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The Extreme Anterior Domain Is an Essential Craniofacial Organizer Acting through Kinin-Kallikrein Signaling

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SUMMARY

The extreme anterior domain (EAD) is a conserved embryonic region that includes the presumptive mouth. We show that the Kinin-Kallikrein pathway is active in the EAD and necessary for craniofacial development in Xenopus and zebrafish. The mouth failed to form and neural crest (NC) development and migration was abnormal after loss of function (LOF) in the pathway genes kng, encoding Bradykinin (xBdk), carboxypeptidase-N (cpn), which cleaves Bradykinin, and neuronal nitric oxide synthase (nNOS). Consistent with a role for nitric oxide (NO) in face formation, endogenous NO levels declined after LOF in pathway genes, but these were restored and a normal face formed after medial implantation of xBdk-beads into LOF embryos. Facial transplants demonstrated that Cpn function from within the EAD is necessary for the migration of first arch cranial NC into the face and for promoting mouth opening. The study identifies the EAD as an essential craniofacial organizer acting through Kinin-Kallikrein signaling.

INTRODUCTION

The face derives from both neural crest and nonneural crest derivatives. The presumptive mouth arises from a conserved extreme anterior domain (EAD) where ectoderm and endoderm are juxtaposed (Dickinson and Sive, 2006). The cranial neural crest (NC) migrates into the future facial region to abut the EAD during tail bud stages in Xenopus. At mouth opening, the cranial NC has begun differentiating into cranial nerves, melanocytes, connective tissue, and chondrocytes that contribute to the jaws and other facial bones (Santagati and Rijli, 2003). The EAD expresses signaling regulators (Dickinson and Sive, 2009), which suggested that the region might act as a facial organizer. We addressed this possibility using transplant assays where EAD lacking the secreted Wnt regulators Frzb1 and Crescent replaced the EAD of a control embryo. Not only did the mouth fail to form, but surrounding facial regions appeared abnormal, suggesting more global activity of the EAD. However, this putative organizer activity was not extensively explored for other factors impacting mouth formation and cranial NC migration.

Molecular rules for NC movement have been extensively described and include contact inhibition of locomotion, coattraction, chase-and-run strategies (Theveneau et al., 2013), and guidance through interaction with extracellular matrix, semaphorins, and Eph/Ephrin signals (Mayor and Theveneau, 2013). Despite these elegant conclusions, the mechanisms that direct the cranial NC into the face primordium, and the identity of localized guidance signals that facilitate this migration are not known.

In a microarray screen to identify regulatory genes expressed in the EAD that may regulate mouth and other aspects of face formation, we isolated carboxypeptidase N (cpn), kininogen (kng), and neural nitric oxide synthase (nNOS). These genes are members of the Kinin-Kallikrein pathway (Kakoki and Smithies, 2009), a regulator of blood pressure (Sharma, 2009) that also participates in inflammation (Bryant and Shariat-Madar, 2009) and renal function. This pathway had not been described as necessary for craniofacial development in any animal. In the adult mammalian Kinin-Kallikrein pathway (Figure 1A), Kallikrein, a protease, cleaves KNG to yield Bradykinin, a 9 amino acid (9AA) peptide. Bradykinin is a vasodilator that binds the Bradykinin B2 (BKB2) G-protein-coupled receptor. BKB2 receptor activates NOS, which converts L-Arginine (Arg) to nitric oxide (NO) and citrulline. Bradykinin can also be cleaved by CPN, yielding 8AA desArg-Bradykinin and Arg that can be converted to NO (Moncada and Higgs, 1995). The BKB2 receptor is constitutively expressed in adult mammals and binds Bradykinin, but not desArg-Bradykinin, to activate NOS (Kakoki and Smithies, 2009). A BKB1 receptor is conditionally expressed during inflammation and binds desArg-Bradykinin but not Bradykinin. Angiotensin Converting Enzyme (ACE) degrades both Bradykinin and desArg-Bradykinin.
In addition to its role in the Kinin-Kallikrein pathway, NO participates in multiple processes including wound healing, tissue regeneration (Filippin et al., 2011), angiogenesis (Cooke, 2003), neurotransmission (Contestabile and Ciani, 2004), and possibly malignancy (Olson and Garban, 2008). NO has been implicated in developmental contexts including neuronal development (Bradley et al., 2010), bone growth regulation, (Yan et al., 2010), cardiac endothelial-to-mesenchymal transition (Chang et al., 2011), and control of organ size and developmental timing (Kuzin et al., 1996). Elevated NO production has been found in developing epithelial tissues, ganglia, and the notochord (Lepiller et al., 2007). In Xenopus, NO is a potent parthenogenetic activator of Xenopus eggs (Jeseta et al., 2012) and is correlated with movement in tadpoles (McLean and Sillar, 2000).

The strong expression of \( kng \), \( cpn \), and \( nNOS \) in the EAD led us to hypothesize that the Kinin-Kallikrein pathway is active during embryogenesis and required for facial development. We present data that support this hypothesis, and additionally show that

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**Figure 1. Mammalian Kinin-Kallikrein Pathway and Putative Pathway Genes Are Expressed in the Developing Face**

(A) Adult mammalian Kinin-Kallikrein pathway (Kakoki and Smithies 2009).

