Two developmental modules establish 3D beak-shape variation in Darwin's finches

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Bird beaks display tremendous variation in shape and size, which is closely associated with the exploitation of multiple ecological niches and likely played a key role in the diversification of thousands of avian species. Previous studies have demonstrated some of the molecular mechanisms that regulate morphogenesis of the prenasal cartilage, which forms the initial beak skeleton. However, much of the beak diversity in birds depends on variation in the premaxillary bone. It forms later in development and becomes the most prominent functional and structural component of the adult upper beak/jaw, yet its regulation is unknown. Here, we studied a group of Darwin's finch species with different beak shapes. We found that *TGFβIIr*, *β-catenin*, and *Dickkopf-3*, the top candidate genes from a cDNA microarray screen, are differentially expressed in the developing premaxillary bone of embryos of species with different beak shapes. Furthermore, our functional experiments demonstrate that these molecules form a regulatory network governing the morphology of the premaxillary bone, which differs from the network controlling the prenasal cartilage, but has the same species-specific domains of expression. These results offer potential mechanisms that may explain how the tightly coupled depth and width dimensions can evolve independently. The two-module program of development involving independent regulating molecules offers unique insights into how different developmental pathways may be modified and combined to induce multidimensional shifts in beak morphology. Similar modularity in development may characterize complex traits in other organisms to a greater extent than is currently appreciated.

craniofacial evolution | evolvability | variability | evolutionary developmental biology | natural variation

Modern evolutionary developmental biology postulates that adaptive morphological changes in adult organisms ultimately originate by alterations in particular developmental programs (1, 2). Thus, exploring cases in which the developmental pathways responsible for evolutionary changes can be identified and characterized is pivotal to our understanding of the origin of morphological diversity (3, 4). In this study, we aimed to understand how changes in developmental controls of a morphological trait may constrain or facilitate diversification. To this end, we focused on unraveling the molecular and developmental mechanisms responsible for patterning the differences in avian beak shapes—which are usually associated with differences in diet and ecological niche—by taking advantage of the natural diversity of beak shapes in the iconic Darwin's finches.

Bird beaks are 3D structures that show a tremendous amount of variation in size and shape along the depth, width, and length axes. Variation in beak shape has profound impacts on the ability of an organism to survive and reproduce in the wild and, thus, has played a major role in the radiation of thousands of species of birds, the most diverse group of land vertebrates (5, 6). Therefore, its adaptive significance coupled to the extreme levels of diversity observed in nature make this trait ideal for tackling developmental and evolutionary questions about morphological diversification in general.

Adult beak morphology is determined by the development of two components, the prenasal cartilage (pnc; the ethmoid process of the nasal septum) followed later in development by the premaxillary bone (pmx). Recent research suggests that in contrast to endochondral mode of bone formation, in which bone develops from a cartilage template (7–10), craniofacial bone (dermatocranium) and cartilage (chrondrocranium) skeletons may represent largely independent tissue modules, which are controlled by different genes and form from separate condensations (11–17). Although the extent of such modularity requires further investigation, it provides a useful framework to study beak development. Our previous studies of pnc formation in Darwin's finches identified two signaling molecules, *Bmp4* and *CaM* that regulate early differences in beak morphogenesis (18, 19) and so provide a partial explanation for beak-shape differences between finch species (19). Comparable studies of the pmx are lacking, and are greatly needed for three reasons. First, it is the most prominent functional and structural component of the adult bird upper beak/jaw, and much of beak diversity in birds depends on variation in this structure (20). Second, it is not known whether molecules such as *Bmp4* and *CaM* have the same roles at this crucially important stage of development or what the relative importance of pnc and pmx tissue modules is in establishing the species-specific differences. Third, previous morphological and genetic studies have failed to explain how beak depth and width became uncoupled in the evolutionary radiation of Darwin's finches (19, 21). Therefore, we undertook an investigation of the developmental mechanisms responsible for generating variation in the pmx of closely related species of this important group of birds. In doing so, we address fundamental questions concerning the evolution of beak-shape diversity.

