Bmp4 and Morphological Variation of Beaks in Darwin's Finches

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Supporting Online Material
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Bmp4 and Morphological Variation of Beaks in Darwin's Finches

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Darwin's finches are a classic example of species diversification by natural selection. Their impressive variation in beak morphology is associated with the exploitation of a variety of ecological niches, but its developmental basis is unknown. We performed a comparative analysis of expression patterns of various growth factors in species comprising the genus *Geospiza*. We found that expression of *Bmp4* in the mesenchyme of the upper beaks strongly correlated with deep and broad beak morphology. When misexpressed in chicken embryos, *Bmp4* caused morphological transformations paralleling the beak morphology of the large ground finch *G. magnirostris*.

Darwin's finches are a group of 14 closely related songbird species on the Galápagos Islands and Cocos Island (*1*–*3*) collected by Charles Darwin and other members of the *Beagle* expedition in 1835 (*4*). Many biology textbooks use these birds to illustrate the history of evolutionary theory as well as adaptative radiation, natural selection, and niche partitioning (*5*–*7*). The diverse shapes and sizes of the finch beaks are believed to be maximally effective for exploiting particular types of food, including seeds, insects, and cactus flowers (*3*, *7*). The external differences in beak morphology reflect differences in their respective craniofacial skeletons (*3*, *8*). The specialized beak shapes are apparent at hatching (*3*, *8*) and thus are genetically determined.

To study the craniofacial development of Darwin's finches, we first developed a staging system by which we could compare them to each other and to the chicken, the existing avian model system (fig. S1) (*9*). We used this system to compare beak development in six species of Darwin's finches belonging to the monophyletic ground finch genus *Geospiza*. The sharp-beaked finch *G. difficilis*, with a small symmetrical beak, is the most basal species (Fig. 1A) (*10*). The other species fall into two groups: three species with broad and deep beaks used for crushing seeds (small, medium, and large ground finches—*G. fuliginosa*, *G. fortis*, and *G. magnirostris*) and cactus finches with long pointed beaks used for reaching into cactus flowers and fruits (cactus and large cactus finches—*G. scandens* and *G. conirostris*) (Fig. 1A) (*7*, *10*).

We compared beak development in embryos of all six species. Species-specific differences in the morphological shape of the beak prominence are first apparent by embryonic stage 26 (Fig. 1, B and C, and fig. S2). We therefore expected factors involved in directing the differential aspects of beak morphologies to be expressed at or before this time. We also expected such species-specific differences to reside in the mesenchyme on the basis of recent transplantation experiments between quail and duck embryos (*11*).

We analyzed expression patterns of a variety of growth factors, which are known to be expressed during avian craniofacial development (*12*–*14*), among the different *Geospiza* species, using in situ hybridizations on equivalent medial sections (as revealed by the presence of Rathke's pouch; fig. S3) of stage 26 and stage 29 embryos (Fig. 1, B and C, and fig. S4) (*15*). We looked for factors whose expression in the mesenchyme of the beak prominence correlated with the increasing depth and width of beaks seen as one compares *G. difficilis* to *G. fuliginosa*, *G. fortis*, and *G. magnirostris*. To eliminate changes in expression that were merely related to the overall size of the bird and not to changes in beak morphology, we also compared expression patterns in *G. scandens* and *G. conirostris,* which are similar in size to *G. fortis* and *G. magnirostris*, respectively, but share the more pointed beak morphology (Fig. 1A).

Most factors examined either showed no difference between Darwin's finches species (including *Shh* and *Fgf8*) (*15*) or, in the case of *Bmp2* and *Bmp7*, correlated with the size of the beak but not its shape (fig. S4). In contrast, we observed a striking correlation between beak morphology and the expression of *Bmp4* (Fig. 1, B and C). In *G. difficilis*, *Bmp4* expression is first seen at low levels in the subectodermal mesenchyme at stage 26 (Fig. 1B). Once the cartilage condensation has occurred at stage 29, *Bmp4* continues to be expressed in mesenchymal cells surrounding the most rostral part of the prenasal cartilage. When the embryos of the three ground finch species were examined, we noted a dramatic increase in the level of *Bmp4* expression in *G. magnirostris* at stage 26, whereas *Bmp4* expression in all the other species was more or less equivalent to that in *G. difficilis* (Fig. 1B). By stage 29, however, all three ground finch species displayed elevated levels of *Bmp4* expression, with *G. magnirostris* being the strongest and *G. fuliginosa* the weakest of these. *G. scandens*, a relatively pointed-beaked species of similar size to *G. fortis*, and *G. conirostris*, which is similar in size to *G. magnirostris*, did not show this increase in relative levels of *Bmp4* expression (Fig. 1C). The expression patterns of all factors were examined in three or four independent embryos for each species (except for *G. scandens*, for which two embryos were examined), and the results were consistent. Thus, the species with deeper, broader beaks relative to their length express *Bmp4* in the mesenchyme of their beak prominences at higher levels and at earlier stages (a heterochronic shift) than species with relatively narrow and shallow beak morphologies. Moreover, the differences in *Bmp4* expression are coincident with the appearance of species-

