Formation of a “Pre-mouth Array” from the Extreme Anterior Domain Is Directed by Neural Crest and Wnt/PCP Signaling

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Formation of a “Pre-mouth Array” from the Extreme Anterior Domain Is Directed by Neural Crest and Wnt/PCP Signaling

Graphical Abstract

Highlights

- A Xenopus pre-mouth cell array splits down the midline to surround the oral opening
- The pre-mouth array forms by convergent extension of EAD ectoderm
- EAD morphogenesis is directed by adjacent cranial neural crest
- Wnt/PCP signaling is necessary and sufficient to elicit EAD convergent extension

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In Brief

Jacox et al. identify a precise cellular organization of extreme anterior domain (EAD) ectoderm—the “pre-mouth array”—that contributes to the future mouth opening in Xenopus. Their data indicate that the pre-mouth array forms by convergent extension, under control of adjacent neural crest and Wnt/PCP signaling.
Formation of a “Pre-mouth Array” from the Extreme Anterior Domain Is Directed by Neural Crest and Wnt/PCP Signaling

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SUMMARY

The mouth arises from the extreme anterior domain (EAD), a region where the ectoderm and endoderm are directly juxtaposed. Here, we identify a “pre-mouth array” in Xenopus that forms soon after the cranial neural crest has migrated to lie on either side of the EAD. Initially, EAD ectoderm comprises a wide and short epithelial mass that becomes narrow and tall with cells and nuclei changing shape, a characteristic of convergent extension. The resulting two rows of cells—the pre-mouth array—later split down the midline to surround the mouth opening. Neural crest is essential for convergent extension and likely signals to the EAD through the Wnt/planar cell polarity (PCP) pathway. Fz7 receptor is locally required in EAD ectoderm, while Wnt11 ligand is required more globally. Indeed, heterologous cells expressing Wnt11 can elicit EAD convergent extension. The study reveals a precise cellular mechanism that positions and contributes to the future mouth.

INTRODUCTION

The extreme anterior domain (EAD) is the earliest facial element. It is present across the anterior midline and comprises directly juxtaposed anterior ectoderm and endoderm that develop into the mouth opening and oral cavity (Dickinson and Sive, 2006, 2009; Jacox and Sindelka et al., 2014). In Xenopus, the EAD is also a craniofacial organizer required for cranial neural crest (NC) ingression toward the midline (Jacox and Sindelka et al., 2014). This results in NC coming to lie on either side of the EAD prior to differentiating into facial tissues (Dickinson and Sive, 2007; Spokony et al., 2002).

EAD development from the EAD occurs in multiple steps (Dickinson and Sive, 2006, 2007) that include specifying a broad anterior domain characterized by the expression of pitx genes at neurula stages and the disappearance of basement membrane (BM) between EAD ectoderm and endoderm at late tailbud. The stomodeal invagination then forms, and ectoderm undergoes a burst of apoptosis that thins the tissue (Dickinson and Sive, 2006). Thinned ectodermal and endodermal layers intercalate to form the buccopharyngeal membrane that perforates as the mouth opens at swimming tadpole stage.

We previously noted that the EAD ectoderm forms a discrete epithelium two to four cells wide (Jacox and Sindelka et al., 2014). Here, we explore this observation further and show that the EAD ectoderm elongates into a “pre-mouth array” that later contributes to the oral opening. Cell sheet elongation can be driven by convergent extension, a process of cell intercalation that lengthens epithelial sheets and may require the Wnt/planar cell polarity (PCP) pathway (Roszko et al., 2009). We address the hypothesis that the pre-mouth array forms by convergent extension (CE) and examine the signaling role of adjacent NC in directing EAD ectoderm morphogenesis. The data reveal a precise cellular organization and identify the associated control mechanisms that position the future mouth opening.

RESULTS

EAD Ectoderm Becomes Longer and Narrower to Form a Pre-mouth Array that Later Opens into the Mouth

To confirm that the Xenopus EAD gives rise to the mouth, and to trace the origin and other derivatives of the EAD, we extended previous fate-mapping analyses (Dickinson and Sive, 2006). Dil (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanin) tracing from mid-neurula (stage 18) to mouth opening at swimming tadpole stage (stage 40) demonstrates that EAD ectoderm originates from the anterior neural ridge and contributes to the mouth (oral cavity), nostrils, and anterior pituitary (AP) (Figure S1), consistent with data from other species (Chapman et al., 2005; Couly and Le Douarin, 1985; Eagleson et al., 1995; Osumi-Yamashita et al., 1994; Schwind, 1928).