Darwin's finches (Thraupinae, Passeriformes) of the Galápagos and Cocos Islands comprise a monophyletic group of 14 closely related species that represent a classic example of adaptive radiation, niche partitioning, and rapid morphological evolution (22–25). In a relatively short period (2–3 my), this group has evolved a diversity of bill shapes adapted to exploiting different food items, particularly under conditions of food scarcity (25). Within the monophyletic genus *Geospiza*, the small, medium, and large ground finches (*G. fuliginosa*, *G. fortis*, and *G. magnirostris*, respectively), which we refer to as "ground finches" in this article, have evolved a series of deep and broad beaks increasing in size that are used to crush seeds. This series of ground finches contrasts with the more elongated and narrow

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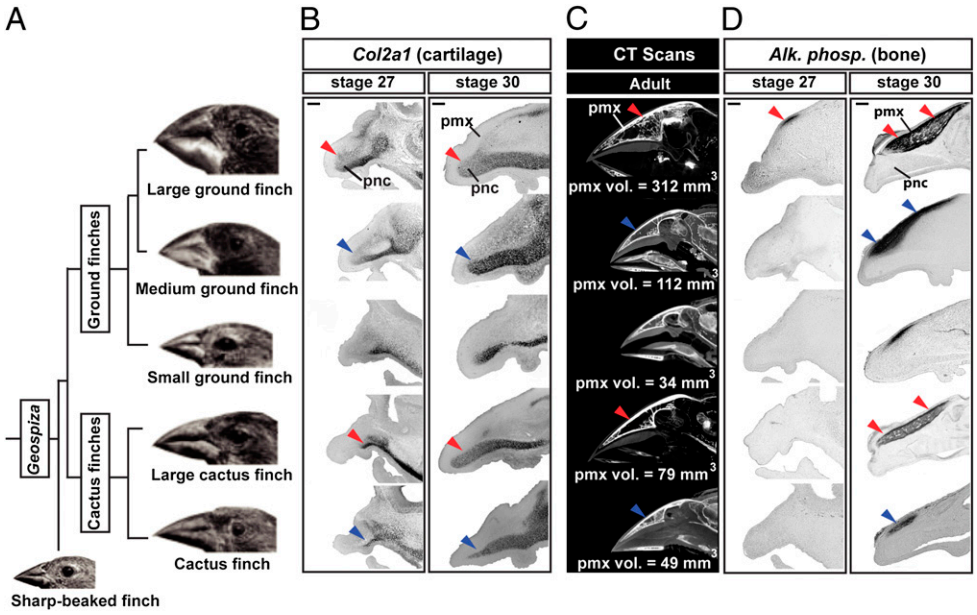

Fig. 1. Contribution of the different skeletal structures to beak variation in Darwin's finches. (*A*) The large, medium, and small ground finches have deep beaks used to crack seeds. In contrast, the large cactus and cactus finches use their elongated beaks to feed on pollen and nectar from flowers. (*B*) At stage (st.) 27 the prenasal cartilage (pnc) condensation, labeled with *Col2a1*, occupies a significant portion of the beak primordia in the large and medium ground finches. By st. 30, the pnc, labeled with *Col2a1*, occupies a smaller proportion of the beak relative to the developing premaxillary bone (pmx). (*C*) Interspecific variation in Darwin's finches is caused mainly by differences in the amount of the pmx. The large and medium ground finches have larger pmx volumes than their size-matched large cactus and cactus finches, respectively. (*D*) These differences in adult pmx volume correlate with the time and place of expression of the osteogenic marker alkaline phosphatase (*Alk. Phosp.*) during embryonic development. Arrow colors in *B*–*D* indicate species that have comparable body sizes but differ in beak morphology. [Scale bars: 0.15 mm (stage 27) and 0.2 mm (stage 30) in *B*; 0.2 mm in *D*.] Molecular tree is from ref. 26.

beaks used by the large cactus and cactus finches (*G. conirostris* and *G. scandens*) to probe deeply into flowers for nectar and pollen (Fig. 1*A*).

Here, we present the results of a microarray screen and comparative gene expression analyses in Darwin's finch embryos, followed by functional experiments in the chicken model system, to determine the molecular and developmental mechanisms responsible for beak-shape differences among species. We find that the pnc in early development and the pmx during late development are regulated by two different sets of molecules. In combination they can alter growth along each beak axis independent of the others, thereby generating variability in both size and shape of this ecologically important trait. Our results suggest that a full understanding of the evolutionary diversification of beak form in birds requires taking into account the regulation of both pnc and pmx tissue modules.

Results and Discussion

During beak development, the pnc and the pmx condensations are established when the beak primordia form (20). The prenasal cartilage is the first skeletal structure to mineralize and establish species-specific beak shapes during early embryonic development (18, 20). As revealed by the expression pattern of the chondrogenic marker *Col2a1*, at embryonic stage 27 (st. 27), the pnc occupies a large portion of the developing upper beak primordia and explains differences in beak shape of the large and medium ground finches at this stage (Fig. 1*B*). However, its relative contribution to forming overall adult beak dimensions is significantly diminished by st. 30 (Fig. 1*B*) (18). At this later stage, the pmx begins to expand from its own condensation and it is this structure that ultimately determines the size and shape of the adult beak (20).

