embryos by injected mRNAs as indicated. mRNAs of chicken Dkk2 (1 ng per embryo), Dkk3 (6 ng per embryo) or Frzb (2 ng per embryo) were injected at 4-cell stage. The numbers of embryos with indicated phenotypes are also shown. (3/33) stands for 3 out of 33 injected embryos showed indicated phenotype. Control animals were injected with 250 pg mRNA of *preprolectin* gene. (C) Summary of the *Xenopus* injection experiment.

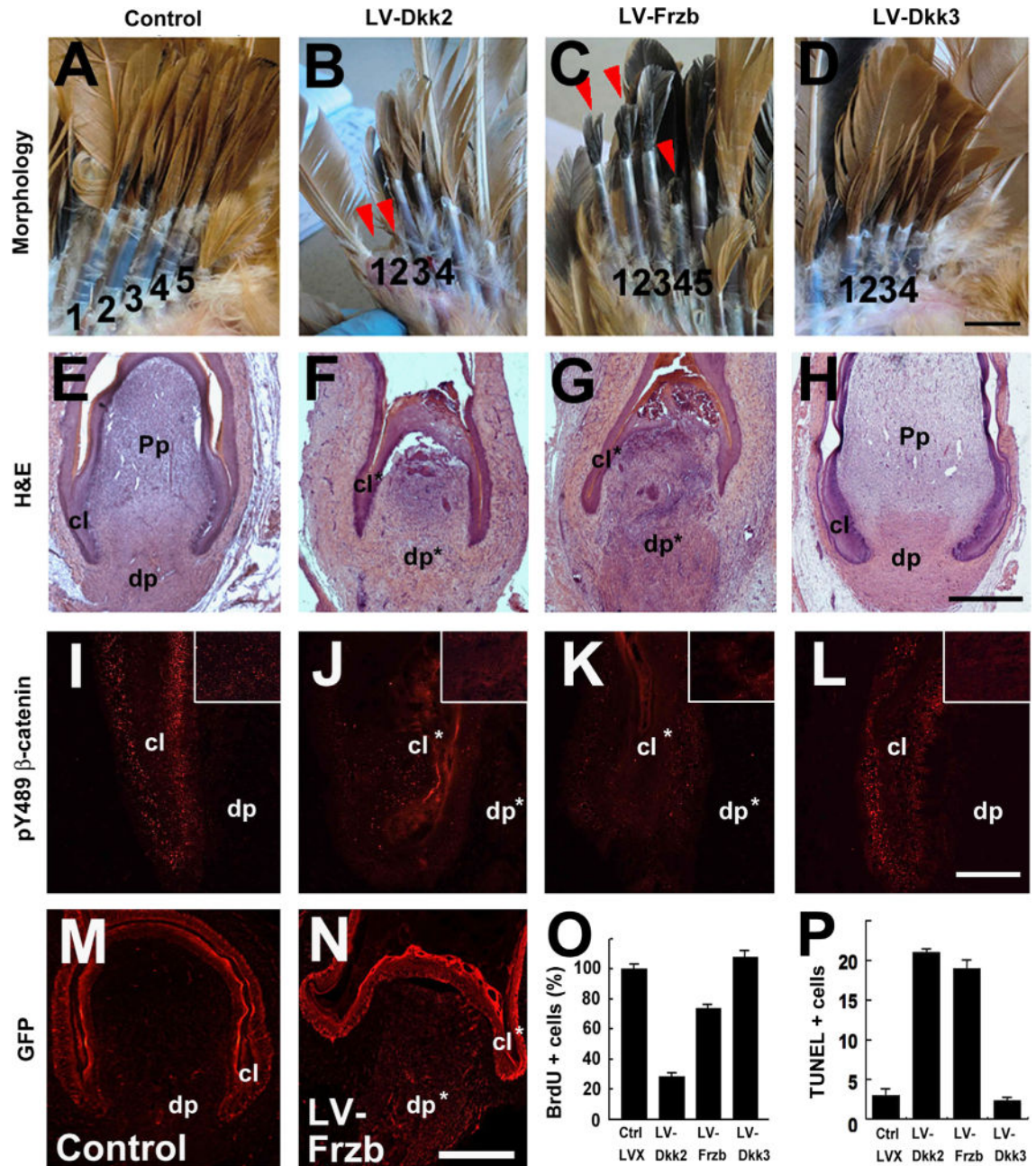


Fig. 4. Overexpression of *Dkk2/Frzb*, but not *Dkk3*, disrupts feather regeneration. (A–D) Representative samples of lentivirus (LV) mediated gene overexpression in the feather follicle. A control virus carrying GFP only was also shown. Four to five wing flight feather follicles in their resting phase were plucked to induce regeneration and infected with the virus, and photographed 