LETTERS

The calmodulin pathway and evolution of elongated beak morphology in Darwin's finches

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A classic textbook example of adaptive radiation under natural selection is the evolution of 14 closely related species of Darwin's finches (Fringillidae, Passeriformes), whose primary diversity lies in the size and shape of their beaks[1–6]. Thus, ground finches have deep and wide beaks, cactus finches have long and pointed beaks (low depth and narrower width), and warbler finches have slender and pointed beaks, reflecting differences in their respective diets[6]. Previous work has shown that even small differences in any of the three major dimensions (depth, width and length) of the beak have major consequences for the overall fitness of the birds[3–7]. Recently we used a candidate gene approach to explain one pathway involved in Darwin's finch beak morphogenesis[8]. However, this type of analysis is limited to molecules with a known association with craniofacial and/or skeletogenic development. Here we use a less constrained, complementary DNA microarray analysis of the transcripts expressed in the beak primordia to find previously unknown genes and pathways whose expression correlates with specific beak morphologies. We show that calmodulin (CaM), a molecule involved in mediating Ca^{2+} signalling, is expressed at higher levels in the long and pointed beaks of cactus finches than in more robust beak types of other species. We validated this observation with *in situ* hybridizations. When this upregulation of the CaM-dependent pathway is artificially replicated in the chick frontonasal prominence, it causes an elongation of the upper beak, recapitulating the beak morphology of the cactus finches. Our results indicate that local upregulation of the CaM-dependent pathway is likely to have been a component of the evolution of Darwin's finch species with elongated beak morphology and provide a mechanistic explanation for the independence of beak evolution along different axes. More generally, our results implicate the CaM-dependent pathway in the developmental regulation of craniofacial skeletal structures.

To understand the genetic basis of the species-specific beak morphologies, we previously performed a comparative candidate gene analysis with developmental genes known to be associated with craniofacial development. We found that a broader and earlier domain of bone morphogenetic protein 4 (BMP4) expression in the distal neural-crest-derived mesenchyme correlated with the very deep and wide beak morphology of the ground finches[8]. This expression difference was shown to be functionally significant by misexpression analysis in chick embryos[8].

However, the candidate gene approach did not yield any candidates for pathways that could be involved in evolution of the longer beak morphology characteristic of the cactus finch species. To identify pathways involved in the evolution of long beaks, cDNA microarrays were used for a direct comparison of the gene expression profiles of several thousand transcripts in stage 26 frontonasal processes (which give rise to the upper beak) of five species of genus *Geospiza*: the sharp-beaked finch (*Geospiza difficilis*), the medium and large ground finches (*G. fortis* and *G. magnirostris*), and the cactus and large cactus finches (*G. scandens* and *G. conirostris*) (Fig. 1a; Methods). We first used hierarchical clustering to inspect whether the overall expression profiles clustered according to species (Methods). The resultant tree illustrates that most of the individual