According to recent mechanical models, the pmx is the principal element of the adult bird upper beak responsible for dissipating and distributing forces generated during consumption of hard seeds (27, 28). Correspondingly, our analyses of microcomputed tomography (CT) scan data showed that the adult large and medium ground finches have considerably larger pmx volumes than the cactus finches and are, thus, ideal for analysis of variation in the pmx (Fig. 1*C* and Table S1). To determine when the species-specific differences in pmx are first established, we examined the expression of alkaline phosphatase, an osteogenic marker, in embryos of five species from the genus *Geospiza* at two critical stages of beak development, st. 27 [embryonic day (E) 5.5] and st. 30 (E6.5) (18, 19). In the species with the largest pmx volume, the large ground finch, alkaline phosphatase was expressed in the condensation of the pmx earlier than in any

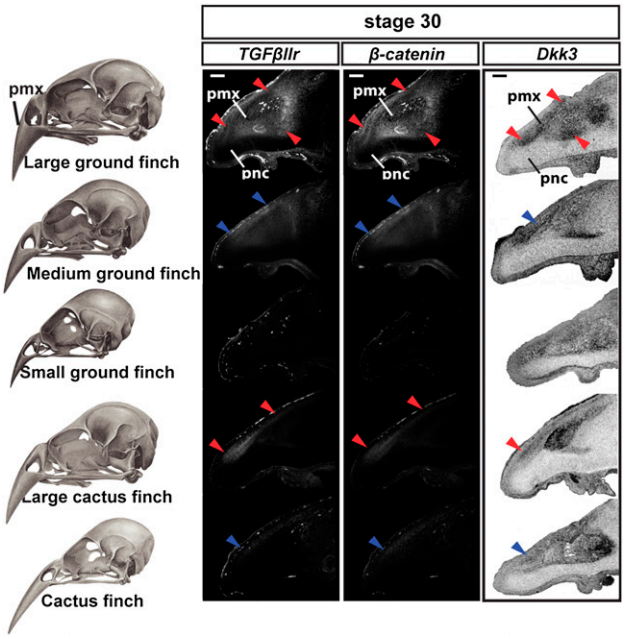

Fig. 2. Variation in the pmx in *Geospiza* correlates with the expression of *TGFβIIr*, *β-catenin*, and *Dkk3*. At st. 30, the large and medium ground finches have high expression levels of *TGFβIIr*, *β-catenin*, and *Dkk3* in strong correlation with the volume of the developing pmx. Arrow colors indicate species that have comparable body sizes but differ in beak morphology. (Scale bar, 0.2 mm.) Images of skulls are from ref. 24, with permission from the author. pmx, premaxillary bone; pnc, prenasal cartilage.

other species (st. 27), indicating that this species undergoes a heterochronic shift in the osteogenesis of this tissue. At the later st. 30, the pmx condensation in the large and the medium ground finches expands to occupy most of the upper beak primordium and expresses higher levels of an osteogenic marker than size-matched cactus finches (Fig. 1*D*). Thus, results from this analysis show that differences in adult pmx volume in Darwin's finches correlate with the time and place of expression of osteogenic markers during embryonic development.

Previously, we showed that two different molecules, *Bmp4* and *Calmodulin* (*CaM*), regulate growth along different dimensions of the developing beak in Darwin's finches (depth/width and length, respectively) by patterning the pnc element (18, 19). However, our functional tests showed that *Bmp4* and *CaM* do not regulate morphogenesis of the pmx directly (18, 19). To identify genes, in an unbiased manner, that might explain the variation seen in the pmx of different species, we took advantage of the previously conducted cDNA microarray-based screen in which we directly compared expression of several thousand transcripts from st. 26 upper beak primordia in Darwin's finches (19). We searched for transcripts whose expression levels correlated with the beak shapes of the large and the medium ground finches as they have considerably deeper and larger pmx than the other species (Fig. 1*C* and Table S1). We identified three transcripts, *TGFβ receptor type II* (*TGFβIIr*), *β-catenin*, and *Dickkopf-3* (*Dkk3*), that were expressed at 4- to 10-fold higher levels in the large ground finches than in the reference species, the sharp-beaked finch (*G. difficilis*) (Table S2). These three new candidates represented significant developmental pathways and were not housekeeping or ribosomal genes.