We assessed the narrowing of the EAD ectoderm previously and noticed its relationship to mouth opening (Jacox et al., 2014b) through a histological time course from late neurula stages and the disappearance of basement membrane (BM) between EAD ectoderm and endoderm at late tailbud. The stomodeal invagination then forms, and ectoderm undergoes a burst of apoptosis that thins the tissue (Dickinson and Sive, 2006). Thinned ectodermal and endodermal layers intercalate to form the buccopharyngeal membrane that perforates as the mouth opens at swimming tadpole stage.
(stage 20) to swimming tadpole (stage 40), analyzing adherens junctions through β-catenin and BM through staining for laminin (Figures 1 and S2). In coronal sections, the EAD ectoderm showed high levels of membrane-bound β-catenin (Figures 1A–1E) and was bordered by BM that separated it from NC, the brain and cement gland (Figures 1 J–1M). Lateral BM was patchy prior to NC ingression toward the EAD ectoderm but became continuous as NC migrated into the face at stage 24 (Figures 1J, 1K, and 3B–3D) and persisted subsequently (Figures 1L and M). Prior to NC ingression, a "pouch" devoid of cells is present on either side of the EAD ectoderm (white box) (Figures 1B–1E, 1J–1K).

At early tailbud (stage 22), the EAD ectoderm comprises a wide and short region (eight to nine cells wide and nine to ten cells high), while at late tailbud stage (stage 28), it forms a tall, thin column of cells (2–3 cells wide and 20–21 cells high) (Table 1; Figures 1B–1M). We call this ~2 × 20-cell organization the pre-mouth array (Figures 1E and 1M, asterisk). BM surrounds EAD ectoderm, laterally (Figures 1J–1M), dorsally, and ventrally (Figures S2; Movie S1). At hatching stage (stage 35–36), the two rows of β-catenin-positive cells separate down the middle and the oral opening is complete in the tadpole (stage 39–40) still surrounded by BM (Figures 1P–1S and 1X–1a). In contrast to BM, the apical marker ZO-1 appeared just before the cell rows separate at hatching stages (Figure S3). Using Claymation, we animated a putative sequence of EAD ectodermal elongation and mouth opening (still frames Figures 1F–1I and 1T–1W; Movie S2).

Figure 1. Coronal Anatomy of *Xenopus* Face and EAD Ectoderm between Late Neurula and Swimming Tadpole


(B–E and P–S) Coronal sections with β-catenin immunolabeling (two independent experiments; stage 22, n = 10; stage 24, n = 11; stage 26, n = 14; stage 28, n = 14). Midline region (bracket) with bright β-catenin labeling is EAD ectoderm of the pre-mouth array. Bracket: region of 10× image (B–E and P–S) enlarged in 25× view (B′–E′ and P′–S′). Asterisk in (E), pre-mouth array at stage 28, enlarged in (E′), cg, cement gland.

(F–I and T–W) Still frames from Claymation of mouth opening found in Movie S2. (I) Pre-mouth array stage of Claymation.

(J–M and X–a) Coronal sections with laminin (green) immunolabeling with propidium iodide (PI) nuclear counterstain (red) (two independent experiments; stage 22, n = 10; stage 24, n = 4; stage 26, n = 6; stage 28, n = 7). Bracket: region of 10× image (J–M and X–a) enlarged in 25× view (J′–M′ and X′–a′). Asterisk (M), pre-mouth array at stage 28, enlarged in (M′).

(B′, C′, J′, and K′) White boxes surround lateral regions next to EAD, which fill with NC cells between stages 22 (B′ and J′) and 24 (C′ and K′). Scale bars represent 170 μm (10×) and 68 μm (25×).
These data identify a previously undescribed stage of mouth development, the ‘pre-mouth array’ that derives from organization of the EAD ectoderm and later contributes to the oral opening.

EAD Ectoderm Reorganization Is Consistent with Convergent Extension

To examine EAD ectodermal reorganization at higher resolution and assess underlying mechanism, we tiled cells in the region and quantified height, width, and depth. As noted at lower magnification, the EAD ectoderm begins as a wide and short region (eight or nine cells wide and nine or ten cells high) at early tailbud stage (stage 22), while at late tailbud stage (stage 28), it forms a tall, thin column of cells (2–3 cells wide and 20–21 cells high) (Table 1; Figures 2A–2D). Analysis of cell shape showed that early-tailbud-stage cells were laterally elongated (bipolar) with laterally elongated nuclei, whereas later-tailbud-stage cells were columnar with round nuclei and formed two parallel rows (Figures 2A–2H). These differences are consistent with convergent extension, where cells rearrange to lengthen and narrow the EAD ectoderm while undergoing stereotypical cell and nuclear shape changes (Wallingford et al., 2002; Yin et al., 2009). The EAD ectoderm is six or seven cells deep at stage 22 and deepens slightly to eight or nine cells at stage 28 (Table 1; Figures 2E–2H). Two models that could account for observed morphogenetic changes of EAD ectoderm were considered. In model 1 (Figures 2I and 2J), lateral EAD ectodermal cells die, narrowing the tissue, while midline cells divide with their mitotic axes oriented to promote tissue elongation. For this model to account for changes in height and width between stages 22 and 28, 67% of EAD ectodermal cells would need to die and each midline EAD ectodermal cell would need to divide once, doubling EAD height (Table 1). In model 2 (Figures 2K and 2L), convergent extension transforms EAD ectoderm from a square to an elongated rectangle due to cellular intercalation and shape change with minimal cell division and death.

