Bmp4 and Morphological Variation of Beaks in Darwin's Finches

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Supporting Online Material

MATERIALS AND METHODS

Staging system for analyzing song-bird development

A straightforward staging system already exists for the domesticated chicken, described by Hamburger and Hamilton (*S1*), and we based our finch staging system on it. However, the chicken (a precocial bird, order Galliformes) is quite divergent from songbirds, such as the finches (altricial birds, order Passeriformes). Not only is their incubation period different, but also various aspects of embryogenesis proceed at distinct relative rates in the finches compared to the chicken. Therefore, we used DIG-labeled antisense RNA probes against various genes known to be involved in craniofacial development to establish stages when, in particular, the finch craniofacial primordia corresponded to various stages in chick development as reflected in expression patterns. To have access to large numbers of embryos, we made use of a readily available species of songbirds, the Zebra Finch (*Taeniopygia guttata*) that has an identical incubation period to Darwin's Finches. While we originally intended to isolate Finch probes for this purpose, we discovered that probes directed against chick genes readily cross-reacted with Zebra Finch embryos, so those were employed for all hybridizations. Based on

these molecular data as well as morphological craniofacial features, we were able to develop a robust staging system for Finch craniofacial development (Fig. S1; S2). Subsequent examination of Darwin's Finch embryos verified that their craniofacial development was very similar to that of *T. guttata* by both morphological and molecular criteria (data not shown).

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 *Geospiza magnirostris, G. fortis, G. fuliginosa, G. scandens, G. conirostris, G. difficilis* and *Certhidea olivacea* on the islands of Santa Cruz and Genovesa. Singing males and their nests were identified at the beginning of the wet season. After breeding had begun nests were checked every day. Darwin Finches females lay clutches of 3-5 eggs, one per day. To avoid disrupting breeding, we collected only the third egg to be laid and incubated it at 100ºF. The embryos were harvested at E5 (st.26) and E6.5 (st.29) according to our altricial avian development staging series. The staging series for songbird development will be described in detail elsewhere. Embryonic material was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2-3 hours at ambient temperature and stored in RNAlater reagent (Ambion) at about 5ºC for 2-5 weeks. The heads were rehydrated in PBS, frozen in OCT and saggitally cryosectioned medially (Fig. S3). Chick antisense riboprobes were prepared and used on Darwin's Finch embryos as previously described (*S2*)(Fig. S4). We analyzed 19 heads of Darwin's Finches: *G. magnirostris* (N=3), *G. fortis* (N=4), *G. fuliginosa* (N=4), *G. conirostris* (N=3), *G. scandens* (N=2), and *G. difficilis* (N=3). A chick *Bmp4* probe was used for *in situ* hybridizations.

Chicken embryo manipulations and statistical analysis

Fertilized eggs were obtained from SPAFAS (Norwich, CT), incubated at 100F, and the embryos were staged according to Hamburger and Hamilton (*S3*). The RCAS::*Bmp4* and RCAS::*Noggin* constructs have been previously described (*S4,S5*). To infect embryos for in vivo studies we either injected the distal part of the frontonasal process of st.24 chick embryos or pooled high titer concentrated virus into the semi-enclosed space surrounding the heads of stage 15 embryos. RCAS(B)::AP (alkaline phosphatase) virus of similar high titer displayed infection of the epithelium and underlying dermis of the head after 36 hours of infection (stage 20) and 48 hours (stage 22)(not shown) that ranged from patchy (10-20% of head surface) to thorough (60-70% of head surface). The infected embryos were collected at stages 30 and 36, fixed overnight in 4% paraformaldehyde in PBS and frozen in OCT for sagittal sectioning. The embryonic heads were photographed and measured in NIH Image 1.62. These arbitrary units were used for the Analysis of Variance function (ANOVA toolbox) in Excel X to calculate standard deviations and *P-values* for the data. For BrdU labeling eggs of stage 30 wild-type and infected chicken embryos was injected with 300μl of 50μg/ml BrdU and incubated for 60 minutes.

SUPPORTING FIGURE LEGENDS

Supplemental Figure 1.

