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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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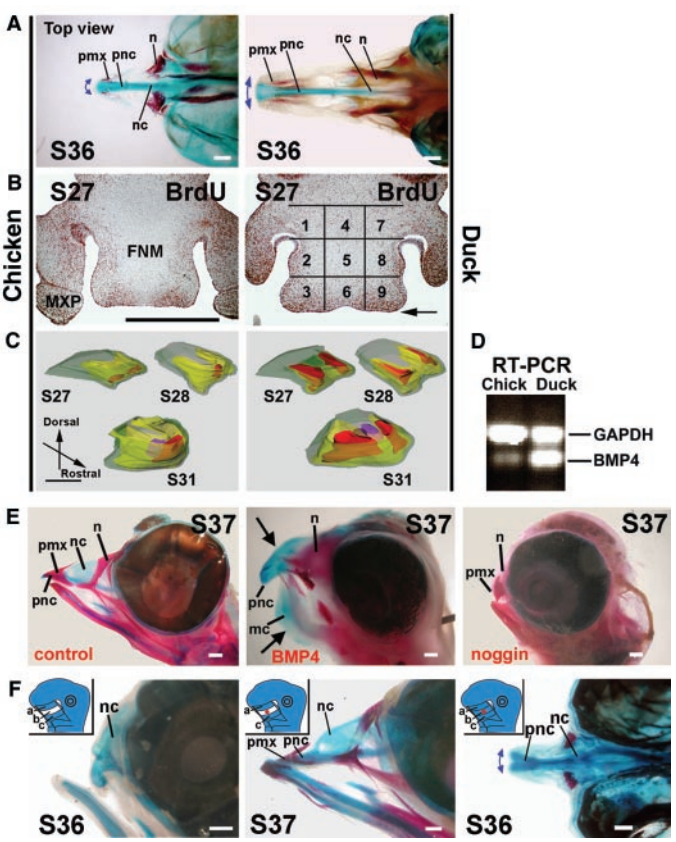


Fig. 1. Cell proliferation and BMP4 function in chicken and duck beak morphogenesis. (**A**) Stage 36 chicken and duck beaks, top view. Blue, cartilage; red, bones. Double-headed arrows indicate beak tip width (fig. S2E). (**B**) Stage 27 frontal sections after 1.5 hours of BrdU labeling. See fig. S1C 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.

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FNM. After stage 31, the basic beak structures were determined. The percentage of BrdU-positive cells was quantified in nine separate FNM regions (Fig. 1B and table S1). A growth zone shift is clearly seen in the three-dimensional reconstruction (Fig. 1C).

Bone morphogenetic protein 2 (BMP2), BMP4, and BMP receptors are expressed in chicken beak prominences (*5*–*7*). We observed higher expression of BMP4 transcripts in ducks than in chickens, using reverse transcription polymerase chain reaction (RT-PCR) with primers conserved between chickens and ducks (Fig. 1D). Furthermore, BMP4 in the mesenchyme was closely associated with the shifting growth zones (fig. S1, C and E). Comparisons showed more diffuse BMP4 expression in the duck than in the chicken, and duck MXPs were larger than chicken MXPs (fig. S1F).

We examined the roles of BMP4 in shaping beaks with two different strategies. To test whether BMP4 drives beak growth, we injected replication-competent avian sarcoma retrovirus (RCAS)-BMP4 into all beak prominences of stage 22 and stage 23 chicken embryos. This treatment resulted in larger beaks with significant increases in length, width, and depth (Fig. 1E, middle; percentage of experiments showing indicated phenotypes = 100%; total number of experiments $n = 15$, $P < 0.001$) compared with controls (Fig. 1E, left). To determine whether BMP is physiologically involved, we misexpressed noggin, the BMP antagonist. Treatment with RCAS-noggin resulted in miniaturized beaks (Fig. 1E, right, 100%; $n = 9$, $P < 0.001$). Analysis of histological sections showed that BMP4 caused enhanced cell proliferation and skeletal differentiation as seen by hematoxylin and eosin, BrdU, and collagen-I staining (fig. S3, A to D), consistent with results obtained using RCAS-BMP receptors (*7*). Noggin had the opposite effect, causing reduced cell proliferation and skeletal differentiation (fig. S3, A and C).

The "frontonasal ectodermal zone" flanked by fibroblast growth factor 8 (FGF8) and Sonic hedgehog (Shh) was shown to direct beak outgrowth (*8*) (fig. S1B). We microsurgically ablated this region (including the epithelium and mesenchyme; region b in fig. S2B), and growth was arrested, with the distal cartilage elements missing (Fig. 1F, left, 70%; $n = 20$). BMP4-coated beads could partially restore growth even when this mesenchymal zone was deleted (Fig. 1F, middle, 62%; $n = 13$). Implanting a BMP4 bead in the nonablated FNM region induced a new growth zone, resulting in a wider beak (Fig. 1F, right, 33%; $n = 12$). The moderate percentage of unablated beak samples responding to BMP beads may be due to a slight shift of bead positions in ovo, because the BMP bead effect appears to be highly location specific (*6*). The width of the upper beak tip was significantly increased ($P < 0.01$) and resembled that of the duck (fig. S2F). Albumin-coated beads did not produce this effect (fig. S2C). Within 24 hours, BMP4-coated beads induced surrounding mesenchymal cell proliferation (*9*).

Our results show that BMP4 is one of the major driving forces building beak mass. The number and activity of localized growth zones in the prominence confer the specific beak shape. Darwin's finches in the Galapagos Islands exhibit different-sized and -shaped beaks (*1*), as a result of a process regulated in part by BMP4 (*10*). We produced beaks that phenocopy those in nature by modulating BMP activities. It is likely that beak shape diversity is achieved by modulating prototypical molecular modules (*11*), and proteins of the BMP pathway may mediate a spectrum of morphological designs for selection.

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Activation of Apoptosis in Vivo by a Hydrocarbon-Stapled BH3 Helix

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BCL-2 family proteins constitute a critical control point for the regulation of apoptosis. Protein interaction between BCL-2 members is a prominent mechanism of control and is mediated through the amphipathic α-helical BH3 segment, an essential death domain. We used a chemical strategy, termed hydrocarbon stapling, to generate BH3 peptides with improved pharmacologic properties. The stapled peptides, called "stabilized alpha-helix of BCL-2 domains" (SAHBs), proved to be helical, protease-resistant, and cell-permeable molecules that bound with increased affinity to multidomain BCL-2 member pockets. A SAHB of the BH3 domain from the BID protein specifically activated the apoptotic pathway to kill leukemia cells. In addition, SAHB effectively inhibited the growth of human leukemia xenografts in vivo. Hydrocarbon stapling of native peptides may provide a useful strategy for experimental and therapeutic modulation of protein-protein interactions in many signaling pathways.

BCL-2 is the founding member of a protein family (*1*–*3*) composed of pro- and anti-apoptotic molecules that serve as an essential control point in apoptosis, governing susceptibility to cell death (*4*–*6*). The BCL-2 family is defined by the presence of up to four conserved BCL-2 homology (BH) domains, all of which include α-helical segments. Anti-apoptotic proteins (for example, BCL-2 and BCL-X_L) display sequence conservation in all BH domains, whereas pro-apoptotic proteins are divided into multidomain members (such as BAX and BAK), and BH3-only members (such as BID and BAD) that display sequence similarity only to the BH3 α-helical domain. The amphipathic α-helical BH3 segment of pro-

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