TGFβIIr, an integral gene of the TGFβ pathway, is a serine/threonine protein kinase that upon ligand binding initiates a series of phosphorylation events that can lead to the regulation of gene transcription (29). *TGFβIIr* is important for craniofacial skeletal development in mammals, and mutations in this gene are associated with certain human craniofacial abnormalities (30) but its function in morphogenesis of bird beaks has not been previously reported. *β-catenin* is a subunit of the cadherin protein complex and an integral component of the Wnt signaling pathway (31). Whereas nuclear translocation of *β-catenin* in the osteogenic cells is both required and sufficient for terminal bone cell differentiation, the relationship between its expression level and osteogenic potential is unknown (32). *Dkk3* encodes a secreted protein, which is the most divergent member of the *Dkk* family in terms of sequence and function, and, unlike the other members of the *Dickkopf* family, is not known to regulate Wnt signaling (33). Although *Dkk3* is known to be expressed during craniofacial development in mouse embryos (34), its role in bird beak morphogenesis has never been established.

We observed a striking correlation between adult beak morphology and expression of our three new candidate genes. The three genes were expressed in broader domains in the large and the medium ground finches than in cactus finches, especially in the large ground finch, in which all three genes were expressed in

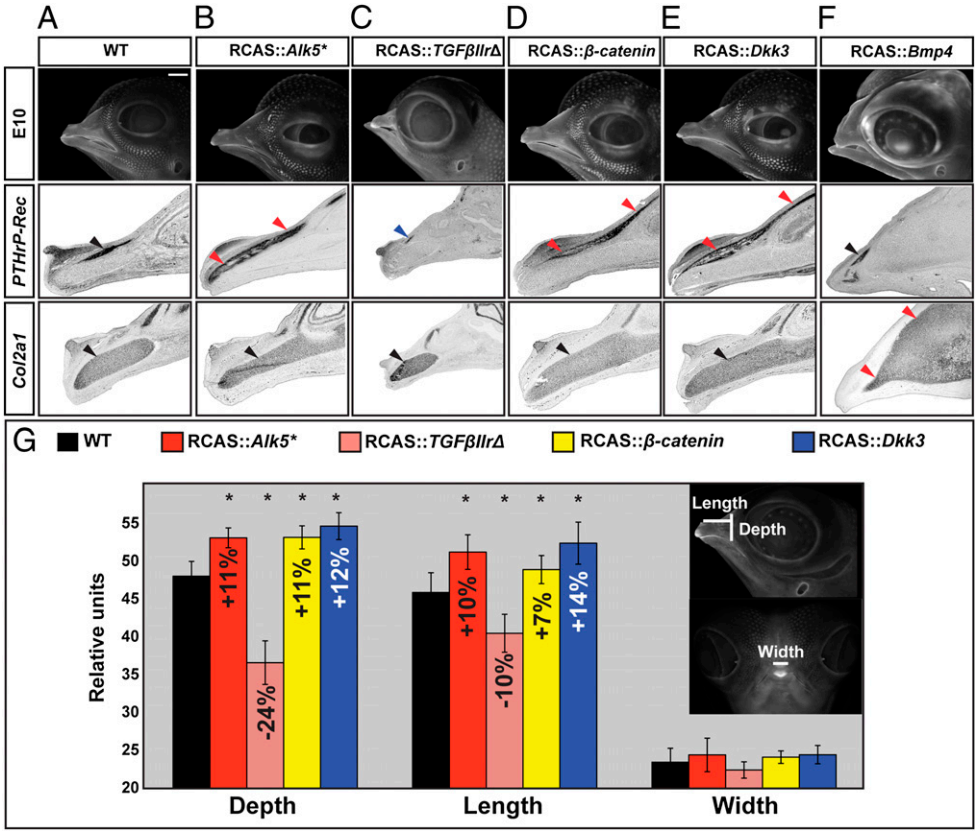

Fig. 3. Functional analysis of *TGFβIIr*, *β-catenin*, and *Dkk3* in the chicken model system. (*A–F*) UV pictures of embryonic day 11 (HH st. 37). (*A*) Wild-type chicken embryos and embryos infected with (*B*) RCAS::*Alk5**, (*C*) RCAS::*TGFβrΔ*, (*D*) RCAS::CA-*β-catenin*, (*E*) RCAS::*Dkk3*, and (*F*) RCAS::*Bmp4* constructs. We used *PTHrP-Rec* and *Col II* probes to reveal early osteoblasts (*PTHrP-Rec*) and chondrocytes (*Col2a1*) (also see Fig. S3). Blue arrows indicate lower expression relative to wild-type specimens, red arrows indicate higher expression, and black arrows indicate no change. (*G*) Histogram showing beak variation in wild-type and RCAS-infected chicken embryos. Embryos infected with RCAS::*Alk5** ($n = 8$), RCAS::*TGFβrΔ* ($n = 9$), RCAS::CA-*β-catenin* ($n = 9$), and RCAS::*Dkk3* ($n = 15$), showed a significant change in their depth and their length relative to wild-type controls ($n = 9$), whereas the width remained (asterisks denote significance at $P < 0.05$, *t* test; error bars represent SD values). (Scale bars: 200 mm in whole-head images and 0.4 mm in sections *A–F*.)