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specific differences in beak morphology. This observed correlation was specific to *Bmp4* expression in the upper beak, whereas expression of *Bmp4* in the lower beak remains constant in spite of the fact that lower beak morphology varies in concert with that of the upper beak (*15*).

We next tested whether the observed change in *Bmp4* expression could be partially responsible for the differences in beak morphology in ground finch species. *Bmp4* has been previously shown to be important for the production of skeletogenic cranial neural crest cells and capable of affecting patterning, growth and chondrogenesis in derivatives of the mandibular and maxillary prominences (*16*–*19*). However, the expression of *Bmp4* is quite dynamic during craniofacial development and might be expected to play different roles at various times.

During craniofacial development, *Bmp4* is first expressed in the epithelium of the maxillary and lateral frontonasal prominences in early embryos. The same factor is later expressed in the distal mesenchyme of the upper beak of embryos at stage 29 and later (Fig. 2, A and B). We took advantage of the ability to misexpress genes during chicken development with the RCAS replication-competent retroviral vector to test the effect of increasing BMP4 levels in both of these domains. Because the RCAS vector does not spread across basement membranes, we were able to confine misexpression to either the facial ectoderm or mesenchyme (Fig. 2, C and E). Infection of the facial ectoderm with the RCAS::*Bmp4* virus caused smaller and narrower upper beaks (fig. S5). Ectodermally infected beaks also showed a dramatic loss of chondrogenesis in the adjacent mesenchyme (Fig. 2, D and F, and fig. S5), indicating a role in epithelial–to-mesenchymal signaling early in head morphogenesis.

In a second set of misexpression experiments designed to mimic the elevated levels of *Bmp4* seen in *G. magnirostris*, we injected RCAS::*Bmp4* virus into the mesenchyme of the frontonasal process of chicken embryos at stage 23 to 24. Because of the time required for viral infection and spread, this results in robust misexpression in the distal frontonasal process around stage 26 (*15*), which is the time when elevated *Bmp4* levels are first seen in *G. magnirostris*. The phenotypes we obtained were quite different from those resulting from epithelial misexpression, showing that *Bmp4* expression has distinct functions in the epithelium and mesenchyme. Rather than diminished beaks, beaks resulting from infection of the mesenchyme were reminiscent of those of the ground finches with deep and broad beaks. These morphological changes in beak morphology were observed before the onset of skeletogenesis, as revealed by *Col II* expression (*15*). By stage 36, the infected beaks ($n = 13$) were on average about 2.5 times as wide (±21%) and 1.5 times (±16%) as deep as uninfected control beaks ($n = 11$; $P < 0.003$) (Fig. 3, A, B, D, and E). The more massive *Bmp4*-infected beaks had a corresponding increase in the size of the skeletal core (Fig. 3, G and H, and fig. S6), again in parallel to a larger beak skeleton of *G. magnirostris*. This skeletal phenotype was observed in the majority of the infected embryos ($n = 11$ out of 13). These data suggest that BMP4 may have a proliferating effect on skeletal progenitors in the upper jaw. Indeed, we find that cell proliferation, as assessed by bromodeoxyuridine (BrdU) labeling, is highest in a zone of the upper beak process where *Bmp4* is expressed (Fig. 3, J to L; marked with arrow in J, asterisks in L and O). Moreover, this zone of high cell proliferation expands and shows a higher level of proliferation after RCAS::*Bmp4* misexpression (Fig. 3, M to O). A similar phenotype was observed in a study reported in an accompanying paper, where *Bmp4* was misexpressed as part of a study comparing its role in the development of the beak in ducks and chickens (*20*). In contrast, mesenchymal injection of the RCAS::*Noggin* virus, which antagonizes BMP2/4/7 signaling, led to a dramatic decrease in the size of the upper beak and to much smaller skeletal elements inside the upper beak ($n = 7$ out of 9; $P < 0.002$) (Fig. 3, C, F, and I).