(B–G) In situ hybridization for \( kng \) (B, B’, E, and E’), \( cpn \) (C, C’, F, and F’), and \( nNOS \) RNA (D, D’, G, and G’) (RNA is purple). Cement gland marker (\( cg \)) is red. Arrow: presumptive mouth. \( cg \), cement gland. (B–G) frontal views; (B’–G’) sagittal sections. Scale bars, 200 \( \mu m \).
Figure 2. *kng*, *cpn*, and *nNOS* Are Required for Mouth Opening and Face Formation

(A–D) *kng*, *cpn*, and *nNOS* loss of function (LOF) using antisense morpholinos. Embryos assayed at stage 40, in four independent experiments. Arrow: mouth region. Bracket: unopened mouth. cg, cement gland. Scale bar in (A–D), 2,000 μm. Scale bar in (A′–D′), 200 μm. (A and A′) Control morphants (100% normal, n = 97). (B–D′) *kng*, *cpn*, and *nNOS* morphants (*kng* [B′] 0% normal, n = 102; *cpn* [C′] 2% normal, n = 105; *nNOS* [D′] 0% normal, n = 129).

(E–H′) Kinin-Kallikrein pathway morphants at stage 22 express presumptive mouth markers, *frzb1* and *xanf1*. Scale bars, 200 μm.

(I–P and I′–L′) Histology of *kng*, *cpn*, and *nNOS* LOF. Coronal sections (I–L, control morphant 100% normal, n = 5; each Kinin-Kallikrein morphant, 0% normal, n = 9) assayed at stage 26 in two independent experiments with β-catenin immunolabeling. Parasagittal sections with anterior to the left (I′–L′, control morpholino 100% normal, n = 5; each Kinin-Kallikrein pathway morpholino, 0% normal, n = 12).

* p < 0.004 compared to control
Kinin-Kallikrein signaling localized to the EAD is necessary for movement of the first arch cranial NC into the face, and for mouth formation. The study identifies the EAD as an essential craniofacial organizing center acting through Kinin-Kallikrein signaling.

RESULTS

**kininogen, carboxypeptidase N, and neural nitric oxide synthase Are Expressed in the EAD during Initial Stages of Craniofacial Development**

*kng*, *cpn*, and *nNOS* expression was identified in the Xenopus EAD region (Dickinson and Sive, 2009; Figure S1A), suggesting activity of an embryonic Kinin-Kallikrein pathway (Figure 1A). Protein alignment showed high conservation of Cpn and nNOS activity of an embryonic Kinin-Kallikrein pathway (Figure 1A), suggesting *kng* are expressed in the EAD during initial stages (Dickinson and Sive, 2009; Figure S1A), suggesting kng is expressed in the prechordal plate with anterior expression adjacent to the EAD (Figures 1B, 1B’, 1E, and 1E’). At stage 20, *cpn* was expressed in deep EAD layers (Figures 1C and 1C’) and by stage 26 at low intensity in the first branchial arch (Figures 1F and 1F’). *nNOS* RNA is present in outer ectoderm of the face, excluding hatching and cement glands (Figures 1D, 1D’, 1G, and 1G’). Later, *nNOS* is expressed in the head and notochord (Peunova et al., 2007). These data show that putative Kinin-Kallikrein pathway genes are simultaneously expressed in adjacent regions of the presumptive face.

**Putative Kinin-Kallikrein Pathway Genes Are Required for Mouth Formation and Neural Crest Development**

A requirement for *kng*, *cpn*, and *nNOS* during craniofacial development would be consistent with activity of the Kinin-Kallikrein pathway. This was tested by loss of function (LOF) using injection of morpholino antisense oligonucleotides (morpholinos, MOs) at the one-cell stage. Specificity of MO targeting was demonstrated by using two MOs, or more importantly, by “rescue” assays where a normal phenotype was observed when MO was co-injected with cognate mRNA lacking the MO target site (Figures S2A–S2D and S2D’). For *kng* and *nNOS* MOs targeting splice sites, qPCR showed a strong decrease in endogenous RNA levels (Figures S2E and S2F). At first hatching stage (stage 40), LOF animals (“morphants”) displayed abnormal body morphology and no open mouth, with a small stomodeal invagination (Figures 2A–2D’, bracket). Nostrils were absent, eyes were small, pigment was reduced, and the face was narrow (Figures 2A’–2D’). Morphant phenotypes were apparent at early tail bud (stage 22, Figures S3A–S3L) and were accompanied by elevated cell death but normal proliferation (Figures S3M–S3V). Despite abnormal mouth phenotypes, the EAD was correctly specified as shown by expression of *frzb1* and *xanf1* (Figures 2E–2H’).

To understand LOF defects, we analyzed tail bud embryos (stage 26) for β-catenin indicating adherens junctions, and laminin indicating basement membrane using immunostaining. In coronal (frontal) sections, controls displayed a narrow midline strip of β-catenin-positive cells running from brain to cement gland, two to four cells wide (Figure 2I). However, in morphants this strip was six to eight cells wide, indicating abnormal epithelial organization (Figures 2J–2L), also apparent in parasagittal sections (Figure 2J’, bracket) where morphants showed a deep region of β-catenin-positive tissue (Figures 2J’–2L’). In morphants, Laminin localization was largely absent from the basement membrane extending from brain to cement gland and separating epidermis and deep ectoderm (Figures 2M–2P, arrows). These data demonstrate epithelial and basement membrane abnormalities at tail bud after *kng*, *cpn*, and *nNOS* LOF.