most of the dorsodistal part of the upper beak primordium that accommodates the pmx condensation (Fig. 2 and Figs. S1 and S2). More specifically, at st. 27, the three molecules were expressed throughout most of the beak mesenchyme (except in the prenasal cartilage) in the large ground finches, whereas they were confined to a much smaller region in the size-matched large cactus finches (Figs. S1 and S2). By st. 30, both the large and medium ground finches expressed these molecules in broader domains in the osteogenic beak mesenchyme than the corresponding large cactus and cactus finches, respectively (Fig. 2 and Fig. S2). Notably, *TGFβIIr* and *β-catenin* accumulated in a restricted domain at the distal beak region in the large cactus and cactus finches in contrast to the broad domains for these genes found in the large and medium ground finches (Fig. 2 and Fig. S2).

To determine the functional significance of the observed correlations, we used the replication-competent retroviral vector (RCAS) in the chicken embryo model to mimic the broader and stronger expression patterns of *TGFβIIr*, *β-catenin*, and *Dkk3* seen in the large and medium ground finches (Fig. 3). Infection with a constitutively active version of the *TGFβ* type I receptor (RCAS::*Alk5**), with a construct-driving expression of the stabilized version of *β-catenin* (RCAS::CA-*β-catenin*), and with a construct carrying the full-length chick homolog (RCAS::*Dkk3*), all led to a significant increase in both beak depth and length, relative to the uninfected controls, whereas beak width remained relatively unchanged (Fig. 3 *A*, *B*, *D*, *E*, and *G*). Most if not all increase in beak dimensions resulted from changes in the pmx element, as revealed by chondrogenic and osteogenic markers (Fig. 3 *A*, *B*, *D*, and *E* and Figs. S3 and S4). In addition, when we infected chicken embryos with a dominant-negative construct to decrease the levels of TGFβ signaling (RCAS::*TGFβr*Δ), we found a significant decrease in beak depth and length, whereas there was little effect on beak width (Fig. 3 *A*, *C*, and *G*). Likewise, this decrease in depth and length was a result of the diminished pmx dimensions (Fig. 3 *A* and *C*). Together, these data suggest that *TGFβIIr*, *β-catenin*, and *Dkk3*, in good correlation with their spatial and temporal expression, act by positively regulating the size and shape of the pmx.

These results differed from the significant joint increase in beak depth and width observed when *Bmp4* signaling is up-regulated in the chicken embryonic beak with the RCAS::*Bmp4* viral construct (Fig. 3 *A* and *F* and Figs. S3 and S4). Misexpression of the three new candidate molecules did not produce a marked effect on the development of pnc (Fig. 3 *A*–*E* and Fig. S3), whereas increased levels of *Bmp4* led to a drastic expansion of the cartilage element and a decrease in pmx production and dimensions (Fig. 3 *A* and *F* and Figs. S3 and S4) (18). This effect results from a greater recruitment of mesenchymal cells for cartilage formation, which in turn causes the depletion of the cells available for the formation of bone, even when bone itself is not infected (18, 19). Therefore, the effect of *Bmp4* up-regulation on the final beak shape must be indirect, perhaps by providing extensive matrix support for the nascent pmx later in development. Because the pnc does not directly cause the pmx to form, the massive expansion of this latter tissue module is likely to be a combination of the early increment in the pnc matrix support and later autonomous effects of *TGFβIIr*, *β-catenin*, and *Dkk3*, which positively regulate its size. Because our current functional tools do not allow us to turn off high levels of *Bmp4* in infected developing beaks after early and midstages of development and after pnc expansion, it is not possible to establish the role of pnc expansion by itself on later pmx development. However, the fact that high levels of *Bmp4* reduces pmx expansion in later beak development in infected embryos underscores how differently the two tissue modules are regulated.