We have identified variation in the level and timing of *Bmp4* expression that correlates with variation in beak morphology in Darwin's finch species. We are tempted to speculate that differences in the cis-regulatory elements of *Bmp4* may underlie the distinct expression patterns, although alternatively they could be explained by differences in the timing or amounts of upstream inductive factors or differences in the transduction of such signals. Two such potential upstream signals are *Sonic hedgehog* (*Shh*) and *Fibroblast growth factor 8* (*Fgf8*), which are expressed in the beak epithelium. Beak outgrowth occurs at the location where their expression domains meet, and

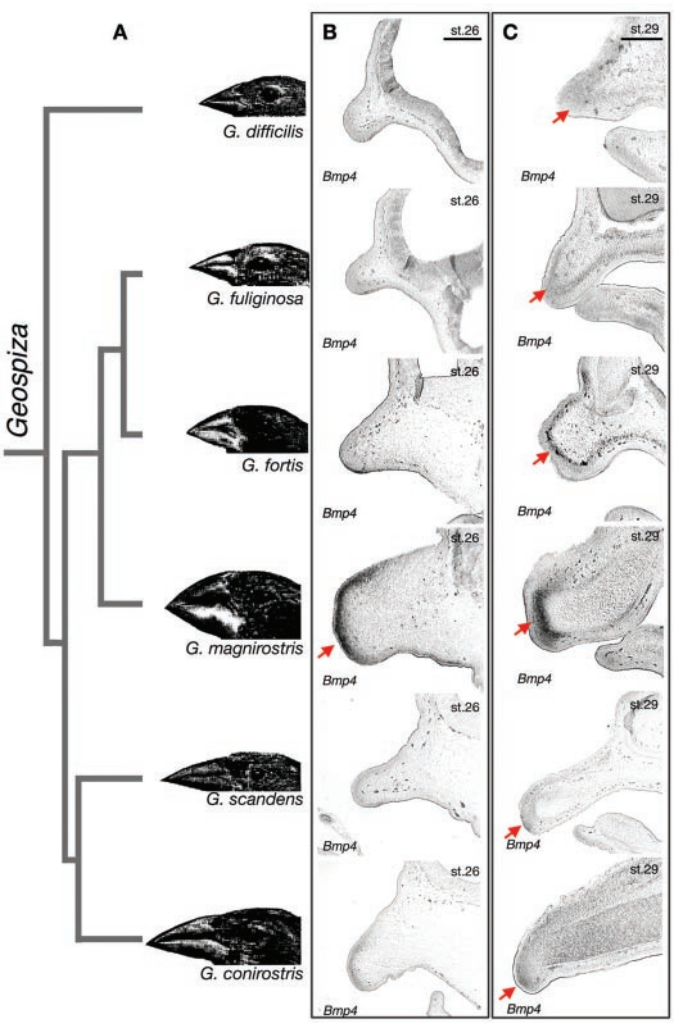

Fig. 1. (**A**) Previous studies suggest that *G. difficilis* is the most basal species of the genus *Geospiza*, and the rest of the species form two groups: ground and cactus finches, with distinct beak morphologies. (**B**) At stage (st.) 26, *Bmp4* is strongly expressed in a broad distal-dorsal domain in the mesenchyme of the upper beak prominence of *G. magnirostris* and at significantly lower levels in *G. fortis* and *G. conirostris*. No *Bmp4* was detected in the mesenchyme of *G. difficilis*, *G. fuliginosa*, and *G. scandens*. (**C**) At stage 29, *Bmp4* continues to be expressed at high levels in the distal beak mesenchyme of *G. magnirostris*. Broad domains of *Bmp4* expression are detectable around prenasal cartilages of *G. fuliginosa* and *G. fortis*. A small domain of strong *Bmp4* expression is also found in the most distal mesenchyme of *G. conirostris*, and weaker expression is seen in *G. scandens* and *G. fortis* (red arrows). Scale bars: 1 mm in (B) and 2 mm in (C).