Reduction of pigment and narrow faces in morphants suggested cranial NC may be abnormal, and, consistently, RNA expression of cranial NC markers *sox9* and *sox10* (Aoki et al., 2003; Mori-Akiyama et al., 2003) was reduced at early tail bud (stage 22) and at late tail bud (stage 26) (Figures 2O–2X) as assayed by in situ hybridization. This was confirmed by qPCR, with >50% reduction in RNA levels (data not shown). Frontal views of control embryos at stage 26 showed a midline strip negative for NC markers (Figure 2J, bracket) that was not apparent or wider in morphants (Figure 2V–2X). These data suggest cranial NC induction, survival, proliferation, or migration is abnormal.

To assay NC induction in morphants, expression of *slug* (LaBonne and Bronner-Fraser, 1998) was examined at early neurula (stage 15) (Figures 2Y–2d). Although *nNOS* and *cpn* morphants displayed normal *slug* expression (Figures 2Y, 2Z, 2c, and 2d), *kng* morphants showed a decrease that was prevented by coinjection of *kng* mRNA (Figures 2a and 2b). Because *cpn* morphants show normal NC induction but a later deficit in NC marker expression, morphants were analyzed for alterations in proliferation and cell death. Axial sections of *sox10* in situ embryos confirmed NC identity (Figures 2e–2h). PH3 labeling demonstrated 50% reduction in mitotic cells (Figures 2i–2j) and
2l) and TUNEL demonstrated a 100% increase in dying cells in cpn morphants relative to controls (Figure 2m) that was partially prevented by coinjecting cognate mRNA (Figures 2k–2m). The data show a requirement for kng, cpn, and nNOS during craniofacial development, including mouth opening. After LOF, multiple changes are observed, in epithelial organization and NC induction, proliferation, or survival, consistent with an active embryonic Kinin-Kallikrein pathway.

kng and cpn LOF Phenotypes Are Prevented by Xenopus Bradykinin Peptides

In the adult, the Kng precursor is processed to release a 9AA peptide, Bradykinin (Bdk) and desArg-xBdk, an 8AA peptide, after cleavage by Cpn. Xenopus Bdk (xBdk) peptide was predicted by aligning Kng protein sequence across species and identifying putative Kallikrein cleavage sites (Figure 3A) (Borgoño et al., 2004). Considering the adult mammalian pathway, we predicted that both the 9AA and 8AA peptides should prevent the kng LOF phenotype, whereas only the 8AA peptide should prevent the cpn LOF phenotype (Figure 1A). Beads soaked in peptides were implanted medially in the future facial region of kng or cpn LOF embryos at tail bud (stage 22), which were scored at tadpole (stage 40) for mouth and facial phenotypes (Figure 3B). Relative to a scrambled xBdk peptide (Figures 3C and 3F), 9AA and 8AA peptides prevented the kng morphant phenotype (Figures 3D, 3E, and 3I), as predicted. In cpn morphants, mouth opening was restored by 8AA but not by 9AA peptide, consistent with the adult model (Figures 3G–3I). However, both peptides restored normal pigment, overall facial symmetry, and head size to cpn morphants (Figure 3I).

To investigate whether xBdk peptide could restore NC development after kng LOF, 9AA scrambled or xBdk soaked-beads were implanted medially (Figures 3J–3L) or anterolaterally below the eye (Figures S4A–S4C). Normal sox9 expression was observed with 9AA xBdk beads (Figures 3J–3L). Consistent data were obtained with lateral implants (Figures S4B–S4C); however, these failed to rescue mouth formation at stage 40 (Figures S4D–S4F). These data support the hypothesis that xBdk peptides derived from Kng direct mouth and NC formation.

Figure 3. Bradykinin-like Peptides Prevent cpn and kng Loss-of-Function Phenotypes

(A) Amino acid sequence alignment of region around Bdk-l peptide. Gray highlights: Bdk-l peptide sequence; red: conserved amino acids; black arrows: Kallikrein and Cpn cleavage sites. Bdk-l (9AA) and Des-Arg xBdk peptides (8AA) used.

(B) Experimental design.

(C–H) Abnormal mouth phenotype after kng LOF prevented by 9AA and 8AA peptides, whereas in cpn morphants was prevented only by the 8AA peptide. (C) kng morphants implanted with 9AA scrambled (9AAscr) peptide bead (28% normal, n = 60). Embryos scored as abnormal if mouth failed to open, was tiny or asymmetric, nostrils failed to form, pigment was absent, or face was abnormally narrow. (D and E) kng morphant implanted with 9AA (D, 60% normal, n = 105) or 8AA bead (E, 57% normal, n = 75). (F) cpn morphants implanted with 9AAscr bead (mouth: 43% normal, n = 67; face: 27% normal, n = 67). (G and H) cpn morphants implanted with 9AA bead (mouth: 41% normal, n = 54; face: 44% normal, n = 54) or 8AA bead (mouth: 65% normal, n = 79; face: 51% normal, n = 79). Scale bar, 200 μm.