3 weeks afterwards. Delayed feather regeneration was indicated by arrow heads. (E–H) H&E analysis of virus-infected feather follicles 4 days post-infection. (I–L) pY489 β -catenin antibody staining (red spots) showing the reduced Wnt signaling after *Dkk2/Frzb* overexpression. The epithelia in the collar regions are shown, and the DP regions are shown in inserts. (M and N) Anti-GFP immunostaining (red) showing lentiviral-

mediated GFP expression in the feather follicles. (O and P) Quantification of BrdU and TUNEL staining results in the feather follicles. The dp, Pp and collar (cl) are labeled; * indicates the disrupted structures. Bar = 1 cm (A–D, shown in D), 1 mm (E–H, shown in H; M and N, shown in N), and 200 μm (I–L, shown in L).

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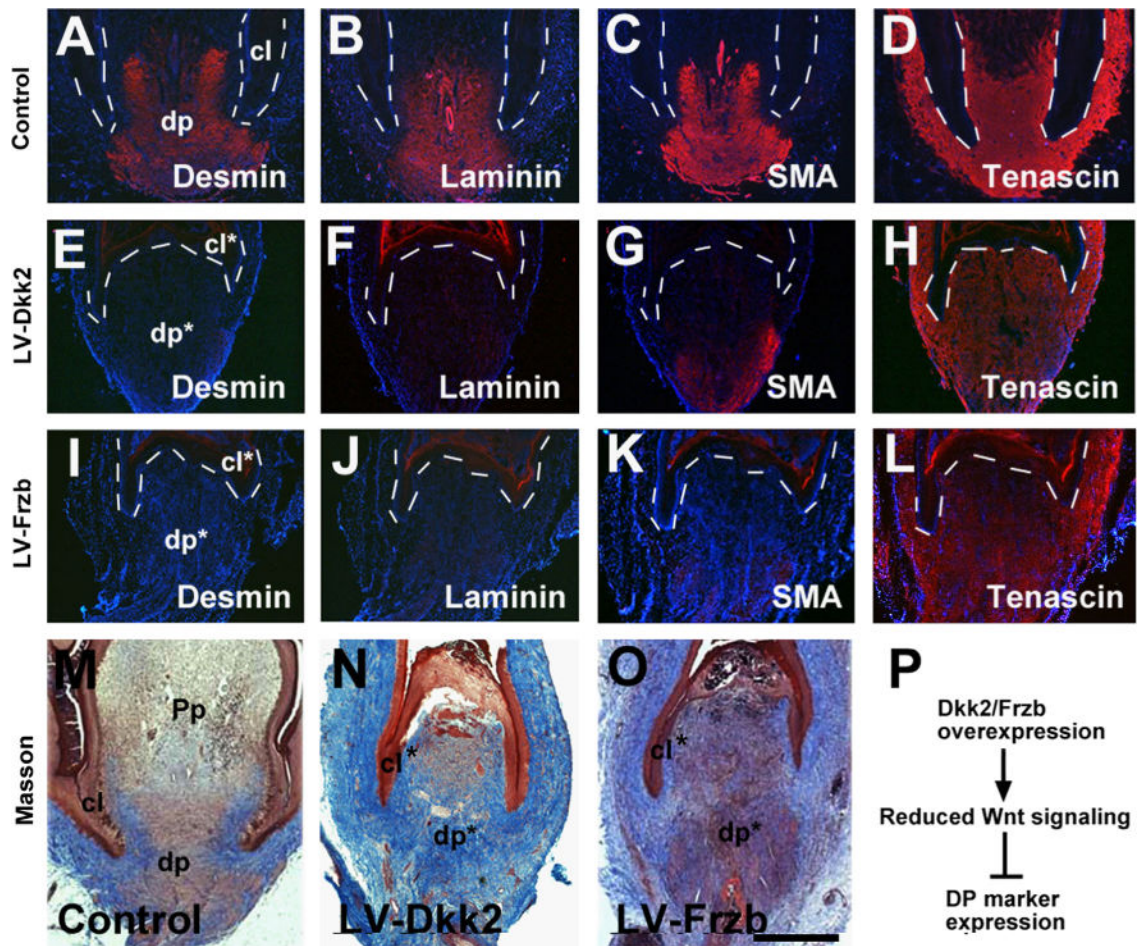