Model 1 is not supported by the data, as fewer than 1% of EAD ectodermal cells die (Dickinson and Sive, 2006; Figure S6), and there are infrequent cell divisions, on average one to three mitoses throughout the EAD ectoderm between stages 23 and 26, with random axis orientation (Figure S6). In contrast, observed tissue and cell changes are consistent with model 2, and we conclude that convergent extension is the likely mechanism by which EAD ectodermal elongation into the pre-mouth array occurs.

### Table 1. Average Cell Number of EAD Tissue Dimensions

<table>
<thead>
<tr>
<th>Stage</th>
<th>Height (n = 35, SD 2.1)</th>
<th>Width (n = 12, SD 2.3)</th>
<th>Depth (n = 23, SD 1.3)</th>
<th>Total (Height x Width x Depth)</th>
<th>Endoderm Depth (n = 16, SD 1.2)</th>
<th>Outer Ectoderm Depth (n = 16, SD 0.7)</th>
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<tr>
<td>Stage 22</td>
<td>9.5</td>
<td>8.2</td>
<td>6.2</td>
<td>485</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Stage 24</td>
<td>10.9</td>
<td>6.7</td>
<td>8.0</td>
<td>579</td>
<td>2.1</td>
<td>1.7</td>
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<tr>
<td>Stage 26</td>
<td>15.6</td>
<td>3.8</td>
<td>8.7</td>
<td>521</td>
<td>2.3</td>
<td>1.4</td>
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<tr>
<td>Stage 28</td>
<td>20.3</td>
<td>2.7</td>
<td>8.2</td>
<td>446</td>
<td>1.9</td>
<td>1.4</td>
</tr>
</tbody>
</table>

EAD Ectoderm Convergent Extension Is Associated with Neural Crest Ingress and Dependent on Neural Crest

Previous analyses (Jacox and Sindelka et al., 2014) suggested that EAD ectoderm elongation is correlated with NC migration into the region. To examine the timing of NC association with EAD ectoderm morphogenesis, we performed detailed immunohistochemistry where mGFP-labeled NC was transplanted into unlabeled recipients, which were later immunostained for β-catenin (Figures 3A–3D). By late neurula, the NC arrived at the EAD, but it did not completely fill the space adjacent to it, and regions of EAD ectoderm were not touching the NC. Later, NC cells closely abutted the entire EAD ectoderm, which became narrower and taller by late tailbud stages. NC cells did not mingle with EAD ectoderm but remained a distinct group with a sharp border at the EAD, suggesting this region may act as a barrier.

To characterize dynamics of NC/EAD localization, live lineage analysis was performed using a mApple-labeled EAD transplanted into a recipient that included mGFP-labeled NC (Figure S3III). Consistent with histology, the EAD transplant changed from a rectangular shape at late neurula to a narrow and tall shape as NC came to lie on either side of the EAD (Figure S3III). Consistent results were obtained by double in situ hybridization with the EAD marker enin and an NC marker, sox9 (Figure S3III).

The close association of NC ingress and EAD elongation suggested that NC might be required for EAD ectoderm morphogenesis. NC lineages were ablated by loss of function (LOF) for the essential gene sox9 (Spokony et al., 2002) by injection of morpholino-modified antisense oligonucleotides (MOs). Resultant embryos did not show EAD ectoderm elongation, indicating a requirement for NC in this process (Figures 3G, 3H, and 3J). A titration of sox9 MOs identified a threshold requirement for proper EAD ectoderm convergent extension (Figure S4). Older embryos did not recover midline convergent extension at stage 32 (Figure S7) and failed to form a mouth and nostrils at stage 40 (Figures 3E and 3F).

These data show a close association of EAD ectoderm morphogenesis and adjacent NC and further demonstrate that NC is required for EAD convergent extension.

A Localized Requirement for Wnt/PCP Signaling from the Neural Crest in EAD Ectoderm Convergent Extension

Convergent extension is dependent on Wnt/PCP signaling in other morphogenetic events (Roszko et al., 2009) (Figure 4P), and we therefore explored necessity of this pathway during EAD ectoderm elongation. We tested whether the frizzled 7 receptor (fzd7), intracellular signaling mediator dvl, and Wnt/PCP ligand wnt11 were required specifically in the EAD for convergent extension. These assays were based on expression of fzd7 RNA throughout the face, wnt11 RNA in the NC, but not in the EAD (Figure S4), and on whole-embryo LOF phenotypes (Figures S5–S7). A facial transplant protocol was used (Jacox et al.,...
Figure 2. Detailed Anatomy and Modeling of Xenopus EAD Ectoderm between Late Neurula and Swimming Tadpole

(A–D) Coronal sections with β-catenin (green) immunolabeling with PI nuclear counterstain (red) from stages 22–28 (two independent experiments; stage 22, n = 10; stage 24, n = 11; stage 26, n = 14; stage 28 n = 14).