(J–L) Expression of neural crest marker sox9 in kng morphants implanted with 9AA bead. Arrow: normal extent of first arch cranial NC. (J) Wild-type expression of sox9 (100% normal, n = 13). kng morphant with a 9AAscr bead (K, 8% normal, n = 12) and 9AA bead (L, 39% normal, n = 13). Scale bar, 200 μm.
Nitric Oxide Prevents kng, cpn, and nNOS LOF Phenotypes, and Endogenous NO Production Is Regulated by xBdk

In mammals, the Kinin-Kallikrein pathway leads to production of the signaling molecule NO. We therefore hypothesized that LOF phenotypes would be prevented by application of the NO donor S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP). SNAP was co-injected with MO at the one-cell stage or injected into the face at late neurula (stage 20). When applied at the one-cell stage, SNAP prevented craniofacial and whole-body phenotypes (Figures 4A–4D; Figures 5A–SSG, SSB–SSD’, and SSJ) and corrected β-catenin and laminin localization and sox9 expression (Figures 4E–4P). When injected into the presumptive facial region, SNAP improved facial development (Figures 5A–SSD’) indicating NO can act at later stages. This rescue was not due to a general effect on all MOs, because the parT phenotype (Ossipova et al., 2005) was not prevented by SNAP (Figures S5H–S5J). Consistent data were obtained using the NO antagonist TRIM applied at stage 20, resulting in abnormal mouth, face, and sox9 expression (Figures 4Q–4R). Although the Kinin-Kallikrein pathway has a role in angiogenesis (Westermann et al., 2008), craniofacial phenotypes did not result from altered blood flow, shown by a head extirpation assay. Thus, an open mouth region of kng, cpn, and nNOS LOF (Figures 5C and 5C0) is necessary to guide the cranial NC into the face. At tadpole (stage 28) (Figures 6B and 6B0), control NC transplanted into control embryos, followed by a control or cpn morphant EAD transplant (Figure 6D). Relative to controls (Figures 6E–E0), cpn EAD LOF showed reduced NC migration at late tail bud (stage 28) (Figures 6F–6F0). In particular, first arch NC showed highly reduced migration anteriorly and medially (Figures 6J and 6J0), demonstrating that cpn expression in the EAD is necessary to guide the cranial NC into the face. At tadpole (stage 40), control transplants developed a normal mouth and face with extensive NC-derived tissue (Figures 6G–6G0) and a normal cartilaginous skeleton (Figures 6K and 6K0). However, cpn EAD LOF transplants failed to form normal mouths or faces (Figures 6H and 6H0) and had substantially less NC-derived tissue (Figure 6H0) with deformed Meckel’s and ceratohyal cartilages (Figures 6L and 6L0). These data demonstrate that local Cpn activity in the EAD is required for migration of the first branchial arch into the face, putatively through processing of Kng-derived peptides.

Localized cpn Activity in the EAD Is Necessary for Migration of First Arch Neural Crest into the Face

The reduction in sox9 expression with cpn LOF suggested that cpn expression is required for NC migration. To analyze migration, fluorescent cranial NC was transplanted into control or cpn morphant hosts at neurula (stage 18) and scored at late tail bud (stage 28) (Figure 6A). Although control transplants displayed three or four distinct branchial arches at late tail bud (stage 28) (Figures 6B and 6B0), control NC transplanted into cpn morphants failed to segregate into branchial arches and did not migrate (Figures 6C and 6C0), indicating a requirement for Cpn in cranial NC migration.

We extended this to ask whether local cpn expression is required for cranial NC migration, using double NC and EAD transplants, where control cranial NC was first transplanted into control embryos, followed by a control or cpn morphant EAD transplant (Figure 6D). Relative to controls (Figures 6E–E0, 6I, and 6I0), embryos with a cpn LOF EAD showed reduced NC migration at late tail bud (stage 28) (Figures 6F–6F0, 6J, and 6J0). In particular, first arch NC showed highly reduced migration anteriorly and medially (Figures 6J and 6J0), demonstrating that cpn expression in the EAD is necessary to guide the cranial NC into the face. At tadpole (stage 40), control transplants developed a normal mouth and face with extensive NC-derived tissue (Figures 6G–6G0) and a normal cartilaginous skeleton (Figures 6K and 6K0). However, cpn EAD LOF transplants failed to form normal mouths or faces (Figures 6H and 6H0) and had substantially less NC-derived tissue (Figure 6H0) with deformed Meckel’s and ceratohyal cartilages (Figures 6L and 6L0). These data demonstrate that local Cpn activity in the EAD is required for migration of the first branchial arch into the face, putatively through processing of Kng-derived peptides.

Conservation of kng Function during Craniofacial Development in Zebrafish

To investigate whether the function of kng in face formation is conserved, we used antisense MOs to target zebrafish (Danio) kng and assayed facial cartilages in 5 day postfertilization embryos by Alcian blue staining (Figures 7A–7E and 7F; Figures S6A–S6C’ and S6D). The MOs used target the kng1 isoform, the only transcript that includes the 9AA Bdk-I peptide. Zebrafish kng is expressed during NC development and mouth opening.
(Figures S6E and S6F), kng LOF led to abnormally shaped Meckel's and ceratohyal cartilages, or abnormal spacing between Meckel's cartilage and the ethmoid plate. As in Xenopus, LOF led to absence of an open mouth (Figures 7G–7I). The LOF phenotype was prevented by coinjection of zebrafish kng that does not bind the MO (Figures 7D and 7D') or by human KNG RNA indicating specificity (data not shown). Morphants injected with Xenopus laevis kng RNA showed no rescue (Figures 7E and 7E') consistent with the greater identity between human and zebrafish Bradykinin than with Xenopus (Figure 3A).