Fig. 5. Overexpression of *Dkk2/Frzb*, but not *Dkk3*, reduces DP marker gene expression. (A–L) DP marker gene expression showing reduced Desmin/Laminin/SMA, but not Tenascin in LV-*Dkk2* and LV-*Frzb* transduced feathers. An empty pLVX virus was used as control. Samples were collected at day 4 post-infection. For a comparison of staining intensity, equal exposure time was used when taken pictures. (M–O) Masson staining showing altered DP characteristics in *Dkk2/Frzb* overexpressed follicles. (P) Summary of the events after *Dkk2/Frzb* overexpression. Dashed lines indicate the epithelial–mesenchymal borders. The dp and collar (cl) are labeled; *indicates the disrupted structures. Bar = 1 mm (shown in O).

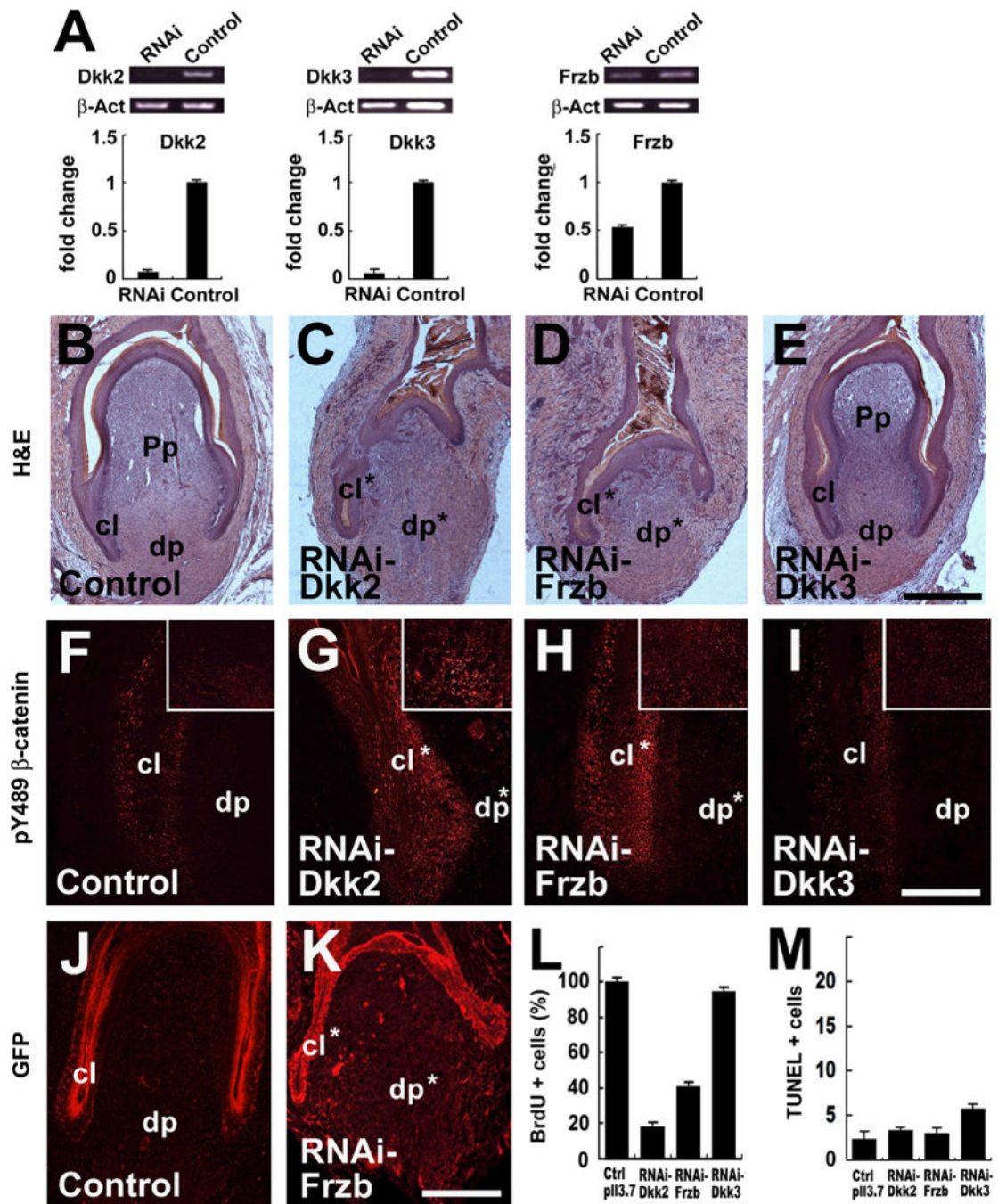


Fig. 6. RNAi knockdown of *Dkk2/Frzb*, but not *Dkk3*, disrupts feather regeneration. (A) Efficiency of shRNA tested in DF-1 cells. Semi-quantitative RT-PCR and qPCR results were shown. A construct targeting a random sequence was used as control. Endogenous mRNA levels were quantified. (B–E) Representative samples of lentiviral-mediated shRNA knockdown in the feather follicles 4 days post-infection. A shRNA virus targeting a random sequence was used as control. (F–I) pY489 β -catenin antibody staining (red spots) showing the increased Wnt signaling after *Dkk2/Frzb* knockdown. The epithelia in the collar regions are shown,

and the DP regions are shown in inserts. (J–K) Virus expression monitored by GFP staining (red). (L and M) Quantification of cell proliferation (BrdU staining) and apoptosis (TUNEL) in the feather follicles. The dp, Pp and collar (cl) are labeled; *indicates the disrupted structures. Bar = 1 mm (B–E, shown in E; J and K, shown in K), and 200 μ m (F–I, shown in I).