Comparison of prenatal development of altricial (songbirds, Order Passeriformes) and precocial (fowl, Order Galliformes) and staging series for the songbirds. Size comparisons of embryos of the Zebra Finch (*T. guttata*) and chicken (*G. gallus*) from Hamburger-Hamilton st.18 (A), st.24 (B) and st.26 (C). (D) A part of the Zebra Finch staging series used to stage embryos of Darwin's Finches. Note, that incubation time for *T. guttata* is identical to that of all the species of Darwin Finches. (E,F) Different structures, such as limbs and jaws, develop at different relative rates in song birds and fowl. Therefore, molecular data were used to correlate stages of craniofacial development between *T. guttata* and *G. gallus*, for example, expression pattern of *Shh* in chicken and Zebra Finch embryos. (G,H) Expression patterns of *Tbx2* in the craniofacial structures of chick and zebra Finch embryos. Scale bars: 1mm in A; 3mm in B; 4mm in C; 1mm in E,F; 0.1mm in G,H.

Supplemental Figure 2.

(A-D) Species-specific differences appear relatively early during development and are maintained in the embryos of *G. scandens* as they develop long, shallow and pointed beaks and these features are maintained through later developmental stages. (**B,E,F**) Species-specific characteristics are easily recognizable by stage 33 in embryos of *G. scandens* (**B**), *G. fortis* (**E**), and *G. magnirostris* (**F**). Scale bars are: B-G 5mm.

Supplemental Figure 3.

Embryonic heads of stage 26 embryos of Darwin's Finches sectioned and shown in Figure 1B. (**A-F**) Heads of *G. difficilis, G. fuliginosa, G. fortis, G. magnirostris, G. scandens* and *G. conirostris* embryos were sectioned medially as revealed by the presence of the Rathke's pouch (RP; red arrowhead) and telencephalic opening (te). Both the upper (ub) and lower (lb) beaks are shown. Very high Nomarski was used so that the low-background sections could be photographed at low magnification. The intensity is reflective of cell density and not of *Bmp4* signal. Scale bars: 1mm in A.

Supplemental Figure 4.

Comparative analysis of *Bmp2* and *Bmp7* expression domains in species of *Geospiza*. (**A**) The six species of *Geospiza* display distinct beak shapes and sizes. (**B**) *Bmp2* was expressed in ventral epithelium and immediately adjacent areas of ventral mesenchyme at stage 26 embryos of *G. fortis* and *G. magnirostris,* and *G. scandens* and *G. conirostris*. The expression was strongest *G. magnirostris* and *G. conirostris* embryos, the two largest species. (**C**) *Bmp7* was expressed in the ventral-most mesenchyme of the FNP of all the species sampled (**D**) Domains of *Bmp4* expression in the upper beak prominence of *G. magnirostris* at stages 26 and 29. Scale bars: 1mm in B,C.

Supplemental Figure 5.

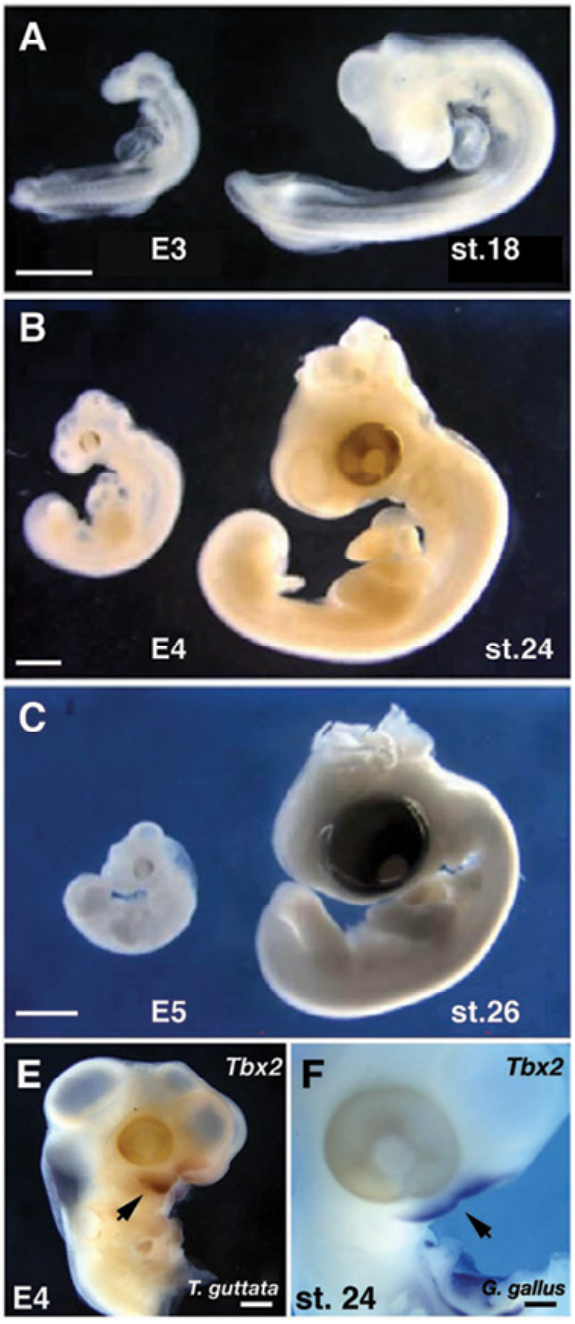
Comparison of *Col II* expression in st. 30 embryos whose epithelium (**A**) and mesenchyme (**B**) were infected with RCAS::*Bmp4* with st. 30 wild-type embryo (**C**). (**D-E**) Epithelial misexpression of RCAS::*Bmp4* causes expansion of the lateral facial