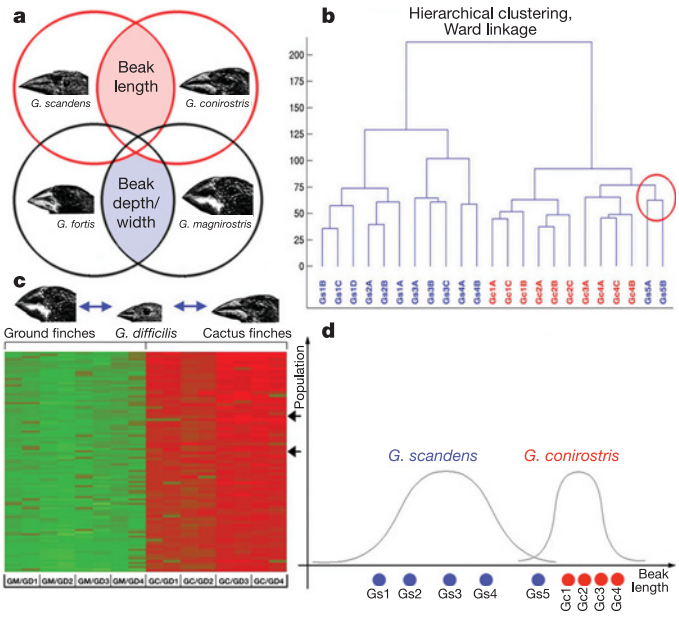

Figure 1 | Microarray analysis in different finch species. a, Clustering strategy to isolate transcripts whose expression correlated with beak morphology. **b**, The Ward linkage tree showed that most of the individual samples clustered by species. Each individual was sampled two to four times. The *y*-axis is euclidian distance between branches. **c**, The final clusters of transcripts, which were upregulated in the comparison between cactus finches and the sharp-beaked finch and were downregulated or remained unchanged in the ground finches compared with the sharp-beaked finch. **d**, Individual expression profiles clustered by species except for the occasional individual profile, probably reflecting a certain overlap in morphology or development between the species. Each spot represents the length of an individual beak.

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expression profiles clustered by species. For example, most individuals of either *G. scandens* (4 of 5) or *G. conirostris* (4 of 4) clustered together with individuals of the same species (Fig. 1b). The expression profile of one particular individual of *G. scandens* (G.s.5) clustered more closely with those of *G. conirostris*, probably reflecting a certain degree of morphological (and thus developmental) overlap between these two species (Fig. 1d). *G. scandens* and *G. conirostris* collected for this study live on different islands (Santa-Cruz and Genovesa, respectively), thus excluding the possibility of species misidentification. We obtained very similar results when we compared *G. fortis* and *G. magnirostris* individuals (largely distinct expression profiles but some individuals clustering with the other species; not shown). It therefore seems that the analysis is sensitive to phylogenetic differences between different species of Darwin's finches.

We then clustered the measurements of signal ratios and intensities for different transcripts to identify genes that were upregulated or downregulated in all individuals of a particular morphology compared with the basal *G. difficilis* reference (Fig. 1, and Supplementary Fig. S1; Methods). All of 100 candidates from the resulting final cluster of cactus finch morphology-specific genes were sequenced to reveal their identity. These genes were screened to produce candidates that were expressed at moderate or high levels of signal intensity on the microarray and were expressed at least fivefold higher in the cactus finches. We found two microarray spots carrying probes with identical sequences for CaM (Supplementary Fig. S2) that were both at a much higher level of average signal in the cactus finch beaks than in the reference sharp-beaked finch (*G. difficilis*) (Supplementary Information). Because these clones represented the most differentially expressed gene that was not an enzyme, a housekeeping gene or a ribosomal gene and the only representative of a signalling transduction pathway, we focused on CaM as our primary candidate associated with the elongated cactus finch beak morphology. CaM is a Ca^{2+}-binding protein that can bind to and regulate many different protein targets and is a key component of a Ca^{2+}-dependent signal transduction pathway[9]. It has not been previously characterized in either craniofacial or skeletal development.

Our microarray data indicate a possible correlation between elevated levels of CaM and the more elongated beak morphology of the cactus finches. To validate this suggestion we performed a comparative *in situ* hybridization on embryos of Darwin's finches (Fig. 2). We found that CaM was indeed expressed at detectably higher levels in the distal–ventral mesenchyme of the frontonasal processes in the cactus finches ($n = 3$) and large cactus finches ($n = 3$) than in similar-sized processes of the ground finches (Fig. 2). Thus, higher expression of CaM is indeed associated with the elongated beak morphology of the cactus finches.

To address whether differential levels of CaM-dependent signalling might be important in the development of distinct beak morphologies in different species of Darwin's finches, we wished to elevate the level of CaM-dependent signalling in the developing chick beak prominence. To accomplish this we used a constitutively activated form of a downstream effector of CaM, CaM kinase kinase (CaMKII; M. J. Taschner, S. Schnaiter and C.H., unpublished observations). In one of the major cellular responses to increased Ca^{2+} levels, CaM activates CaMKII. CaMKII, in turn, activates downstream kinases, enabling them to phosphorylate and hence activate various targets, such as the transcription factors cAMP-response-element-binding protein (CREB), serum response factor and CREB-binding protein.