Sox10::GFP transgenic fish were used to observe NC specification and migration after kng LOF. In both controls and morphants, NC was properly specified at the 10-somite stage (data not shown), and migration to form the first and second pharyngeal arches was normal until 48 hpf (Figures 7J–7Q). However, by 60 hpf, Meckel's cartilage, derived from the first pharyngeal arch, fails to condense in morphants (Knight and Schilling, 2006). We conclude that zebrafish kng is necessary for NC and mouth development, demonstrating a conserved requirement for Kinin-Kallikrein signaling. The phenotypes observed in zebrafish are apparent at a later stage than those observed in Xenopus, indicating that temporal control of facial development by Kinin-Kallikrein signaling may differ between species.

DISCUSSION

This study demonstrates activity of the Kinin-Kallikrein pathway during embryogenesis and localized control of craniofacial development through this pathway. Three major conclusions are reached. First, the embryonic pathway in Xenopus functions through a signaling sequence similar to that described for the adult mammalian pathway, and conservation is present in zebrafish. Second, nitric oxide (NO) production is an outcome of the embryonic pathway in Xenopus, involved in craniofacial development, but a facial phenotype was not explored (Peunova et al., 2007). The requirement for kng in zebrafish facial development implies involvement of NO, and this is in accord with effects of treating zebrafish embryos with a NO inhibitor (Kong et al., 2014). In Zebrafish, NOS isoforms are expressed in the developing face, specifically in the mandibular primordium and surrounding the oral cavity, consistent with this role (Poon et al., 2003, 2008). Another route to NO production is the endothelin pathway and consistent with our results, mice deficient in endothelin-1 have craniofacial abnormalities (Kurhara et al., 1994).

Figure 4. kng, cpn, and nNOS Loss-of-Function Phenotypes Are Prevented by the NO Donor, SNAP, and Kinin-Kallikrein Morphants Show Reduced Nitric Oxide Production that Is Increased by xBdk

(A–D) Facial morphology of kng, cpn, and nNOS loss of function (A–D) and with SNAP (A–D'). Embryos assayed at stage 40 in three independent experiments and scored as abnormal if mouth failed to open, was tiny or asymmetric, nostrils failed to form, pigment was absent, or face was abnormally narrow. Arrow: mouth region. Bracket: unopened mouth. cg, cement gland. (A) Control MO injected (98% normal, n = 427) (B–D) kng, cpn, or nNOS MO injected. (A') SNAP plus control MO, (B–D') kng, cpn, or nNOS MO plus SNAP coinjection (kng [B'] 85% normal, n = 105; cpn [C'] 86% normal, n = 98; nNOS [D'] 90% normal, n = 87). Scale bars, 100 μm.

(E–L') Histology of kng, cpn, and nNOS LOF embryos after SNAP treatment. Parasagittal sections with anterior to left assayed at stage 26 with β-catenin (E–H') and laminin immunolabeling (I–L'). β-catenin: green. Laminin: green, with nuclear propidium iodide: red. cg, cement gland. (E–H') β-catenin in control embryos (E and E'), LOF embryos (F–H), and LOF embryos coinjected with SNAP (F–H') (kng [F'] 100% normal, n = 5; cpn [G'] 100% normal, n = 5; nNOS [H'] 100% normal, n = 5). (I–L') Laminin staining in control embryos (I and I'), LOF embryos (J–L', and LOF embryos coinjected with SNAP (J–L') (kng [J'] 75% normal, n = 4; cpn [K'] 80% normal, n = 5; nNOS [L'] 100% normal, n = 4). Scale bars, 100 μm.

(M–P) Expression of sox9 RNA (in situ hybridization) after SNAP injection into kng (N, N'), cpn (O, O'), and nNOS (P, P') LOF embryos. Lateral view. Scale bar, 100 μm. (Q–R) NOS inhibitor TRIM prevents mouth formation and reduces sox9 expression. (Q and Q) Wild-type embryos (100% normal, n = 6). (R and R') TRIM-treated embryos (17% normal, n = 6). (Q and R') Lateral view of sox9 in situ hybridization at stage 26. Scale bars in (Q) and (R), 100 μm; scale bars in (Q') and (R'), 400 μm.

(S–T') Extipated heads show open mouth and normal pigmentation at swimming tadpole (stage 41). (S and S') Control heads (96% normal, n = 27). (T and T') Isolated heads (92% normal, n = 26), (S' and T') front view, (S' and T') side view. Scale bar, 100 μm.

(U–X) Fluorescence after incubation with NO sensor DAF-FM in control embryos (U), kng (V), cpn (W), and nNOS (X) LOF embryos. cg, cement gland. Sagittal view. Scale bar, 170 μm.