structures. Frontal and side whole-head views of embryo with epithelium infected with RCAS::*Bmp4*. The nasal capsules surrounded by the MXP tissue are indicated with white arrowheads. The upper beak prominence was highly reduced (white arrow). Scale bars: 1mm in A, 2mm in D.

Supplemental Figure 6.

Spread of RCAS infection as shown with RSCH probe in chicken upper beaks illustrated in Figure 4D,H,L. (**A**) No RCAS infection in control wild-type embryos. Strong RCAS::*Bmp4* (**B**) and RCAS::*Noggin* (**C**) infection can be detected with RSCH *in situ* hybridization.

FIGURE S1



D	G. gallus	T. guttata
st. 1	Pre-Streak, 1-2h	Pre-Streak, 1-2h
st. 5	Head-Process, 19-22h	Head-Process, about 20h
st. 9	Seven somites, primary optic vesicles present, 29-33h	Seven somites, primary optic vesicles present, ~30h
st.14	22 somites, axes of forebrain and hindbrain form a right angle, 50-53h	22 somites, axes of forebrain and hindbrain form a right angle, ~55h
st.18	30-36 somites, tail-bud turned to the right at a right angle, MXP visible, 65-69h	30-36 somites, MXP visible, ~70h
st.26	contour of MXP a broken line, 2nd arch overgrown 3rd and 4th arches, 4.5-5 days	contour of MXP a broken line, 2nd arch overgrown 3rd and 4th arches, ~5 days
st.30	distinct egg-tooth, MNP approaches upper beak, 6.5 days	distinct egg-tooth, MNP approaches upper beak, 7 days
st.38	nictitating membrane covers anterior scleral pappilae and approaches cornea, lower lid has grown to level of cornea, lids form a ellipse opening	nictitating membrane covers anterior scleral pappilae and approaches cornea, lower lid has grown to level of cornea
	~11 days	~10 days
Hatching:	20-21 days	11 days