SHH and FGF8 have been shown to synergistically drive outgrowth, and in the process to induce expression of *Bmp4* in subjacent mesenchyme (*21*, *22*). Also, we have not ruled out the possibility that genes expressed in other regions of the face are important for directing morphogenesis. In addition to the correlation between variation in *Bmp4* levels and the development of the beaks of Darwin's finches, we have also found that artificially increasing BMP4 levels in the beak mesenchyme is sufficient to alter beak morphology in the same direction as is seen in the larger ground finches. Thus, although polymorphism in other genes may also contribute to differences in beak morphology, we propose that variation in *Bmp4* regulation is one of the principal molecular variables that provided the quantitative morphological variation acted on by natural selection in the evolution of the beaks of the Darwin's finch species (*23*).

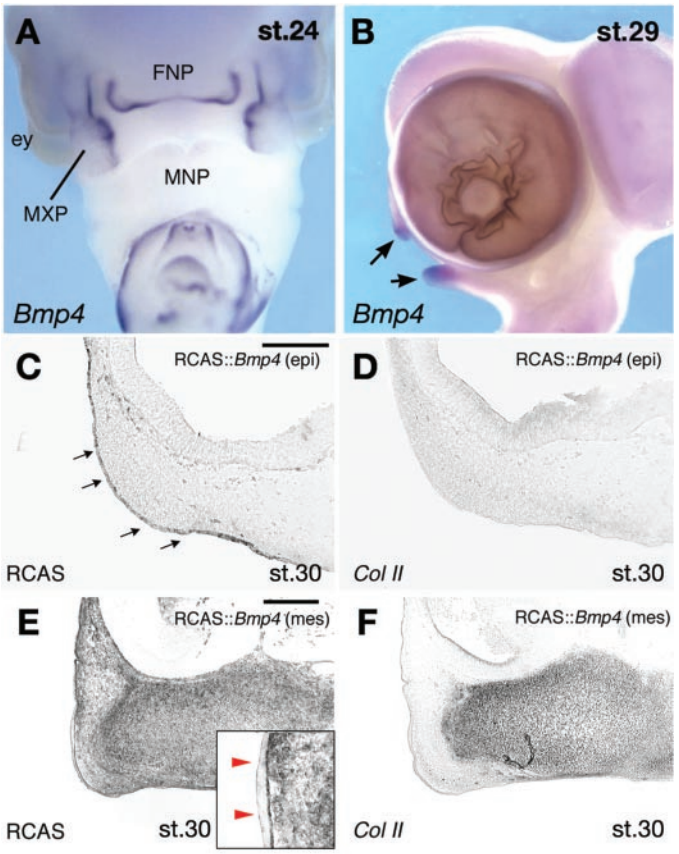

Fig. 2. (**A**) At stage 24, *Bmp4* is expressed in the ectoderm of the maxillary process (MXP) and lateral frontonasal process (FNP). (**B**) By stage 29, *Bmp4* expression expands into the distal mesenchyme of the upper and lower beaks (black arrows). (**C** and **E**) Misexpression of RCAS::*Bmp4* can be targeted to either the facial ectoderm [red arrowheads in inset in (E)] or the mesenchyme (mes) of the frontonasal process of a chicken embryo, as revealed with in-situ hybridization with a virus-specific probe. (**D**) No chondrogenesis is detected in the embryos whose epithelium (epi) was infected with RCAS::*Bmp4* virus. (**F**) In contrast, embryos whose FNP mesenchyme was infected with RCAS::*Bmp4* virus showed high levels of chondrogenesis as revealed with an anti–*Col II* riboprobe. MNP, mandibular process; ey, eye. Scale bars: 1 mm in (C) and (E).