Both CaM and CamKII are expressed at low levels in the mesenchyme of the chicken upper beak process (data not shown). To stimulate higher CaM-dependent CAMKII signalling, we used an avian retroviral vector carrying the constitutively active form of CaMKII (RCAS::CA-CaMKII) to infect the distal mesenchyme of early stage-24 chick frontonasal processes. Although virus injection was targeted to the distal upper beak prominence with relative ease, the resultant infection was somewhat variable. In more than half of the cases, the infection was limited to the distal and ventral mesenchyme surrounding the developing cartilage and did not spread to the skeletal element (Fig. 3d; $n = 9$ of 16; not shown). Because these infected regions closely approximate the domain of elevated CaM expression in the cactus finches (Fig. 2), we restricted our analysis to these embryos. In chicks in which activated CaMKII was misexpressed specifically in the distal/ventral mesenchyme, we observed a significant increase in the length of the beaks (length in arbitrary units: 0.78 ± 0.05 ($\pm$s.d.); $P < 0.0056$, $n = 9$; more than 10% beak elongation relative to control embryos (Fig. 3a–d)), whereas the beak width and depth were not affected (Fig. 3, and Supplementary Fig. S3).

For a better understanding of the phenotype induced by activated CaMKII in beaks, we studied the expression of markers for skeletal cell types, proliferation and cell death (Fig. 3, and Supplementary Fig. S4). *In situ* hybridization analysis with probes directed against skeletogenic genes verified that the cartilage element was indeed longer in the distal/ventrally infected embryos (Fig. 3). We found that