(E–H) Sagittal sections with β-catenin (green) immunolabeling from stages 22–28 (three independent experiments; stage 22, n = 12; stage 24, n = 10; stage 26, n = 12; stage 28, n = 12).

(A′–D′ and E′–H′) Cell membranes traced in white. Blue line separates deep EAD from outer ectoderm. Yellow line separates EAD ectoderm from endoderm.

(A″–D″ and E″–H″) Cell outlines in black. Dotted line is the top of the cement gland (cg).

(D–D″) Pre-mouth array is present and indicated by cell outlines. Scale bar (25x), 88 μm. Scale bars (40x), 43 μm.


(K and L) Model 2. (K) Stage 22. (L) Stage 28.

(M) Quantification of height versus width of EAD (see Experimental Procedures) (three independent experiments; stage 22, n = 12; stage 24, n = 16; stage 26, n = 17; stage 28, n = 47). p values were obtained from unpaired, two-tailed t tests comparing sequential stages. Error bar represents SD.

(N) Diagram demonstrating coronal (A–D′) and sagittal (E–H′) sections.

(O) Diagram showing the change in height (H), width (W), and depth (D) of the EAD ectoderm and its surrounding BM between stages 22 and 28. Blue ovals, EAD ectodermal cells undergoing convergent extension. Orange rectangular prism, laminin BM surrounding EAD ectoderm.
Figure 3. EAD Ectoderm Undergoes Convergent Extension as the Cranial Neural Crest Approaches the Midline and EAD Convergent Extension Fails to Occur in sox9 LOF Embryos

(A) Experimental schematic.

(B–D) Coronal sections with mGFP-labeled NC (green) and β-catenin (red) immunolabeling from late neurula (stage 24) to late tailbud (stage 28) (two independent experiments; stage 24, n = 7; stage 26, n = 7; stage 28, n = 4). Midline region (bracket) with bright β-catenin labeling is EAD ectoderm. Bracket: region of 10x image (B–D) enlarged in 40x view (B’–D’) and 63x view (B”–D”). cg, cement gland.

(E and F) Frontal view of control and sox9 LOF embryos at swimming tadpole (stage 40) assayed in two experiments (control MO in E, n = 24; sox9 MO in F, n = 30.) Dots surround open mouth. Bracket: unopened mouth. Scale bar, 200 μm.

(G–H) Coronal sections assayed in four independent experiments (n = 23) with β-catenin immunolabeling and Hoechst nuclear labeling. Midline region with bright β-catenin labeling is EAD ectoderm. Bracket: region of 10x image (G and H) enlarged in 25x view (G’ and H’). cg, cement gland.

(I) Graph depicting percent of embryos displaying face, mouth, nostril, and pigment formation phenotypes at stage 40 in control and sox9 LOF embryos. p values were obtained from Fisher’s exact probability test.

(J) Quantification of height over width of EAD (see Experimental Procedures). p values were obtained from unpaired, two-tailed t tests. Error bar represents SD. Unless otherwise specified, scale bars represent 170 μm (10x), 68 μm (25x), 43 μm (40x), and 27 μm (63x).
where GFP-labeled EAD was removed from control, Dep+-injected embryos or from fzl7 and wnt11 LOF embryos at early tailbud (stage 22) and transplanted to sibling controls (Figure 4A) (Jacox et al., 2014a). Control transplants underwent normal EAD ectoderm elongation (Figure 4B, 4D, 4H, and 4K; quantified in Figures 4F, 4G, 4N, and 4O); however, when fzl7 LOF or Dep+ EAD was transplanted into control embryos, extension did not occur and small, deformed mouths and nostrils formed (Figures 4C, 4E–4G, 4I, 4L, 4N, and 4O). In contrast, transplant of wnt11 LOF EAD led to normal extension and face formation (Figures 4J and 4M–4O), indicating that regions outside the EAD can supply Wnt11. Local fzl7 LOF did not alter NC development, as measured by sox9 expression (Figure S7).

Failure of EAD ectoderm to undergo convergent extension is correlated with reduced or absent nostrils, but it is unclear whether these events are mechanistically connected. After sox9, wnt, and fzl7 LOF, laminin expression adjacent to the EAD was disordered and spotty (Figure S6), suggesting that NC may be required for formation of BM lateral to the EAD. We further asked whether oriented cell division or death occurred during EAD ectoderm elongation. Phospho-histone-3 (PH3) immunolabeling before or during EAD ectoderm elongation showed few dividing cells, randomly oriented (Figure S6). Minimal cell death was observed in the EAD ectoderm of control or LOF embryos (Figure S6). The very low levels of cell division and death and lack of significant change after Wnt/PCP LOF further support the conclusion that the EAD ectoderm undergoes convergent extension.