(Y–c) Control morphant with no bead (Y). kng morphant with no bead (Z), with 9AA xBdk scrambled bead (a) or 9AA xBdk bead (b). Images collected with same exposure, gain, and fluorescent illumination. kng morphants implanted with 9AA xBdk bead displayed 50% of control fluorescence compared with 23% of control fluorescence in morphants treated with 9AA scr xBdk peptide. Frontal view. Scale bar, 100 μm. (c) Graph showing morphant fluorescence as percentage of control fluorescence; cpn morphants: 49%; kng morphants: 24%; and nNOS morphants: 64%. Each dot represents average of three biological replicates from independent experiments. p values: one-tailed t test.
The demonstration that the EAD is necessary for migration of the first arch NC into the facial region addresses the long-standing question of what region might guide the migratory cranial NC into the face. Our findings not only underscore the organizer capacity of the EAD, but identify cpn locally expressed in the EAD as required for NC ingress, possibly through processing of Kng-derived peptides. Consistent with a guidance function for xBdk, midline or lateral placement (into the EAD) of xBdk-impregnated beads was sufficient to overcome the NC migration defect after Kinin-Kallikrein LOF. Bradykinin is promigratory in other settings, for malignant cells and trophoblasts, whereas NO is involved in inflammation-induced cell migration (Chen et al., 2000; Cuddapah et al., 2013; Erices et al., 2011; Yu et al., 2013). Interestingly, another substrate for CPN is C3a, a small complement peptide required for more local aspects of cranial NC migration (Carmona-Fontaine et al., 2011; Matthews et al., 2004).

In addition to a role for Kinin-Kallikrein signaling in NC migration, kng is necessary for NC induction, whereas cpn is needed later for NC proliferation and survival, highlighting complex spatiotemporal requirements for Kinin-Kallikrein signaling during NC development. Unlike NC specification, mouth specification does not depend on Kinin-Kallikrein signaling. However, mouth opening is tightly linked to NC that abuts the EAD, suggesting that the Kinin-Kallikrein pathway may indirectly regulate mouth opening through the NC. Consistently, application of a xBdk peptide or NO donor after mouth specification and neural tube closure restored a normal NC, normal face morphology, and concomitantly an open mouth to LOF embryos.

Genes that encode Kinin-Kallikrein pathway factors are found in all vertebrates, raising the question of whether activity of this pathway during craniofacial development is conserved. The requirement for kng function during zebrafish NC development and mouth formation supports broad conservation. Additionally, ACE inhibitors that stabilize Bradykinin, used to treat high blood pressure, are teratogens associated with human craniofacial defects (Barr and Cohen 1991). In mammals, single-gene LOF in Kinin-Kallikrein pathway proteins do not obviously result in craniofacial defects (Cheung et al., 1993; Mashimo and Goyal, 1999; Merkulov et al., 2008; Mueller-Ortiz et al., 2009); however, certain double mutants or compound heterozygotes have not been examined. Humans heterozygous for CPN function suffer from angioedema without developmental manifestation; however, no reported patients have complete CPN deficiency, indicating an essential function for this protein (Matthews et al., 2004). A screen for mouse genes involved in craniofacial

Figure 5. Local cpn Expression Is Required for Mouth Opening
Local requirement of kng, cpn, and nNOS expression tested with EAD transplants.
(A) Experimental design: donor morphant tissue was transplanted to un.injected sibling recipients.
(B–E) EAD transplant outcome from control, cpn, kng, or nNOS morphant donor tissue (control [B] 100% normal, n = 11; cpn [C] 28% normal, n = 14; kng [D] 83% normal, n = 24; nNOS [E] 61% normal mouth phenotype, 72% normal facial phenotype, n = 18). (B–E) Overlay of (B)–(E) with GFP fluorescence indicating donor tissue. Dots surround open mouths. Bracket: unopened mouth. Frontal view. Scale bar, 100 μm.
(F) Quantification of structure depending on morphant background of facial tissue.
(G–H) sox9 expression in cpn morphant donor tissue transplants, compared with control morphant transplants. sox9 in situ hybridization in control morphant transplants (G, G’, 70% with normal expression, n = 10) and cpn morphant transplants (H and H’, 36% with normal expression, n = 11). Two representative embryos shown. Scale bar, 100 μm.
(I and J) (I) Summary of urea assay for analysis of Cpn activity. (J) Chart summarizing level of urea derived from free Arg in cpn morphants or morphants coinjected with cpn RNA, as percent of urea derived from free Arg in control morphants. Urea levels in control morphants and wild-type embryos were equivalent. Each dot represents an independent experiment. p value: one-tailed t test.
development identified a Glutamate Carboxypeptidase and a Protein Inhibitor of Nitric Oxide (PIN), suggesting that NO activity is involved in mammalian facial development (Fowles et al., 2003). It is also possible that redundant genes or another pathway such as endothelin signaling work together with Kinin-Kallikrein signaling.

Our study defines the Kinin-Kallikrein pathway and nitric oxide as key for craniofacial development in Xenopus and zebrafish and addresses the long-standing question of how the NC specifically moves into the face. The observations suggest important future directions, including mechanistic studies addressing a putative NC guidance function for xBdk and other EAD-derived
activities, and the relationship between NC migration and mouth formation.