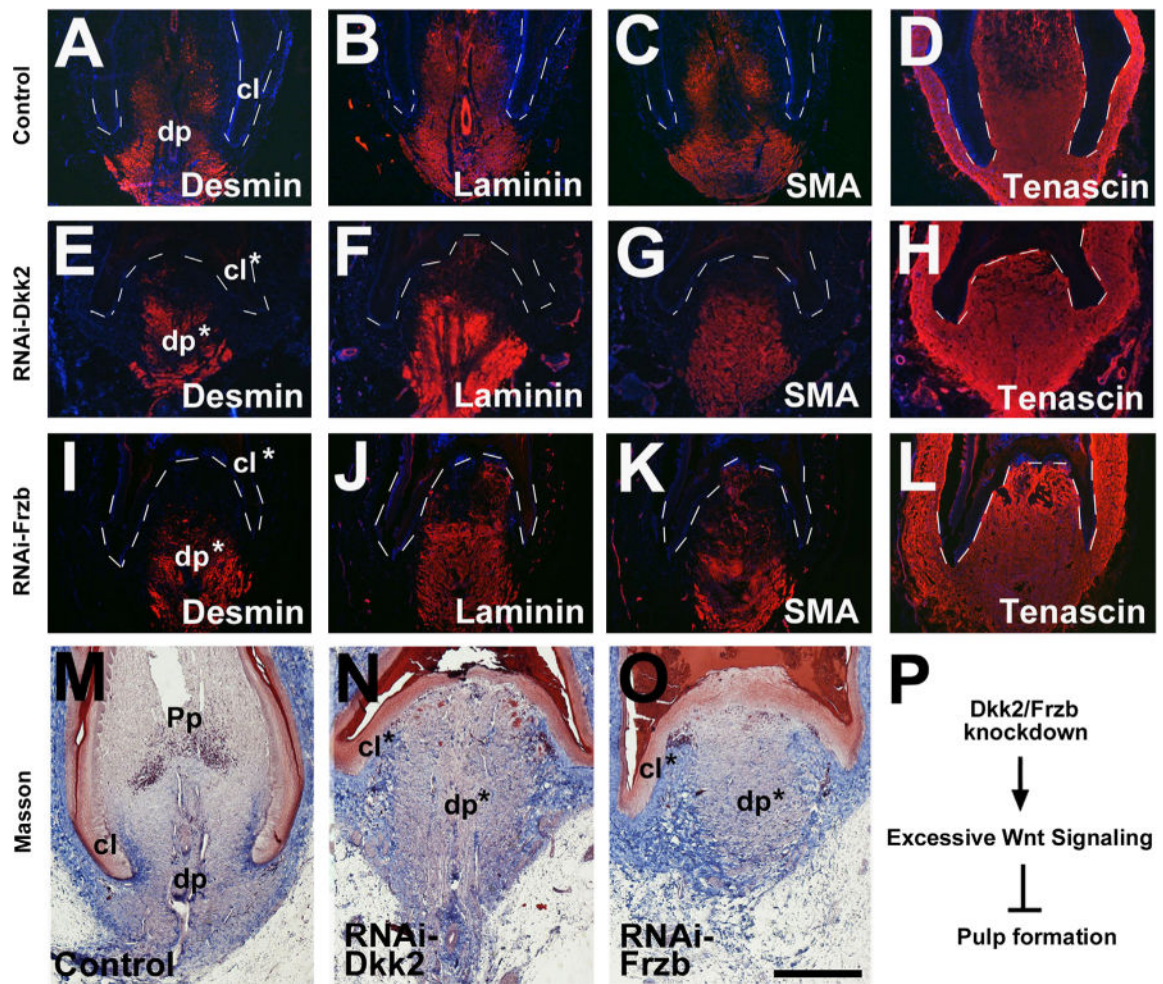


Fig. 7. RNAi knockdown of *Dkk2/Frzb*, but not *Dkk3*, maintains DP properties but reduces pulp formation. (A–L) RNAi-*Dkk2/Frzb* maintains DP marker gene expression. A virus targeting a random sequence was used as control. Samples were collected at day 4 post-infection. For a comparison of staining intensity, equal exposure time was used when taken pictures. (M–O) Masson staining showing *Dkk2/Frzb* knockdown retains DP characteristics but reduces pulp formation. (P) Summary of the events after *Dkk2/Frzb* knockdown. Dashed lines indicate the epithelial–mesenchymal borders. The dp and collar (cl) are labeled; * indicates the disrupted structures. Bar = 1 mm (shown in O).