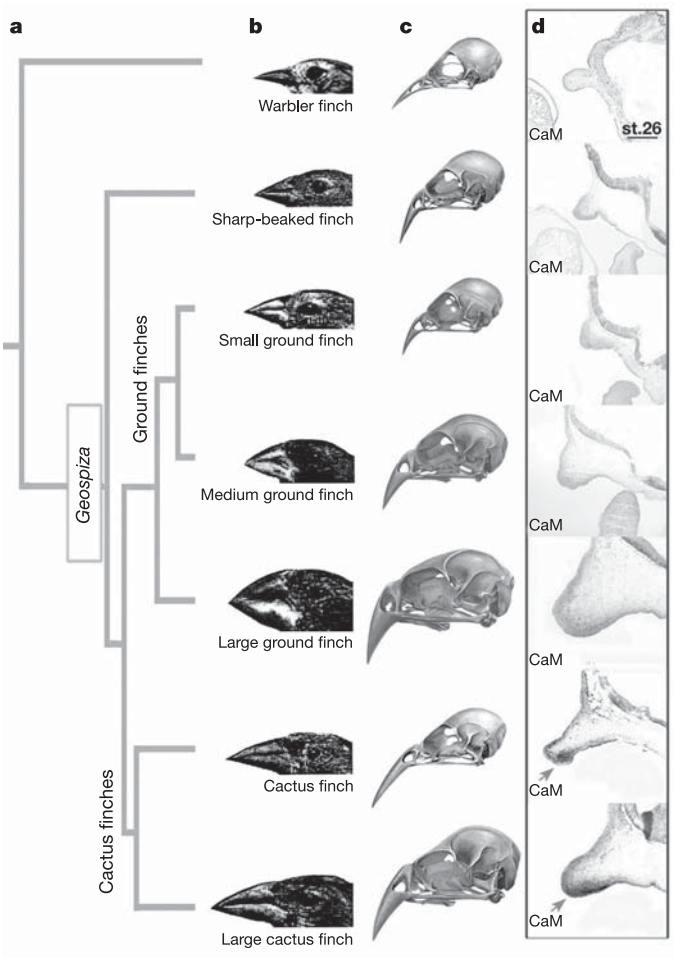

Figure 2 | Comparative analysis of CaM expression in finches. **a**, **b**, *Geospiza* group species displaying distinct beak morphologies form a monophyletic group. **c**, The differences in beak morphology are skeletal. **d**, CaM is expressed in a strong distal–ventral domain in the mesenchyme of the upper beak prominence of the large cactus finch, *G. conirostris*, somewhat lower levels in cactus finch, *G. scandens*, and at significantly lower levels in the large ground finch and medium ground finch, *G. magnirostris* and *G. fortis*, respectively. Very low levels of CaM were detected in the mesenchyme of *G. difficilis*, *G. fuliginosa* and the basal warbler finch *Certhidea olivacea*. CaM expression domains are indicated with short arrows in **d**. Scale bar, 1 mm in **b**. The molecular tree is from ref. 23; images of skulls are from ref. 6, with permission from the author.

cell death, as revealed by the TUNEL (TdT-mediated dUTP nick end labelling) assay, was essentially unchanged relative to the wild-type condition (not shown). Staining for anti-PCNA (proliferating cell nuclear antigen) antibody and labelling with bromodeoxyuridine indicate that proliferation might have been upregulated in the distal half of the pre-nasal cartilage element of infected beaks (not shown). The expression of cell-cycle regulators cyclin D1 and the activator protein 1 (AP1) family members c-Jun and c-Fos was upregulated in the distal parts of pre-nasal cartilage and its perichondrium (arrow in Supplementary Fig. S4J; not shown). As these skeletal tissues were not infected with the retrovirus, this effect was indirect. The same genes were downregulated in the mesenchyme, which forms the premaxillary dermal bone (arrowheads in Supplementary Fig. S4I).