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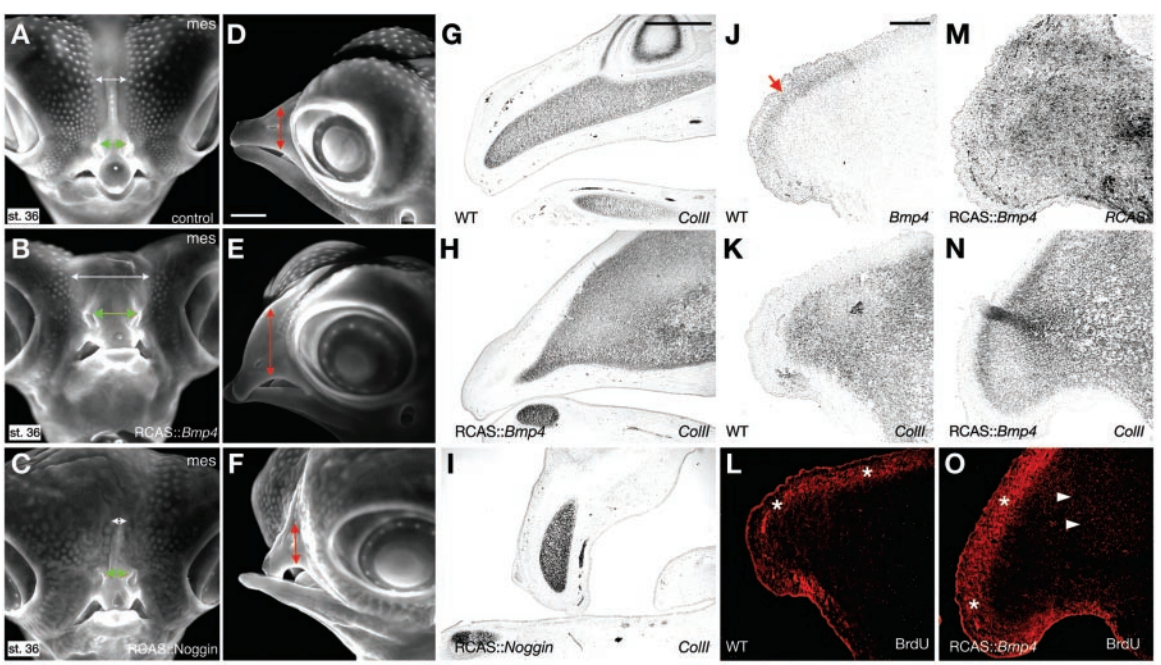

Fig. 3. (**A** and **D**) Ultraviolet pictures of wild-type stage 36 chicken embryonic heads. The width and depth of the beak are shown with white and red double-headed arrows, respectively. The width of the beak tip is indicated with a double-headed green arrow. (**G**) Prenasal cartilage in wild-type chickens forms a narrow protruding skeletal rod. (**B** and **E**) RCAS::*Bmp4* infection in the mesenchyme of the frontonasal process caused a significant increase in the width and depths of the beak. (**H**) These larger upper beaks contained more skeletogenic cells, as revealed with *Col II* in-situ hybridization. (**C** and **F**) In contrast, infection with RCAS::*Noggin* led to narrower and shallower upper beaks. (**I**) Ectopic *Noggin* produced smaller skeletal elements inside the upper beak. BrdU labeling reveals that the *Bmp4* expression domain [red arrow in (**J**)], which is rostral and dorsal to the developing prenasal cartilage (**K**), is closely associated with proliferating cells (**L**) of stage 30 chick embryos. The upper beaks of embryos infected with RCAS::*Bmp4* (**M**) by stage 30 develop larger cartilages (**N**), and there is an up-regulation of cell proliferation both around and within the developing cartilage (**O**). Scale bars: 2 mm in (D); 0.5 mm in (G); and 1 mm in (J).

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Molecular Shaping of the Beak

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Beak shape is a classic example of evolutionary diversification. Beak development in chicken and duck was used to examine morphological variations among avian species. There is only one proliferative zone in the frontonasal mass of chickens, but two in ducks. These growth zones are associated with bone morphogenetic protein 4 (BMP4) activity. By "tinkering" with BMP4 in beak prominences, the shapes of the chicken beak can be modulated.

During bird evolution, the beak emerged as the dominant avian facial feature, adapting birds to diverse eco-morphological opportunities (*1*, *2*). The beak is made up of multiple facial prominences: the frontonasal mass (FNM), maxillary prominences (MXP), lateral nasal prominences (LNP), and mandibular prominence (MDP) (fig. S1A). During development, these prominences are proportionally coordinated to compose a unique beak. Progress in molecular mechanisms underlying early beak morphogenesis has been reviewed recently (*3*, *4*). Here, we focus on later events that mold the shape of the FNM, by comparing proliferation zones of chickens and ducks that have distinct beak shapes (Fig. 1A, fig. S2A).