FIGURE S2

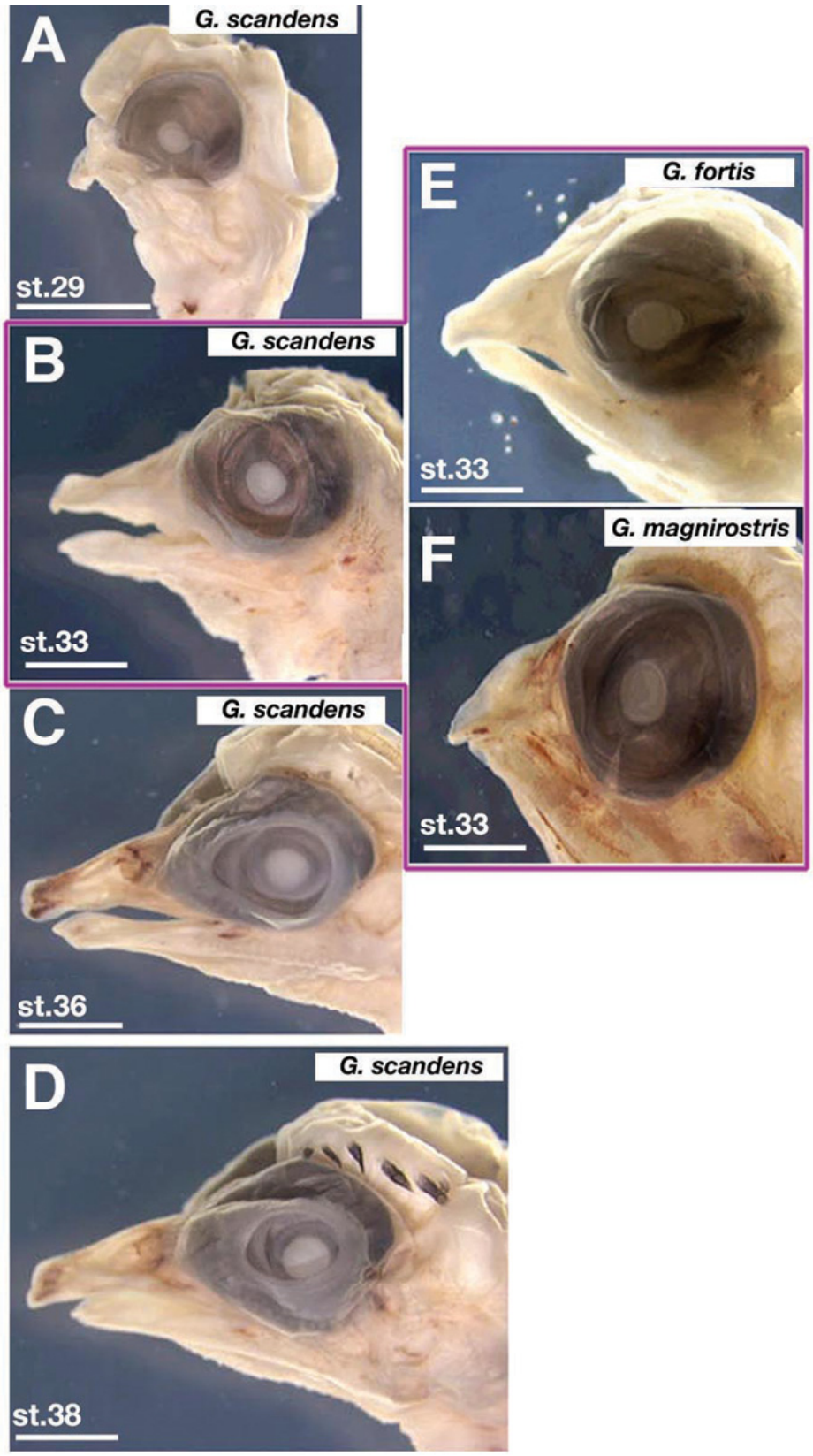

A
G. scandens
st.29
B
G. scandens
st.33
C
G. scandens
st.36
D
G. scandens
st.38
E
G. fortis
st.33
F
G. magnirostris
st.33