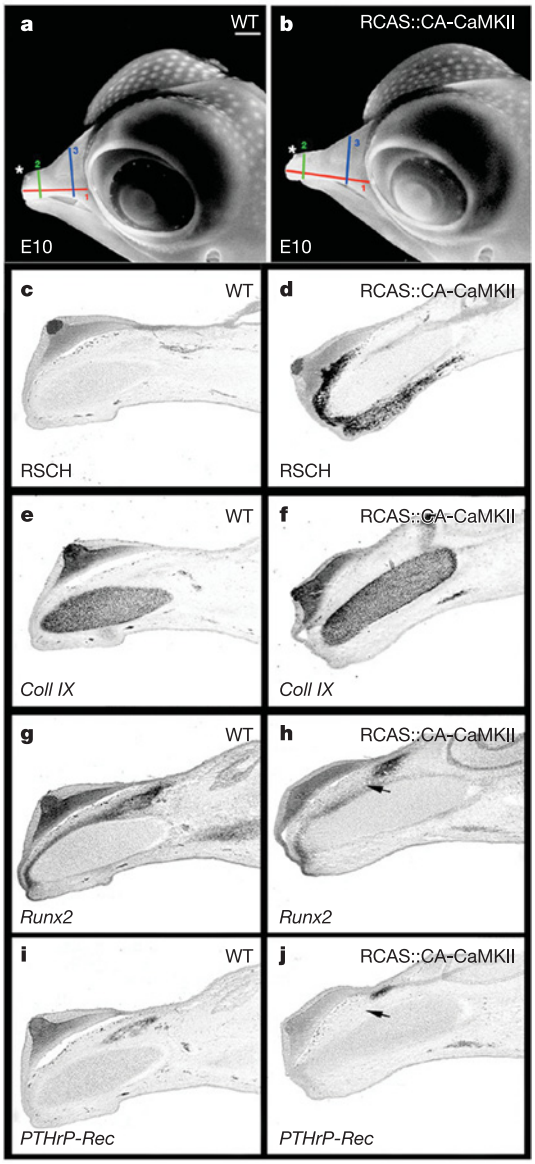

Figure 3 | Functional analysis of CaM-dependent pathway in beak development. **a**, **b**, Whole head views of embryonic day 10 (E10; HH stage 36) wild-type (**a**) and RCAS::CA-CaMKII-infected (**b**) chicken embryos. The length of the beak is shown with a red line; the depth of the beak at the base and the depth of the beak at the tip are shown with blue and green lines, respectively. **c**–**j**, We used RSCH (**c**, **d**), *Coll IX* (**e**, **f**), *Runx2* (**g**, **h**) and *PTHrP-Rec* (**i**, **j**) probes to reveal RCAS infection (RSCH), chondrocytes (*Coll IX*) and early osteoblasts (*Runx2*). **c**, **e**, **g**, **i**, Wild type; **d**, **f**, **h**, **j**, RCAS::CA-CaMKII infected. The star indicates an egg-tooth. Scale bar, 2 mm in **a**.

Consistent with our observations, parallel studies in the laboratory of one of us (C.H.) have also indicated that CaMKII signalling leads to the elongation of limb skeletal elements in a non-cell-autonomous manner (M. J. Taschner, S. Schnaiter and C.H, unpublished observations), in a similar manner to our observation in the beak.

There are few examples of the identification and characterization of developmental pathways responsible for evolutionary morphological change[10–15]. Previous work on Darwin's finches has shown that selection on adult beak variation results in changes in growth in the next generation, as a result of genetic correlations between adult and juvenile expression of the same morphological characters[16,17]. Here, using a microarray approach, we found evidence that a higher level of CaM-dependent signalling is both biologically relevant and functionally important for the morphogenesis of the longer beaks of cactus finches used in a specialized manner to probe cactus flowers and fruit for nectar.

The avian beak varies between species, and indeed between individuals of the same species, along at least three different axes: length, depth and width. In Darwin's finches it can be seen that there is both linkage and independence in the variation along these different axes. For example, in the two species of cactus finches there is a much weaker genetic correlation between adult beak length and depth or width than between depth and width themselves. In contrast, in *G. fortis* all three correlations are strong[18,19]. The data reported here provide at least a partial explanation for this difference between the finch species. Embryos of the cactus finch species, in which beak length shows independence from width and length, strongly express CaM (a factor specifically affecting beak length) during beak morphogenesis, whereas *G. fortis* has minimal CaM expression (Fig. 4).

In considering the independence of beak length from width and depth, it is particularly intriguing that, here and in our previous study, we have shown that two different factors (BMP4 and activated CaMKII), expressed in a similar domain in the beak prominence, result in changes in growth along different dimensions of the developing beak (width/depth and length, respectively) without negatively affecting

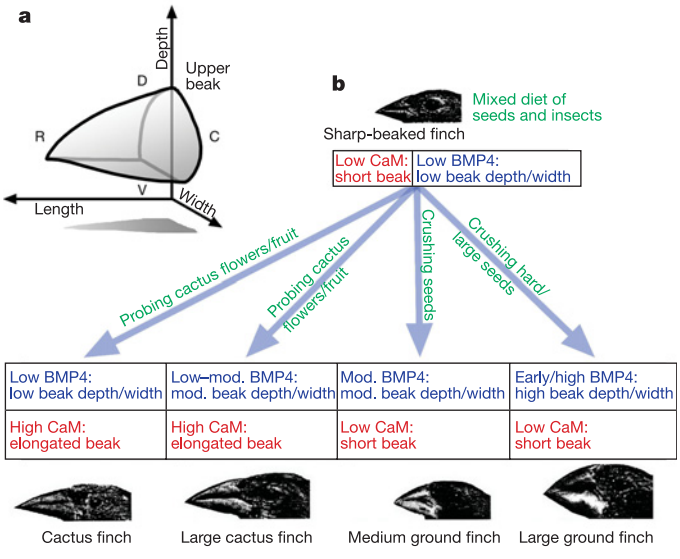

Figure 4 | BMP- and CaM-dependent signalling regulates growth along different axes, facilitating the evolution of distinct beak morphologies in Darwin's finches. **a**, Developing avian beak is a three-dimensional structure that can change along any of the growth axes. **b**, A beak of the sharp-beaked finch reflects a basal morphology for *Geozpiza*. The model for BMP4 and CaM involvement explains development of both elongated and deep/wide beaks of the more derived species. Abbreviations: C, caudal; D, dorsal; R, rostral; V, ventral.