EXPERIMENTAL PROCEDURES

Embryo Preparation

*Xenopus laevis* and zebrafish, *Danio rerio* embryos were cultured using standard methods (Sive et al., 2000; Westerfield et al., 2001). *Xenopus* embryos were staged according to Nieuwkoop and Faber (1994); *Danio* embryos were staged according to Kimmel et al. (1995). Lines used were *Sox10::GFP* (Curtin et al., 2011). All animal use is reviewed and approved by MIT IACUC, under protocol number 0414-026-17.

RNA and qPCR

RNA extraction, cDNA preparation, and qPCR measurements were conducted according to Dickinson and Sive (2009). Primer sequences are available on Figure 7. Function of *kng* in Craniofacial Development Is Conserved in Zebrafish

(A–A') Camera lucida of facial cartilages. E, ethmoid plate; C, ceratohyal cartilage; M, Meckel’s cartilage.

(B–E) *kng* loss of function using splice morpholinos and rescue with zebrafish (zf) *kng* mRNA. Embryonic cartilage scored at 5 dpf after Alcian blue staining in three independent experiments. Scale bar, 250 μm. (B and B') Control morphants co-injected with mRNA were normal (88% normal, n = 50). (C, C', E, and E') *kng* morphants and *kng* morphants co-injected with 200 ng Xenopus *kng* mRNA showed abnormal facial cartilage. Meckel’s cartilage was truncated, boxy, and pointed at an abnormal angle. The ceratohyal cartilage was positioned at an abnormal angle, perpendicular to the midline. (*kng* C and C') 3% normal, n = 61; *kng* mo plus frog mRNA (E and E') 0% normal, n = 65.

(D and D') *kng* morphants co-injected with 200 ng zebrafish (zf) mRNA showed partial rescue. Embryos scored as partially rescued if Meckel’s cartilage was longer, more rounded, and pointed dorsally and if ceratohyal cartilage pointed more anteriorly, compared to *kng* morphants (54% partial rescue, n = 89).

(F) Quantification of phenotypes. p values: one-tailed Fisher’s exact test. N, normal or partially rescued phenotype. A, abnormal phenotype.

(G–I) Ventral views of mApple-injected embryos at 48 hpf. White arrow: open mouth. White bracket: closed mouth. Scale bar, 100 μm. (G) Control morphants (100% normal, n = 5). (H) *kng* splice morphants failed to form open mouths (0% normal, n = 6). (I) *kng* splice morphants co-injected with 200 ng zf mRNA had open mouths (67% normal, n = 6). (J–Q) Confocal images of *Sox10::GFP* zebrafish coninjected with 75 pg mApple and 4 ng control morpholino (100% normal, n = 5) or 4 ng *kng* splice morpholino (0% normal, n = 5). Paired images of the same embryo show GFP signal alone and GFP with mApple. Numbers indicate pharyngeal arches (PA). Bracket: uncondensed/disorganized cartilage. Lateral view. M, Meckel’s cartilage. Scale bar, 100 μm. (J–K) At 36 hpf, NC has migrated into the face of both morphant and control embryos to form first and second PA. (L–M) At 48 hpf, the first PA has begun to extend under eye to form the lower jaw in both morphant and control embryos. (N and N') At 60 hpf, first PA has condensed into Meckel’s cartilage in control embryos. (O and O') At 72 hpf, Meckel’s cartilage is prominent in control embryos. (P and P') At 72 hpf, cartilage of the lower jaw remains disorganized and uncondensed in morphants.
request. Three sets of five heads at stage 22 for sox10 and at stage 26 for sox9 were collected for each of four conditions, including control MO, cpn MO, kng MO, and nNOS MO to provide biological replicates. Equal amounts of RNA were used for reverse transcription (RT) and qPCR to measure sox9 or sox10 RNA. qPCR data from three readings for each of four conditions were averaged, and their distribution was plotted to determine SD. Average morphant qPCR value divided by control morphant qPCR value gave expression level relative to control.

In Situ Hybridization
cDNA sequences used to transcribe in situ hybridization probes including cpn (BC059995), kng (BC083002), nNOS (Punova et al., 2007), sox9 (AY035397), sox10 (Aoki et al., 2003), xanf1 (Ermakova et al., 2007), frzb1 (BC108885), and XCG (Sive et al., 1989). In situ hybridization was performed as described by Sive et al. (2000), without proteinase K treatment. Double-staining protocol adapted from Wiellette and Sive (2003).

Morpholinos and RNA Rescues
Xenopus anteseis morphline-modified oligonucleotides (morpholinos [MOs]) included one start site MO targeting cpn, two splice site MOs against kng and nNOS, and a standard control MO. Sequences are as follows: cpn MO 5’-ACCAACATCCCAGTGCCATTCTCCC-3’, kng MO 5’-TTTACCATTGTCTCTTACCTGTC-3’, nNOS MO 5’-TGCTAAGAAACACACGAGAACATC AA-3’. nNOS MO resulted in an intron inclusion with an early stop codon at AA313, whereas kng MO resulted in an aberrant transcript that could not be amplified by RT-PCR, suggesting it was too large to be amplified or the primer binding sites spanning the MO sequence were missing. qPCR in Figure S2/E confirms a reduction in normal kng mRNA transcript following MO treatment. Danso morpholinos include a start site and a splice site MO targeting kng1. Sequences are kng1 MO 5’-CAAGCTCTTGTCCAGCGCCATTGTC-3’ and kng1 MO 5’-AGCCTGAGGAAACACAAACGCACGT-3’. The splice site Kng1 morphant binds the terminal 22 bp of intron 2 and the first 3 bp of exon 3.

kng cDNA, nNOS cDNA, and cpn cDNA without 5’ UTRs were cloned into the CS2+ vector. RNA was generated in vitro using the mMESSAGE mMACHINE kit (Ambion). RNA (~1 ng) and morpholino (14–18 ng) were coinjected at the one-cell stage to test morpholino specificity via RNA rescue.