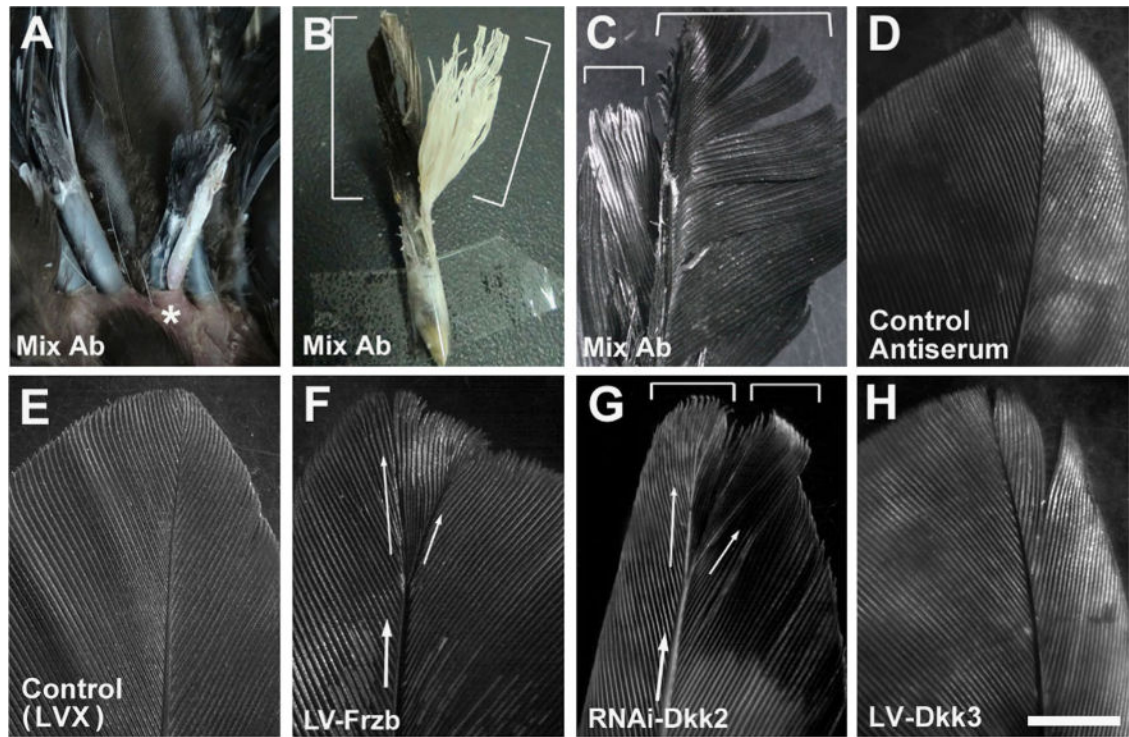


Fig. 8. *Dkk2/Frzb* regulates feather axis formation. (A–D) Micro-bead mediated antibody delivery into the feather follicle induces two feathers from a single follicle (marked by*). Two feather vanes were formed in 20% (3/15) of the cases. Control antiserum delivery caused no abnormality in the feather ($n = 5$). (E–H) Lentiviral-mediated overexpression of *Frzb* or RNAi knockdown of *Dkk2* produced two feather axes, and to a less extent, two feather vanes in 20% of the cases (3/15). No abnormality was produced by an empty viral vector pLVX transduction in the control feather follicles ($n = 10$), or LV-*Dkk3* transduced feather follicles ($n = 10$). Bar = 1 cm (shown in H).

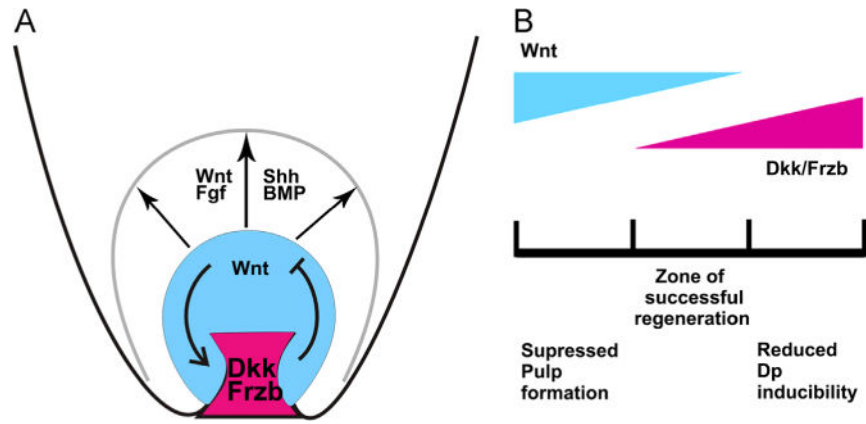


Fig. 9. Wnt/Dkk regulates feather growth and regeneration. (A) The feather DP expresses high levels of Wnt inhibitors including Dkk2 and Frzb. These inhibitors interact with the mostly epithelial/pulp Wnt signaling, and regulate feather regeneration and axis formation. Other signaling molecules are also involved in various stages of feather growth and regeneration, including FGF/BMP./Shh etc. (B) The Wnt signaling must be properly