FIGURE S3

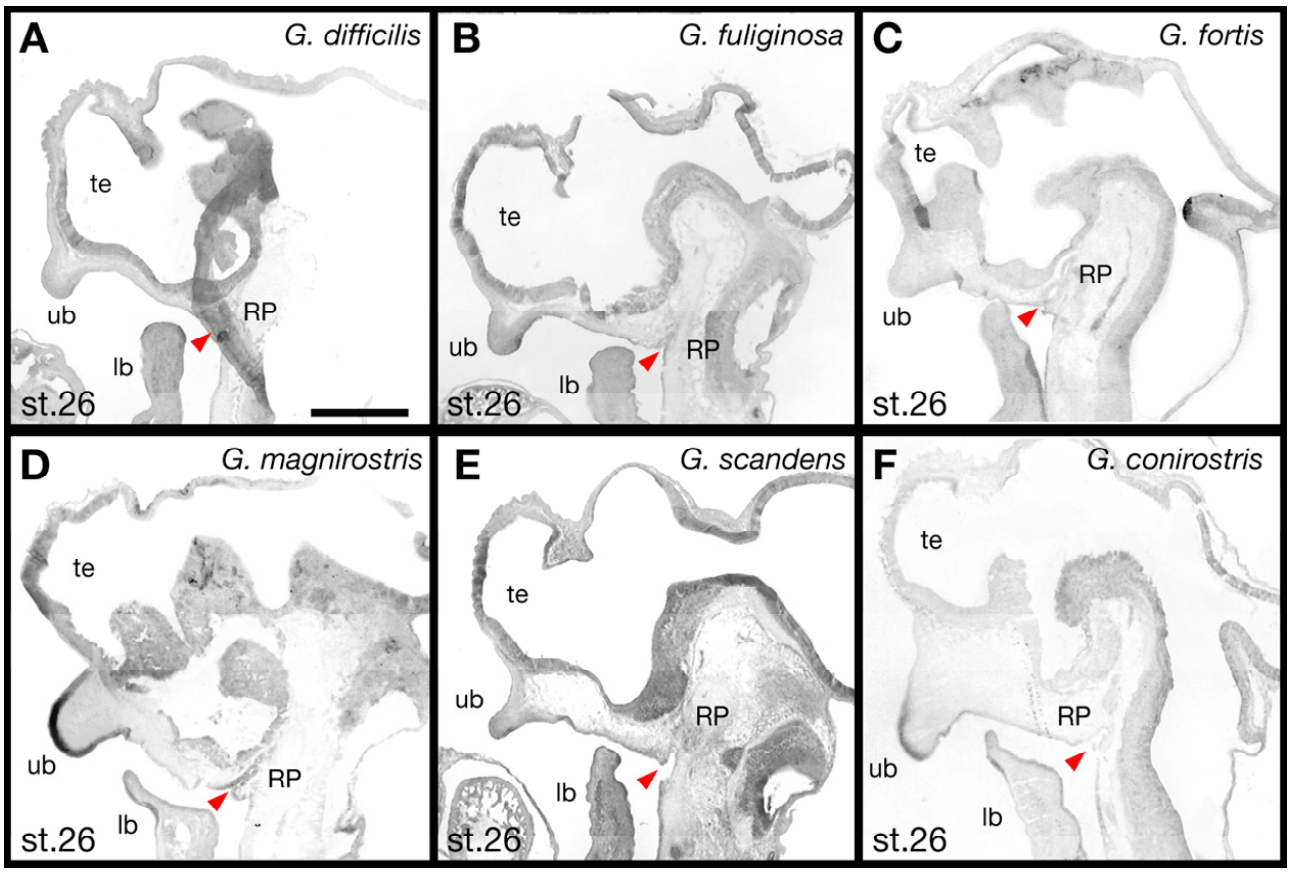

A
G. difficilis
te
ub
RP
lb
st.26
B
G. fuliginosa
te
ub
RP
lb
st.26
C
G. fortis
te
RP
ub
st.26
D
G. magnirostris
te
ub
RP
lb
st.26
E
G. scandens
te
ub
RP
lb
st.26
F
G. conirostris
te
RP
ub
lb
st.26