the other axes, explaining the observed independence of these traits. Moreover, analysis of BMP4 and CaM expression in chick embryos infected with RCAS::CA-CaMKII and RCAS::BMP4, respectively, showed that these two molecules do not regulate each other during beak development (data not shown). These two molecules vary independently of each other in some species as demonstrated by their reciprocal expression levels in the two medium-sized species with thick and with long beak morphologies, whereas they are expressed together in the developing beak of the large cactus finch in good correlation with its robust yet elongated beak morphology (Figs 1, 3 and 4)[6,8]. Theoretically, such modular developmental regulation need not have been necessary to construct a beak. A common set of growth-promoting pathways could, in principle, have led to outgrowth of the frontonasal process along all three dimensions. Indeed, a single factor, BMP4, seems to stimulate growth of the beak along two dimensions, promoting deeper and wider beaks, explaining the linkage in their variation. Using a common signal for outgrowth in different axes still permits evolution of form, for example through the action of localized antagonists. However, the developmental programme using a distinct pathway for the long axis of the beak enhances the 'evolvability' along this dimension, by allowing independent variation along the different axes. Thus, the dissociable regulation of growth in different beak axes is an example of the organization of the biology of the organism facilitating the generation of variation on which natural selection can act[20].

It will be of interest to sample other species of Darwin's finches with elongated beaks such as the woodpecker finch *Cactospiza pallida*, as well as other avian species with long beaks, to discover how generally the CaM-dependent pathway is involved in elongation of the beak skeleton. Upregulation of this pathway at stage 26 is not simply related to low beak depth and width of beaks, because CaM expression was very low in the pointed but slender beaks of the warbler finch embryos (Fig. 3). Upregulation of CaMKII activity in the distal mesenchyme of the upper beak prominence in chicken embryos produced a roughly 10% increase in beak length, primarily due to elongation of the pre-nasal cartilage rod, which is in concert with the differences in relative beak length between the cactus finches and the more basal sharp-beaked finch and other ground finches[6]. This might indicate that additional pathways and mechanisms have a function in generating the even longer beaks found in other avian lineages, such as some shorebirds, hummingbirds and Hawaiian honeycreepers, all highly adaptive.

METHODS

Collection and treatment of embryonic material from Darwin's finches. Under an agreement with the Galápagos National Park, we received quotas for collecting embryos of *G. magnirostris*, *G. fortis*, *G. fuliginosa* and *G. scandens* from Santa Cruz Island, and *G. conirostris*, *G. difficilis* and *C. olivacea* from Genovesa Island. To avoid causing nest defection, only the third egg was collected shortly after it was laid; it was then incubated at 100 °F (37.8 °C). Embryonic material was fixed in 4% paraformaldehyde in PBS for 2 h at ambient temperature and stored in RNAlater reagent (Ambion) at about 5 °C for two to five weeks. Chick antisense riboprobes (*CaM*, *Coll IX*, *Runx2* and *PTHrP-Rec*) were prepared and used on Darwin's finch embryos as described previously[8]. We analysed 26 heads of Darwin's finches: large ground finch ($n = 3$), medium ground finch ($n = 5$), small ground finch ($n = 4$), large cactus finch ($n = 4$), cactus finch ($n = 3$), sharp-beaked finch ($n = 3$) and warbler finch ($n = 4$).

Chicken embryo manipulations and statistical analysis. Fertilized eggs (Spafas) were incubated at 100 °F. Embryos were staged as described in ref. 21. The RCAS::CA-CamKII construct will be described elsewhere (M. J. Taschner, S. Schnaiter and C.H., unpublished observations). To infect embryos for studies *in vivo* we injected the distal part of the frontonasal process of HH stage-24 chick embryos. The infected embryos were collected at embryonic day 10, fixed overnight in 4% paraformaldehyde in PBS, and frozen in OCT (optimal cutting temperature) medium for sectioning. Extent of viral infection was assayed by hybridization *in situ* with a virus-specific RSCH probe. The embryonic heads were photographed and measured in NIH Image 1.62. These arbitrary measurements were used for the analysis of variance function (ANOVA toolbox; *t*-test) in Microsoft Excel X to calculate standard deviations and *P* values for the data.