Figure 4. Fzl7 Is Locally Required in the EAD Ectoderm for Convergent Extension
Local requirement of Dsh, fzl7, and wnt11 expression tested with an EAD transplant technique.

(A) Experimental design: donor LOF tissue was transplanted to uninjected sibling recipients.

(B–C) EAD transplant outcome from control or Dep+ RNA donor tissue assayed in three experiments. (B and B') Control RNA (n = 23). (C and C') Dep+

Figure S7. Additional figures and data.
These results indicate that a Wnt11 signal originating outside the EAD, likely in the NC, engages the Fz17 receptor on EAD ectodermal cells to activate convergent extension via the Wnt/PCP pathway.

The GTPase Effectors Rac and JNK Are Required for EAD Ectoderm Convergent Extension

The Wnt/PCP signaling pathway bifurcates (Wallingford and Haas, 2005), and we asked which branch was involved in EAD convergent extension by assaying relevant GTPases using inhibitors and a bead-implantation assay (Figure 5A). Beads soaked in DMSO control or Rock or Rho inhibitors did not interfere with EAD ectoderm morphogenesis or face formation (Figures 5B, 5E–5G, and 5J–5M), although Rac1 and JNK inhibitors were associated with reduced convergent extension and failure of mouth and nostril formation (Figures 5C, 5D, 5H–5I, 5L, and 5M). Implanting JNK inhibitor beads at stages 22, 24, or 28 caused marked craniofacial abnormalities at stage 40, while inhibitor application after convergent extension at stage 32 resulted in a less severe phenotype, suggesting JNK signaling is crucial specifically during tailbud stages for mouth development (Figure S8). Immunostaining for active, nuclear localized p-JNK (Yin et al., 2009) demonstrated few positive nuclei prior to EAD ectoderm elongation (Figures 5N and 5T) and maximal nuclear localization in EAD ectodermal cells during elongation (stage 23; Figures 5O and 5T). The number of p-JNK-positive nuclei peaked by stage 23, as elongation is beginning, and diminished by stage 26, when extension is largely complete (Figures 5P and 5T). Fz17 LOF embryos demonstrated significantly lower levels of positive nuclei at stage 23 relative to control, concomitant with absence of EAD ectoderm convergent extension (Figures 5Q–5T). When a bead containing the JNK activator anisomycin was implanted in Wnt11 LOF faces, there was an improvement in convergent extension (Figures 5U–5X). These data indicate that the Rac1 and JNK branch of Wnt/PCP signaling is necessary for EAD ectoderm convergent extension.

Wnt11 Can Substitute for the Neural Crest and Is Sufficient to Direct EAD Ectoderm Convergent Extension

Since Wnt11 is necessary for EAD ectoderm elongation, we asked whether Wnt11 is sufficient to elicit this process by testing whether animal caps expressing ectopic Wnt11 could substitute for NC. One-cell embryos were injected with RNA encoding a control secreted protein (MMP11) or Wnt11; animal caps (blastula stage ectoderm) were removed at stage 9 and implanted on either side of the EAD in control or sox9 LOF embryos at stage 22 (Figure 6A). Embryos were assayed at stage 28 when EAD ectoderm convergent extension is normally complete. While caps expressing MMP11 did not alter control embryos (Figures 6B and 6F) or rescue EAD shape in sox9 LOF embryos (Figures 6D and 6H), Wnt11-expressing caps were sufficient to restore EAD ectoderm elongation when implanted into sox9 LOF embryos (Figures 6E, 6I, and 6J). Wnt11-expressing caps reduced EAD ectoderm elongation in control embryos (Figures 6C, 6G, and 6J), consistent with other overexpression phenotypes (De Calisto et al., 2005; Garriock et al., 2005). These data show that Wnt11 signaling is sufficient to direct EAD ectoderm convergent extension and highlight the conclusion that the Wnt/PCP pathway directs the pre-mouth array stage of oral development.

DISCUSSION

Mouth development is a lengthy process (3 days in Xenopus or 5 weeks in humans) and includes many steps that position the mouth-forming region and later define where the opening will occur. This careful sequence of events ensures that when the mouth opens, it connects productively with the digestive system. In this study, we clarify one mechanism underlying mouth formation and reach four major conclusions. First, EAD ectoderm forms an organized cell arrangement, the pre-mouth array, that will later split down the midline and contribute to the oral opening. Second, pre-mouth array formation is elicited by convergent extension. Third, the signal for EAD convergent extension originates in the NC as it comes to lie on either side of the EAD. Fourth, Wnt/PCP signaling is both necessary and sufficient to elicit EAD convergent extension. These findings reveal an unexpected level of precision controlling vertebrate mouth development.