Because *TGFβIIr*, *β-catenin*, and *Dkk3* displayed largely overlapping domains of expression in the beak primordia and were coexpressed in many of the same mesenchymal cells (Fig. S5), they could potentially be regulating each other's expression during beak development. To investigate this possibility, as well as possible interactions between these genes and *Bmp4* and *CaM*, we used chicken embryos to analyze the effects of misexpressing each candidate molecule on the other genes (Fig. 4 *A* and *B* and Fig. S6). We found that expression of *TGFβIIr* did not change in any of the infected embryos relative to controls, as measured by quantitative real-time PCR and in situ hybridization (Fig. 4*A* and Fig. S4). Similarly, *β-catenin* remained consistent and only increased in embryos infected with the RCAS::CA-*β-catenin* construct, suggesting a positive feedback interaction (Fig. 4*A* and Fig. S7). Up-regulation of the TGFβ pathway or *β-catenin* caused a strong up-regulation of *Dkk3* expression (Fig. 4 *A* and *B*). Conversely, down-regulation of the TGFβ pathway produced a decrease in the expression of *Dkk3*, suggesting that *Dkk3* is downstream of both TGFβ and *β-catenin* pathways (Fig. 4*B*). Expression of *Bmp4* remained unchanged across treatments and was only significantly reduced in RCAS::*Bmp4* infected embryos, indicating a negative feedback interaction (Fig. 4*A* and Fig. S4). Furthermore, up-regulation of the TGFβ pathway and of *Dkk3* caused an increase in the expression of *CaM* around the pmx (Fig. 4*A* and Fig. S6). However, because *CaM* expression in Darwin's finches is localized around the pnc before the appearance of *TGFβIIr* and *Dkk3* expression (19), we conclude that *TGFβIIr* and *Dkk3* do not regulate *CaM*-mediated pnc morphogenesis in Darwin's finches. In addition, because the expression domain of *CaM* in ground finches does not extend to the pmx condensation during

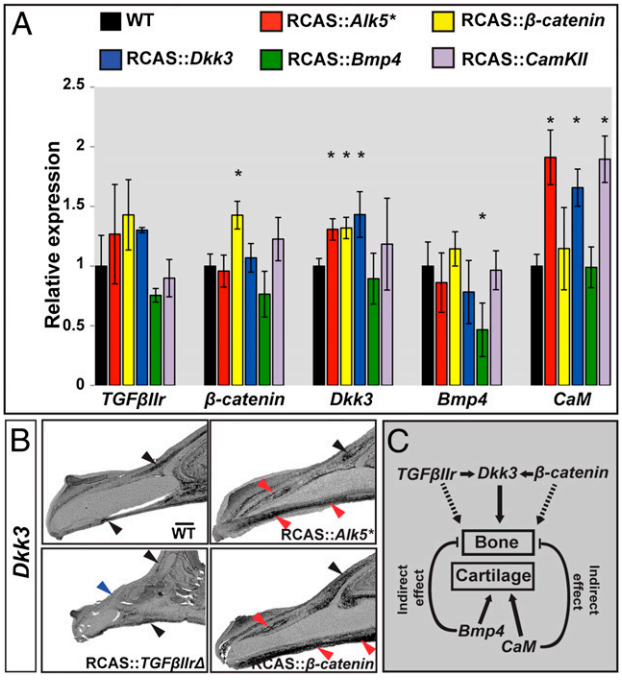

Fig. 4. Interaction of genes regulating beak development. (*A*) Quantitative real-time PCR assays measuring gene expression levels of *TGFβIIr*, *β-catenin*, *Dkk3*, *Bmp4*, and *CaM* in embryos infected with RCAS constructs. Expression levels are shown relative to wild-type uninfected controls (asterisks denote significance at $P < 0.05$, *t* test, $n = 5$; error bars represent SD values). Up-regulation of the TGFβ pathway and of *β-catenin* led to higher expression of *Dkk3* (*A* and *B*). Conversely, down-regulation of the TGFβ pathway caused a decrease *Dkk3* expression (*B*). Blue arrows indicate lower expression relative to wild-type specimens, red arrows indicate higher expression, and black arrows indicate no change (see text for details). (Scale bar, 0.4 mm.) (*C*) A general model of beak development in which *Bmp4* and *CaM* act independently to alter the growth of the prenasal cartilage and *TGFβIIr*, *β-catenin*, and *Dkk3* regulate the premaxillary bone.

the stages studied, its function in bone tissue may be limited to ossification processes at later stages (19). This analysis demonstrates that *β-catenin*, *TGFβIIr*, *Bmp4*, and *CaM* do not regulate each other's expression (Fig. 4*A* and Fig. S6) and can regulate beak development independently by altering different axes of growth. In summary, *Bmp4* and *CaM* play important roles in the early expansion of the pnc skeleton in ground and cactus finches, respectively (18, 19). This sets the stage, likely indirectly, for the later morphogenesis of the pmx, which is patterned through the coordinated action of a network of a different set of interacting regulatory molecules, *TGFβIIr*, *β-catenin*, and *Dkk3* (Fig. 4*E*) (18, 19).