Darwin finch microarray production and usage. A DNA microarray (21,168 spots) was printed from a non-normalized poly(A)-primed cDNA library made from RNA isolated from multiple (12 individuals) frontonasal processes of stage-26 and stage-29 embryos of the medium ground finch, *G. fortis*. We used Cy5-labelled probes made from individual frontonasal processes of the four derived species of *Geospiza* for direct comparisons against a common Cy3-labelled reference sample made from pooled RNA of several (nine individuals) embryos of more basal *G. difficilis* (Fig. 1a, and Supplementary Fig. S1). In most cases we compared four unrelated individuals from each of the derived cactus finch and ground finch species (*G. scandens*, *G. conirostris*, *G. magnirostris* and *G. fortis*) against the pooled common reference (Figs 1a and 4, and Supplementary Fig. S1). RNA from each individual finch beak prominence was independently amplified and labelled in triplicate with a control dye swap. We used the two highest-quality sets of microarray data from each triplicate for clustering. Raw.gpr files were generated with GenePix 3.0 (Molecular Devices). Normalization and statistical analysis of the GPR data files were performed in MatLab (The Math Works). Data were normalized with the Lowess algorithm[22]. Only spots with a signal intensity exceeding the median background +2 s.d. were considered, which left 7,369 spots. The data was $\log_2$ transformed.

Microarray cluster analysis. Clustering analysis and visualization were computed in MatLab. Agglomerative hierarchical clustering was performed by using the euclidean distance measure: the average linkage and Ward heuristics were used to connect the gene clusters. For *k*-means clustering, the *k*-means algorithm partitioned the genes into *k* discrete clusters on the basis of their expression. The number *k* (50) was preselected. The resultant tree illustrates that duplicates of the amplification/labelling experiments from the same individuals clustered together (Fig. 1b).

Measurements of signal ratios and intensities for different transcripts were clustered to identify genes that were upregulated or downregulated in all individuals of a particular species compared with the basal *G. difficilis* reference. Species-specific clusters were further cross-compared to reveal transcripts that were consistently upregulated in frontonasal processes of all individuals of the cactus-finch beak morphology and that remained unchanged or were downregulated in beak primordia of the ground finches. Conveniently, the cactus finch has an upper beak depth similar in size to that of the medium ground finch, whereas the large cactus finch's beak is more similar in size to that of the large ground finch while differing strongly in shape. This allowed us to separate transcripts exhibiting the size-specific regulation from those with shape-specific regulation.

Data analysis: hierarchical clustering. Gene expression patterns of nine experimental samples representing five *G. scandens* and four *G. conirostris* samples were analysed by hierarchical clustering with Ward linkage. The samples were divided into two groups on the basis of differences in gene expression. Figure 2 shows a cluster dendrogram of the two groups, *G. scandens* (blue) and *G. conirostris* (red). Within each branch the siblings were found to cluster tightly together on a sub-branch.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.A. performed embryonic material collection, microarray probe preparation, microarray hybridizations and scanning, sectioning of material, *in situ* hybridizations and CaMKII functional analysis in chicken embryos. W.P.K. conducted all relevant bioinformatics analyses. C.H. constructed the RCAS virus carrying the constitutively active version of CaMKII. B.R.G. and P.R.G. provided logistics and secured permits for the fieldwork on the Galápagos Islands. C.J.T. conceived and supervised the project. A.A., B.R.G., P.R.G. and C.J.T. co-wrote the manuscript. All authors discussed the results and commented on the manuscript.

Author Information The sequence of Darwin's finch CaM has been deposited at GenBank under accession number DQ386479, and the microarray data have been filed with ArrayExpress under the accession number E-MEXP-702. Reprints and permission information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests.
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