Organization of the presumptive mouth ectoderm into two rows of cells that will later open down the apical midline has not been observed previously. We termed this the pre-mouth array to reflect that organization of the Xenopus mouth is established long before it opens. Indeed, the pre-mouth array forms very early, prior to breakdown of the basement membrane between ectoderm and endoderm (Dickinson and Sive, 2009) and persists until stomodeum formation begins, when the apical surfaces of the pre-mouth array cells begin to separate. Localization of apical junction proteins is associated with separation (Figure S3), and future analyses will address when the apical domain appears and whether it is necessary for mouth opening. The pre-mouth array forms from EAD ectoderm that overlies and connects with pharyngeal (gut) endoderm, suggesting that a signal from the underlying endoderm may also be involved in mouth opening.

Morphogenetic mechanisms by which the pre-mouth array forms could not be accounted for by directed proliferation or apoptosis, but EAD ectodermal cell shape and rearrangements appear similar to mediolateral intercalation resulting in convergent extension of axial mesoderm during Xenopus and zebrafish gastrulation (Keller et al., 2000; Tada and Heisenberg, 2012; Yin et al., 2009). We therefore conclude that the EAD ectoderm undergoes convergent extension. Absence of EAD ectoderm convergent extension is tightly associated with an abnormally small or absent mouth opening, suggesting it is a necessary part of mouth development.

The EAD ectoderm that undergoes convergent extension is a multilayered array, six to eight cells deep, which is bounded by BM. Thus, there is BM above the EAD ectoderm (abutting the epidermal ectoderm and brain), below it (abutting the pharyngeal endoderm) and on either side (abutting the NC) (Movie S1). These BMs form a 3D “cage” around the EAD ectoderm and perhaps constrain its morphogenetic possibilities. It will be interesting to examine how EAD morphogenesis is coordinated between ectodermal layers. Interestingly, prior to NC ingress, the region around the EAD ectoderm is devoid of cells, forming a
Figure 5. Inhibition of GTPases JNK and Rac1 Is Associated with a Reduction in EAD Ectodermal Convergent Extension

(A) Experimental schematic of inhibitor loaded bead implantation in the presumptive mouth, EAD region.
(B–F) Frontal view of swimming tadpole (stage 40) embryos with inhibitor loaded beads implanted in presumptive mouths, assayed in three experiments. (B) Control DMSO (n = 97). (C) Rac1 inhibitor (n = 40). (D) JNK inhibitor (n = 44). (E) Rock inhibitor (n = 75). (F) Rho inhibitor (n = 39). Bracket: unopened mouth. Dots surround open mouths. cg, cement gland. Scale bar, 200 μm.

(G–K) Coronal sections, stage 28, assayed in 3 independent experiments. (G and G') Control DMSO (n = 31). (H and H') Rac1 inhibitor (n = 27). (I and I') JNK inhibitor (n = 24). (J and J') Rock inhibitor (n = 9). (K and K') Rho inhibitor (n = 12) with β-catenin immunolabeling. Midline region with bright β-catenin labeling is EAD ectoderm. Bracket: region of 10x image (G–K) enlarged in 25x view (G’–K’). Scale bars represent 170 μm (10x) and 68 μm (25x).

(L) Graph depicting percentage of embryos, displaying face, mouth, nostril, and pigment formation phenotypes at stage 40. p values are from Fisher’s exact probability test.

(legend continued on next page)
pouch that is filled by NC cells as they become tightly apposed to the basal sides of the EAD (Figure 1). The BM along the lateral edges of EAD ectoderm is patchy prior to NC ingress, suggesting that NC may promote BM formation on either side of the EAD.

Given that EAD ectoderm convergent extension does not occur in the absence of NC, the NC likely sends the signal for morphogenesis. The disposition of NC relative to EAD ectoderm suggests that it signals along the basal-apical axis and not along the planar axis, as suggested for convergent extension during gastrulation (Tada and Heisenberg, 2012). It will be important to examine the localization of EAD Wnt/PCP signaling components to further understand the direction of the signal.

The requirement for wnt11 and the ability of Wnt11 to substitute for NC in eliciting EAD ectoderm extension indicates that this is a pivotal signaling factor; however, other Wnt/PCP ligands may also be involved. Wnt/PCP signaling is required for proper NC migration in Xenopus. For example, Wnt/PCP signaling at contact points between NC cells halts its movement and leads to a change in their direction; this contact inhibition promotes coherent NC migration (Carmona-Fontaine et al., 2008). In another example, through a “chase-and-run” reciprocal interaction, placodal cells attract NC, but direct contact facilitates N-cadherin junction formation, activating the PCP pathway that repulses placodal cells (Thervenau et al., 2013; Steventon et al., 2014). NC and placodes remain in close proximity and signal reciprocally to coordinate morphogenesis of sensory systems (Steventon et al., 2014). Wnt/PCP signaling functions in an additional way to modulate EAD ectoderm morphogenesis, although it is unclear whether factors used are identical in each of these cases. Many additional factors are required for mouth formation, including sonic hedgehog (Eberhart et al., 2006; Tabler et al., 2014), retinoic acid (Kennedy and Dickinson, 2012; Dickinson and Sive, 2009) and β-catenin Wnt signaling (Dickinson and Sive, 2009). However, the role of these factors in EAD ectoderm morphogenesis is not known.