The observed differences in beak morphologies among members of the genus *Geospiza* can be better explained by analyzing both the functions and the expression patterns of the genes examined here (Fig. 5 and Fig. S7). For example, during the evolution of a specialized granivore morphology, exemplified for example by *G. magnirostris* and *G. fortis*, there was a similar allometric increase in beak depth and width, relative to the basal condition represented by *G. difficilis* (21, 35). Of all of the genes we have analyzed so far, *Bmp4*, which regulates these two dimensions in a coordinated way, is likely to be the most prominent player in generating this morphology by drastically increasing the size of the pnc along these two axes. However, in the evolution of *G. magnirostris* and *G. fortis*, beak length also increased allometrically, albeit not so markedly, and this change cannot be explained by action of *Bmp4* alone (18), suggesting that the *TGFβIIr*, *β-catenin*, *Dkk3* regulatory network may be regulating growth along this axis. In cactus finches, average beak depth and beak width did not change in the same proportions, relative to *G. difficilis*; depth increased nearly twice as much as width in both *G. conirostris* and *G. scandens* (21, 35). This morphology could have arisen through an initial influence of *CaM* on the pnc (19), and subsequently by the *TGFβIIr*, *β-catenin*, *Dkk3* regulatory network, which causes the pmx to increase in depth as well as length without concomitant change in width. Thus, by examining development of the pmx we have learned how depth and width may be uncoupled in Darwin's finches, e.g., in cactus or tree finches (21). Importantly, we have learned that knowledge of pnc regulation is insufficient for understanding beak variation, but we have also learned that regulation of pmx is not sufficient either. Two regulatory networks are needed to understand the diversity of beak form. Our results are consistent with the hypothesis that beak differences in Darwin's finches are established by combined and complementary changes in regulation of the pnc and the pmx.

The embryonic expression patterns indicate that the differences in the way the pnc is regulated in ground and cactus finches is paralleled by a similar difference in the way the pmx is regulated in these two groups of finches. In ground finches, the main factors regulating pnc and pmx (*Bmp4* and the *TGFβIIr*, *β-catenin*, *Dkk3* network, respectively) are broadly expressed in the dorsal region of the developing beak mesenchyme, whereas in cactus finches, the genes regulating the pnc and pmx (*CaM* and the *TGFβIIr*, *β-catenin*, *Dkk3* network) accumulate in a restricted domain of the distal region (18, 19) (Figs. 2 and 5). This suggests that the incipient species differences that are set up during pnc development become strengthened when the pmx forms via similar regulatory mechanisms in similar locations.

Because natural selection acts on phenotypic variation within populations, detailed studies aimed at understanding variation in intraspecific developmental programs will be fundamental to establish how interspecific differences evolve. Previous studies in Darwin's finch populations of *G. fortis* and *G. scandens* have shown that, whereas beak depth and width are strongly correlated, both phenotypically and genetically, each is correlated less strongly with beak length (35), suggesting that beak length has greater scope for independent evolutionary change than the others. Because the genes controlling the pnc and pmx can affect all three beak dimensions differently, mutational changes in the timing, strength, and/or place of their expression could alter trait relations. If the resulting changes in adult beak proportions are advantageous, the mutations could rise to fixation. In agreement with heritability studies of single populations, which have demonstrated the polygenic nature of beak size and shape variation in Darwin's finch populations (25, 36), we have identified a number of genes that could be responsible for generating such changes, and as a result more fully understand at the molecular level that there is both linkage and independence in the variation along different beak axes (19).