Peptide and NO Donor Rescues
Peptides (Thermo Scientific) were designed according to predicted sequences including 9 amino acid (AA) Xenopus Bradykinin (xBdk) (SYKGLSPFR) and 8AA Des-Arg xBdk (SYKGLSPF) and diluted to 0.1 or 0.2 mg/ml. Affi-gel blue agarose beads (50–100 mesh, Bio-Rad) loaded with peptides were prepared according to Carmona-Fontaine (2011). For rescues, beads resuspended in 0.1 mg/ml peptide solution were implanted in the presumptive mouth region at stage 22 and scored at stage 40. For NC assays, beads resuspended in 0.2 mg/ml peptide solution were implanted in the side of the head or presumptive mouth at stages 20–22. Embryos were fixed at tail bud (stage 26) for in situ hybridization analysis. For peptide-rescue assays, partial LOF morphants were employed to maximize viability.

NO donor, S-Nitroso-N-acetyl-DL-penicillamine (SNAP) (Sigma) was diluted to 100 mM in 50% DMSO solution. For early rescues, 1 nl of SNAP was co-injected with morpholino (14–18 ng) and SNAP, and conducted immunohistochemistry and NO staining (Figures 7 and S6) and contributed to manuscript preparation. A.R. contributed in situ hybridization data (Figures 2Y–2Z and 2a–2g). A.D. identified and conducted all experiments in zebra-fish (Figures 7 and S6) and contributed to manuscript preparation.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.026.

AUTHOR CONTRIBUTIONS
L.J. designed and conducted all bead, extirpation, transplant, migration LOF and rescue assays (Figures 3, 4S–4T, 5, and 6). NO and urea quantification assays (Figures 4Y–4c, and S1–S5), and in situ hybridization experiments (Figures 2Q–2q, 3J–3L, and 5G–5H). L.J. wrote and revised the manuscript drafts. R.S. designed and tested morpholinos, executed LOF rescues with cognate RNA and SNAP, and conducted immunohistochemistry and NO staining (Figures 2A–2D’, 2I–2P, 4A–4L’, 4Q–R’, and 4U–4X), except for Ph3 and TUNEL experiments, conducted by L.J. (Figures 2h–2i). R.S. contributed in situ hybridization data (Figures 4M–4P’ and “E–2H”) and obtained or cloned all plasmids. R.S. and L.J. assembled and modified figures and contributed in situ hybridization data shown in Figure 1.J.C. designed and conducted all experiments in zebrafish (Figures 7 and S6) and contributed to manuscript preparation. A.R. contributed in situ hybridization data (Figures 2Y–2Z and 2a–2g). A.D. identified CPN and kinogen in the EAD and performed initial experiments. H.S. directed and supervised the study and wrote the manuscript.

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Urea Assay
A bovine Arginase solution (2 mg/ml lyophilized bovine Arginase [Sigma # A3233]) in 50 mM MnCl2 was incubated for 1 hr at 37°C. Stage 28–29 embryos were anesthetized and decapitated, with 180 heads per condition. Heads were dounced in 80 μl of water, spun for 10 min at 1,100 rpm at 4°C, and 100 μl of clear, cytoplasmic fraction was mixed with 75 μl of Arginase solution for a 2 hr incubation at 37°C. Urea content was measured using the Abcam Urea Assay Kit (Abcam #AB83362). Absorbance was read on a Tecan “Infinite Pro” microplate reader and calculated as a percentage of wild-type or control morphant level.

Immunohistochemistry
Immunohistochemistry was performed as described (Dickinson and Sive, 2006). Primary antibodies included polyclonal anti-laminin antibody (Sigma L-9393) diluted 1:150 and polyclonal anti--β-catenin (Invitrogen) diluted 1:100. Secondary antibody was Alexa 488 goat anti-rabbit (Molecular Probes) diluted 1:500 with 0.1% propidium iodide as a counterstain. Sections were imaged on a Zeiss LSM 700 and 710 Laser Scanning Confocal microscopes. Images were analyzed using Imaris (Bitplane) and Photoshop (Adobe).

Whole-Mount TUNEL, PH3, and Alcian Blue Labeling
TUNEL and PH3 labeling were performed according to Dickinson and Sive (2006, 2009). Alcian blue staining was performed according to Kennedy and Dickinson (2012).

Transplants and Head Extirpation
EAD transplants were performed according to Jacox et al. (2014); NC transplants were performed according to Cancilla and Mayor (1996). For head extirpation, morphant and wild-type embryos were grown to stage 31–32, when the stomodeum forms. Embryos were anesthetized in Tricaine, and heads were removed below the cement gland excluding the developing heart. Heads were moved to 0.5 × modified Barth’s saline for healing and growth. Whole embryos and heads were scored for facial and mouth development at stage 40.
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REFERENCES