Temporal- and spatial-specific changes of proliferative zones occur within the FNM (Fig. 1B; fig. S1, C and D). In stage 26 chickens, labeling with short pulses of BrdU (5-bromo-2′-deoxyuridine) showed cell proliferation in both FNM lateral edges. At stage 27, the two growth zones shifted toward the rostral margin, flanking the midline. At stage 28, these growth zones gradually converged into one centrally localized zone. In ducks, the two bilaterally positioned growth zones persisted in the lateral edges, widening the developing FNM. These changes precede morphological changes of the

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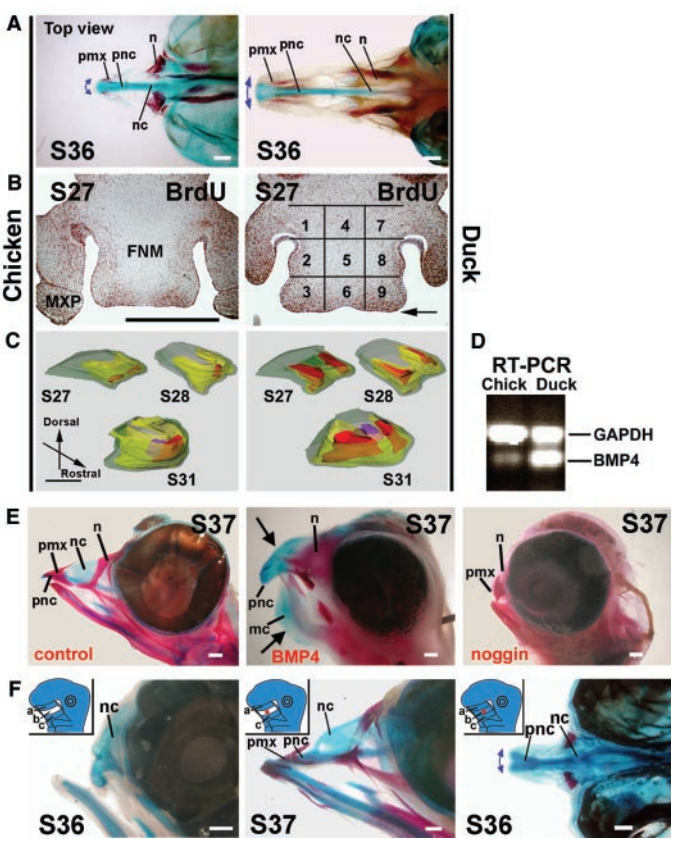

Fig. 1. Cell proliferation and BMP4 function in chicken and duck beak morphogenesis. (**A**) Stage 36 chicken and duck beaks, top view. Blue, cartilage; red, bones. Double-headed arrows indicate beak tip width (fig. S2E). (**B**) Stage 27 frontal sections after 1.5 hours of BrdU labeling. See fig. S1C for stages 26, 28, 29, and 31 BrdU labeling. The percentage of BrdU-positive cells was quantified in nine regions using the grid overlay (*12*) shown in table S1. Arrow indicates the rostral margin. (**C**) Three-dimensional reconstruction of the percentage of BrdU-positive cells in the FNM. Red indicates >20% BrdU-positive cells, yellow 10 to 20%, and green <10%. Viewing the inner red zone through the yellow zone appears orange. Purple indicates proliferation in the cartilage region. (**D**) BMP4 RT-PCR from stage 25 FNMs showed a higher BMP4/GAPDH ratio in ducks than in chickens. (**E**) (Left) Stage 37 control. (Middle and right) RCAS-BMP4 or RCAS-noggin was injected into all beak prominences of chicken embryos and harvested at stage 37. Arrows indicate enlarged skeletal elements. (**F**) (Left) Stage 20 chicken FNM was divided into three regions (a to c, defined in fig. S2B). Excision of region b containing the frontonasal ectodermal zone and subjacent mesenchyme (inset) truncated the upper beak with distal cartilage elements missing as observed at stage 36. Ablation of region a or c showed normal growth (not shown). (Middle) BMP4 beads (inset, red circle) can rescue most growth and cartilage elements from region b–ablated specimens (stage 37). (Right) Addition of BMP4 beads to nonablated FNM resulted in wider upper beaks (stage 36). FNM, frontonasal mass; mc, Meckel's cartilage; MXP, maxillary prominence; n, nasal bone; nc, nasal chonchae; pmx, premaxilla bone; pnc, prenasal cartilage. Scale bars, 1 mm.