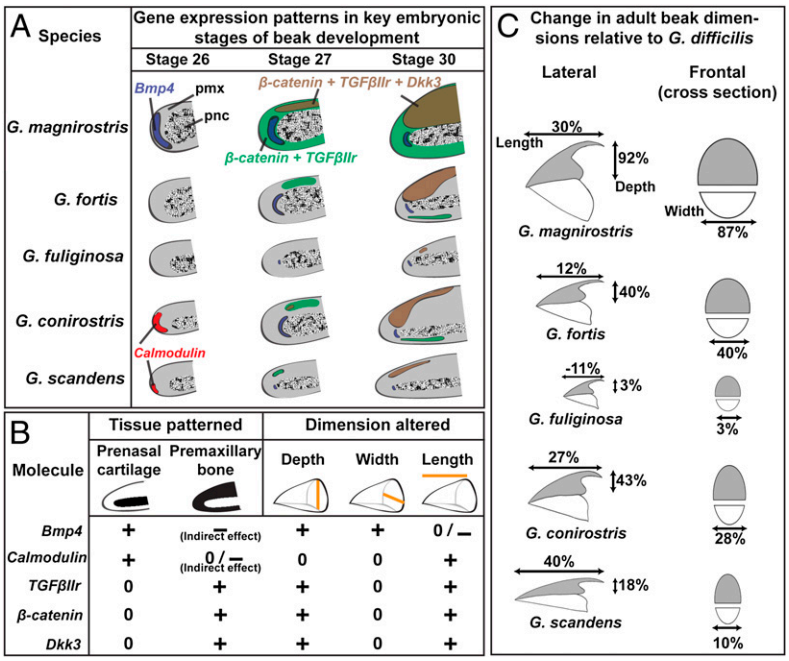

Fig. 5. The distinct beak morphologies in *Geospiza* are generated by differences in the time and place of expression of different genes. (*A*) Species with deep beaks, such as *G. magnirostris* and *G. fortis*, have earlier and broader expression of *Bmp4*, *TGFβIIr*, *β-catenin*, and *Dkk3*, whereas expression of *CaM*, *TGFβIIr*, *β-catenin*, and *Dkk3* is localized distally in species with elongated beaks, such as *G. scandens* and *G. conirostris*. (*B*) Through their action on different skeletal tissues, different genes alter independent dimensions of growth. (*C*) The beak of the sharp-beaked finch, *G. difficilis*, represents a basal morphology for *Geospiza* (26, 37). Expression and function of the genes described here explain changes in beak dimensions of the more derived species. +, positive effect; 0, no effect; –, negative effect; pmx, premaxillary bone; pnc, prenasal cartilage. Measurements in *C* were taken from ref 21, corrected for wing length, and correspond to averages from males that were collected in the islands where we obtained our samples.

The two-module program of development involving independent regulating molecules provides a more comprehensive view of the potential for evolutionary change than has been obtained so far because it allows for multidimensional variability (2, 38, 39). Furthermore it may offer mechanisms to explain how the tightly coupled depth and width dimensions can evolve to some extent independently. Because all modern birds share the same overall beak skeletal structure, although differing remarkably in size, proportions, and curvature, our results provide a general framework for understanding how this great diversity is brought about developmentally. One obvious need for the future is an investigation into the development of other highly divergent beak shapes, including such extreme cases of beak curvature as those of hummingbirds, Madagascan vangas, and some of the Hawaiian honeycreepers (5). A major advance in understanding will come with the identification of mutations in upstream genetic loci responsible for species-specific differences in adult beak form and their relationship to the developmental mechanisms reported here.

Materials and Methods

Please see *SI Materials and Methods* for quantitative real-time PCR analysis, quantification of gene expression area, and micro-CT scans. Details of the microarray production and data analysis are described elsewhere (19) and in the *SI Materials and Methods*.

Darwin's Finches' Embryo Collection and Preparation. Embryos of Darwin's finches were collected according to regulations established by the Galapagos National Park using methods described in detail elsewhere (38). A total of 33 embryos were analyzed: *G. magnirostris* (st. 27, $n = 3$; st. 30, $n = 3$), *G. fortis* (st. 27, $n = 4$; st. 30, $n = 5$), *G. fuliginosa* (st. 27, $n = 3$; st. 30, $n = 3$), *G. scandens* (st. 27, $n = 3$; st. 30, $n = 5$), and *G. conirostris* (st. 27, $n = 2$; st. 30, $n = 2$).

In Situ Hybridizations and Immunohistochemistry. In situ hybridizations were performed as described before (40) using the in situ hybridization antisense probes for chicken. Additional details are described in the *SI Materials and Methods*.

Functional Experiments in Chicken Embryos. CDNA fragments containing a constitutively active form of the *TGFβIr* (*Alk-5*), a dominant negative form of *TGFβIIr*, and the entire coding region of the chicken *Dkk3* were cloned into the *SLAX-13* vector and then subcloned into *RCAS(BP)A* using methods described before (41). RCAS::CA-*β-catenin*, RCAS::*Bmp4*, and RCAS::CA-*CamKII* constructs have been described previously (see *SI Materials and Methods* for RCAS constructs references). Viruses were harvested, concentrated, and titered using methods described before (39). Fertilized eggs were obtained from SPAFAS, incubated at 37 °C, and staged according to Hamburger and Hamilton. Frontal nasal processes were infected at st. 24 and embryos were collected at stage 36, fixed overnight in 4% paraformaldehyde, washed in PBS, stained with SYBR-safe (Invitrogen), and photographed under UV light under a Discovery v8 stereoscope (Carl Zeiss). Frontal and lateral images were taken for each head, and measurements of different upper beak parameters were recorded in triplicate using the Axiovision 4.6.3 software (Carl Zeiss) (19). The extent of viral infection was assayed by in situ hybridization with the viral-specific probe, RSCH. Up-regulation of *β-catenin* and of *Dkk3* was monitored using the antibodies and in situ hybridization probe described above.

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