Previous analyses demonstrated that the EAD is an organizer that promotes ingestion of first-arch NC into the nascent face to lie on either side of the EAD (Jacox et al., 2014b; Figure 3). This study describes a subsequent reciprocal signaling from NC to the EAD ectoderm to elicit convergent extension. There may be additional NC/EAD interactions. We note that NC cells and EAD ectodermal cells do not mingle and the NC does not cross the midline, implying that the EAD is a barrier to incoming NC, perhaps maintaining distinct identities in the two halves of the face.

In sum, we have identified a pre-mouth array stage in oral development that forms through local signaling interactions and readies the embryo to later make an open mouth. The precision involved is striking, and future analyses will define details of the mechanism underlying pre-mouth array formation and mouth opening.

**EXPERIMENTAL PROCEDURES**

**Embryo Preparation**

Xenopus laevis embryos were cultured using standard methods (Sive et al., 2000). Xenopus embryos were staged according to Nieuwkoop and Faber (1994). All animal subjects and protocols used in this publication were overseen and approved by the Massachusetts Institute of Technology Institutional Review Board.

**Dil Mapping and Transplants**

All fate mapping and transplants were done in 0.5× modified Barth’s solution (MBS). Fate mapping was performed by injecting a 5- to 10-nl drop of Dil (2 mg/ml, Molecular Probes) or 3,3′-diododecyloxacarbocyanine perchlorate (DiO, 2 mg/ml; Molecular Probes) into the EAD or anterior neural ridge ectoderm at early or late neurula stages. Embryos were photographed and fixed at tailbud and tadpole stages for immunohistochemistry. EAD transplants were performed according to Jacox et al. (2014). NC transplants were performed according to Mancilla and Mayor (1996). Animal cap transplants were performed on late neurula control or sox9 LOF embryos, injected with 5 ng morpholino. Tissue lateral to the EAD in LOF embryos was extirpated using a 1-mm-diameter capillary tube pulled to a fine point. Animal caps were removed from embryos injected with 1 μg either inactive MMP11 (control) or Wnt1 mRNA plus 1 μg mApple mRNA. Animal cap tissue was transplanted into the face of extirpated LOF embryos, and held in place with glass bridges for 1–2 hr. Transplants were cultured until late tailbud and then photographed, fixed, and sectioned for immunohistochemistry.

Height, depth, and width measurements of the EAD were made as follows: height, number of cells between the top of the cement gland and bottom of the brain, in coronal and sagittal sections; depth, number of cells between the top/ left and bottom/right boundaries of the EAD deep ectoderm with high β-catenin labeling, in sagittal sections; and width, number of cells between the left and right borders of the bright, β-catenin-positive midline or between the left and right midline laminin basement membranes, in coronal sections.

**In Situ Hybridization**

cDNAs were used to transcribe in situ hybridization probes, including cprn (BC059995), sox9 (AY035397), fzl7 (De Calisto et al., 2005), wnt11 (Tada and Smith, 2000), pitx1 (Schweickert et al., 2001), pitx2c (Schweickert et al., 2001), frzb1 (BC108885), and XCG (Sive et al., 1989). In situ hybridization was performed as described by Sive et al. (2000), without protease K treatment. The double-staining protocol was adapted from Wiellette and Sive (2003).

**Morpholinos and RNA Rescues**

Xenopus antisense MO-modified oligonucleotides (morpholinos; MOs) included start site MOs targeting fzl7 (31 ng; Winklbauer et al., 2001), wnt11 (9 ng; Pandur et al., 2002), and sox9 (5 ng; Spokony et al., 2002) injected at the one-cell stage. Murine mRNA (500 ng fzl7 mRNA and 700 ng wnt11 mRNA) was injected into the face of extirpated LOF embryos, and held in place with glass bridges until the mid gastrula (stage 28). Embryos were fixed, and sectioned for immunohistochemistry.

**In Vivo Injection of NC and EAD**

NC and EAD transplants were performed according to Mancilla and Mayor (1996) at tailbud and tadpole stages for immunohistochemistry. EAD transplants were performed according to Jacox et al. (2014). NC transplants were performed according to Mancilla and Mayor (1996).

**Antibody Staining**

Antibodies were used as follows: anti-mApple (green), anti-mCherry (red), and anti-Hoechst (blue). All embryos were staged according to Nieuwkoop and Faber (1956). All antibody staining was performed according to Mancilla and Mayor (1996).

**Immunohistochemistry**

Immunohistochemistry was performed as described by Sive et al. (1996), except that all primary antibodies were diluted 1:1000 and secondary antibodies were diluted 1:1000. Antibodies were used as follows: anti-mApple (green), anti-mCherry (red), and anti-Hoechst (blue). All embryos were staged according to Nieuwkoop and Faber (1956). All antibody staining was performed according to Mancilla and Mayor (1996).