FIGURE S4

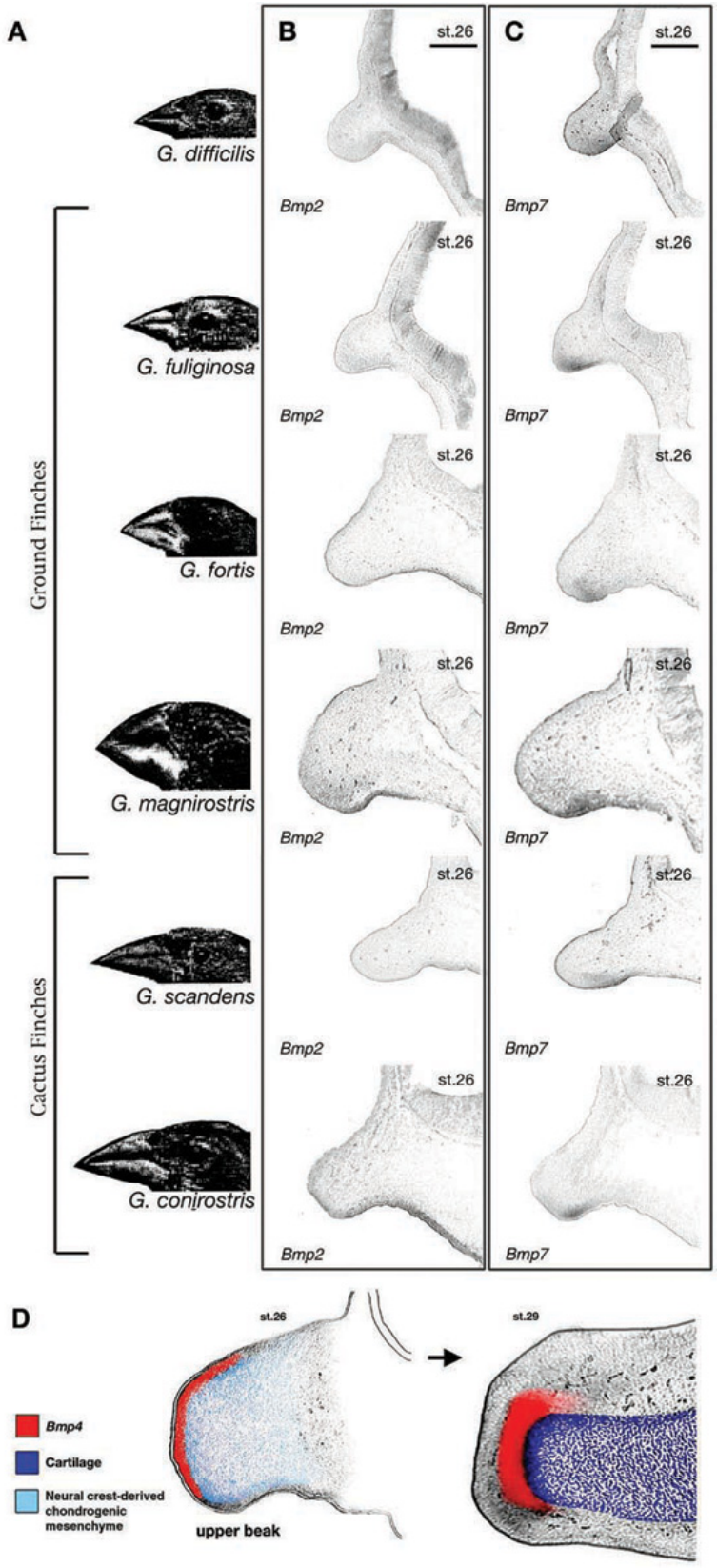

A
B
C
st.26
G. difficilis
Bmp2
Bmp7
G. fuliginosa
G. fortis
G. magnirostris
Ground Finches
G. scandens
G. conirostris
Cactus Finches
D
st.29
Bmp4
Cartilage
Neural crest-derived chondrogenic mesenchyme
upper beak