balanced to promote successful feather regeneration. When *Dkk2/Frzb* is overexpressed (reduced Wnt signaling), there is less DP property; when *Dkk2/Frzb* is knockdown (excessive Wnt signaling), pulp formation is reduced. Both will lead to delayed feather regeneration.

Table 1

Expression of major signaling pathway members in the feather follicle.

	Gene	DP	Pp	Erz
Reference genes	β -Actin	1340	2406	660
	Gapdh	3914	2720	2600
Wnt signaling pathway	Wnt5a	8	90	27
	Wnt5b	1	15	19
	Wnt6	0	12	30
	Wnt11	1	15	0
	Wnt4	4	2	4
	Wnt3a	0	0	1
	Fzd8	36	3	16
	Fzd7	24	8	10
	Fzd2	16	21	1
	Fzd1	20	19	4
	Fzd6	3	33	3
	Fzd9	6	6	30
	Frzb	785	630	4
	Dkk2	230	27	3
	Dkk3	190	173	10
	Sfrp1	158	107	1
	Sfrp2	95	346	0
TGF-β superfamily signaling Pathway	Ltbp1	485	1108	5
	Decorin	285	58	14
	Chordin-like1	110	2	2
	Tgf-b3	48	96	43
	Tgf-b2	12	2	4
	Inhba	7	36	0
	Admp	5	3	110
	Bmp4	256	144	1
	Bmp7	111	28	17
	Bmp2	2	5	1
Other signaling molecules	Noggin2	46	18	0
	Fgfr1	305	215	25
	Fgfr2	42	28	33
	Fgf7	14	2	0
	Fgf12	8	8	3
	Spry2	19	25	5
	Notch1	115	245	262
	Jagged1	40	24	30
	Serrate2	67	42	69

Values are tags-per-million (TPM) counts from gene expression profiling. DP, dermal papilla; Pp, pulp; Erz, ramogenic zone feather epithelium.