**Quantification of Cells with p-JNK Positive Nuclei**

Quantification of cells with p-JNK positive nuclei in the EAD ectoderm. The total number of EAD nuclei was equivalent between stage-matched control and wnt11 LOF embryos. p < 0.0006, control stage 23 compared to stages 20 and 26. p values were obtained from unpaired, two-tailed t tests.
mRNA, generated from pRKs plasmids, gifts of Chris Garcia and Jeremy Nathans. Addgene plasmids 42259 and 42290 (Yu et al., 2012) and morpholinon (2′I MO 31 ng, wnt11 MO 9 ng) were coinjected at the one-cell stage to test morpholino specificity via RNA rescue. wnt11 mRNA (De Rienzo et al., 2011), truncated Dep+ disheveled mRNA (Sokol, 1996; Tada and Smith, 2000), and noncatalytic mmp11 (gift of Malcolm Whitman) were generated from plasmids. RNA was generated in vitro using the mMESSAGE mMACHINE kit (Ambion).

**Immunohistochemistry**

Immunohistochemistry was performed as described previously (Dickinson and Sive, 2006). Caspase-3 and PH3 labeling was performed according to Kennedy and Dickinson (2012) and Dickinson and Sive (2009). Primary antibodies included a rabbit polyclonal anti-laminin antibody (Sigma L-9393) diluted 1:150, rabbit polyclonal anti-β-catenin (Invitrogen) diluted 1:100, a mouse monoclonal anti-β-catenin (Abecco Dickinson 347580) diluted 1:750, and a mouse monoclonal anti-ZO-1 antibody (Invitrogen) diluted 1:100. Secondary antibodies included Alexa 488 and Alexa Fluor 647 goat anti-rabbit (Molecular Probes) and Alexa 488 goat anti-mouse (Molecular Probes) diluted 1:500 with 0.1% propidium iodide (Invitrogen) or Hoechst (Life Technologies) as a counterstain. Phalloidin (Life Technologies), an actin dye, was used in combination with Dil labeling. Sections were imaged on a Zeiss LSM 710 laser scanning confocal microscope. Images were analyzed using Imaris (Bitplane) and Photoshop (Adobe).

**Wnt/PCP Inhibitor and Activator Assays**

Rock inhibitor (Calbiochem Y-27632, stock: 20 mM in DMSO, working: 200 μM in 2% DMSO, −80°C storage), Rac1 inhibitor (Santa Cruz N223766, stock: 37 mM in DMSO, working: 200 μM in 2% DMSO, −20°C storage), Rho inhibitor (Calbiochem CCG-1423, stock: 22 mM in DMSO, working: 200 μM in 2% DMSO, −20°C storage), and JNK inhibitor (Sigma-Aldrich SP600125, stock: 20 mM in DMSO, working: 200 μM in 2% DMSO, 4°C storage) were resuspended in DMSO, aliquoted, and stored at stock concentrations until incubation with beads. The JNK activator anisomycin (Santa Cruz Biotechnology sc-3524, stock 67 μg/ml, working: 67 ng/ml in water, 4°C storage) (Liao et al., 2006) was resuspended in water, aliquoted, and stored at 1,000× stock concentrations until incubation with beads. AG 1-XX resin beads (Bio-Rad 140–1,231, 50–100 mesh) were washed in ethanol, dried, mixed with diluted, working concentration inhibitor solution, and incubated overnight at 4°C. Affi-gel blue agarose beads (Bio-Rad 50–100 mesh) loaded with anisomycin activator were prepared according to Carmona-Fontaine (2011). Late neurula embryos had a small incision cut in their facial midline where a bead was inserted into the foregut behind the EAD. Embryos were grown to late tailbud for fixation and immunohistochemistry and to swimming tadpole for live imaging.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes eight figures and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.073.

**AUTHOR CONTRIBUTIONS**

H.S. directed and supervised the study. L.J. carried out all experiments, except for those conducted by J.C. H.S. directed and supervised the study. L.J. and H.S. designed the experiments. L.J. and H.S. wrote and revised the manuscript with input from all authors. L.J. and J.C. contributed to Figures S4 A–S4P and S7. H.L.-M. and L.J. created Movie S1 using data collected by L.J. L.J. and H.S. contributed to Figures S5A–S5R. Technical assistance was provided by A.R. for in situ hybridizations and immunohistochemistry in Figures 2, S3II, G–S3II, S4Q–S4X, S5I–S5J, S5P–S5R, S6A–S6L, and S7. H.L.-M. created the Claymation movie demonstrating EAD convergent extension shown in Figures 1 F–I and 1 T–1W and Movie S2. H.L.-M. and L.J. created Movie S1 using data collected by L.J. L.J. and H.S. wrote and revised the manuscript with input from all authors.

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