FIGURE S5

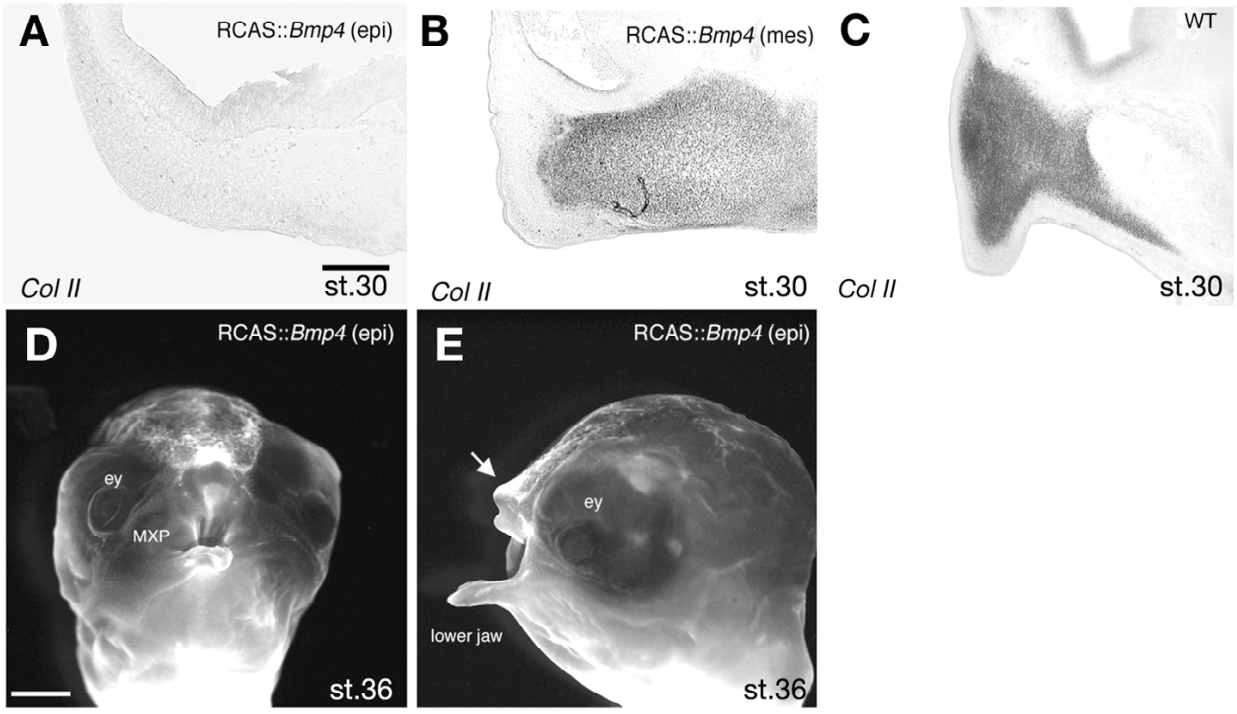
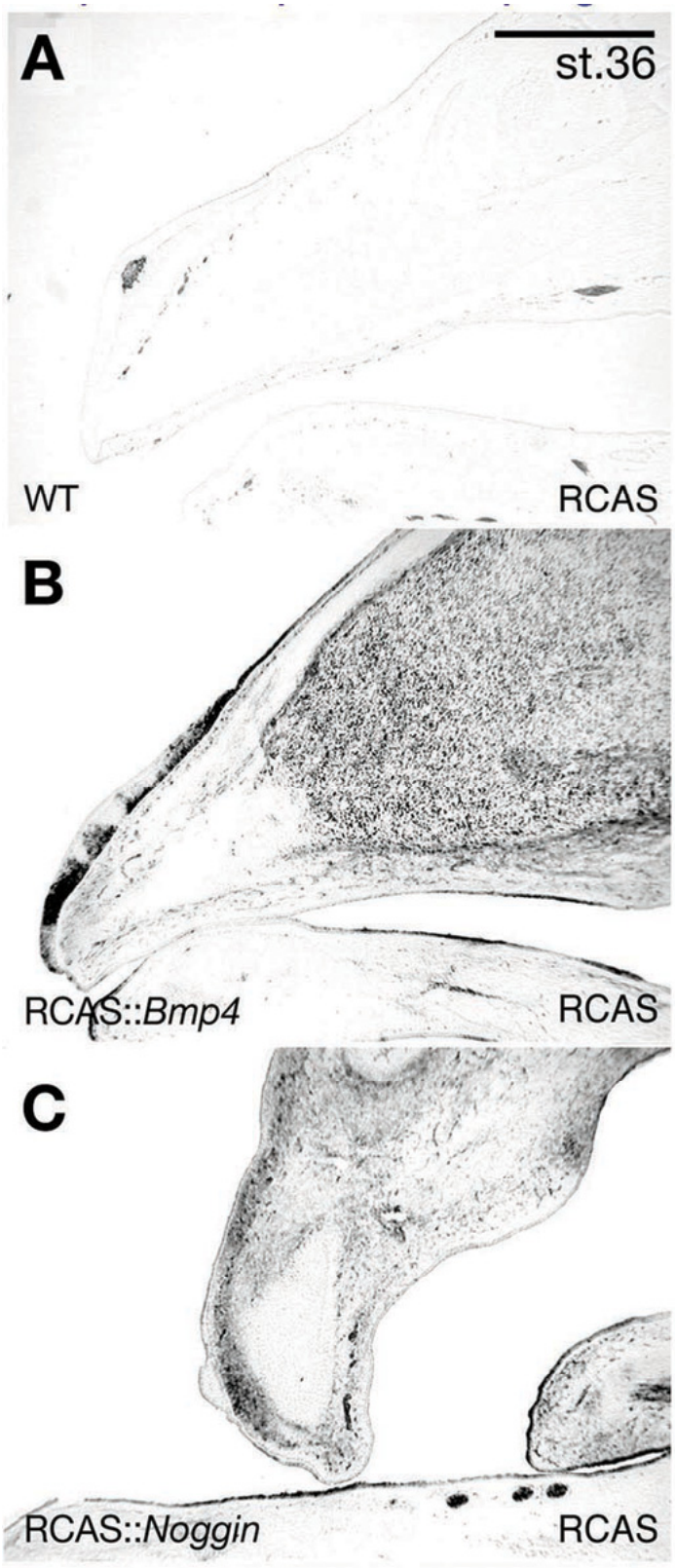

A
RCAS::Bmp4 (epi)
Col II
st.30
B
RCAS::Bmp4 (mes)
Col II
st.30
C
WT
Col II
st.30
D
RCAS::Bmp4 (epi)
ey
MXP
st.36
E
RCAS::Bmp4 (epi)
ey
lower jaw
st.36

FIGURE S6



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