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 (Dickinson and Sive, 2007; Spokony et al., 2002) 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
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 *xanfl*1. Scale bars, 200 μm.

(I–P) 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).

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Protein alignment showed high conservation of Cpn and nNOS hybridization and quantitative RT-PCR (qPCR) (Figures 1B–1G) activity of an embryonic Kinin-Kallikrein pathway (Figure 1A). EAD region (Dickinson and Sive, 2009; Figure S1A), suggesting kng of Craniofacial Development Are Expressed in the EAD during Initial Stages of Craniofacial Development. Gene expression was examined by in situ hybridization and quantitative RT-PCR (qPCR) (Figures 1B–1G and S1E–S1G). At tail bud (stages 20 and 26) when the EAD is present and cranial NC is migrating, 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). This data shows 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 S2B'). For kng and nNOS MOS targeting splice sites, qPCR showed a strong decrease in endogenous RNA levels (Figures S2E and S2F). At late hatching stage (stage 40), LOF animals (“morphants”) displayed abnormal body morphology and 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 xantf1 (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 2I', 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 2Q–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

(M–P) Parasagittal sections with laminin immunolabeling (M–P) assayed at stage 26 in two independent experiments (control morphant 100% normal, n = 10; each Kinin-Kallikrein morphant 8% normal, n = 12). β-catenin: green; laminin: green; nuclear propidium iodide: red. Bracket: presumptive mouth region. cg, cement gland. Scale bar, 170 μm. (Q–T) kng, cpn, and nNOS morphants showed reduced expression of neural crest markers sox10 and sox9. (Q–T) sox10 in situ hybridization at stage 22. (U–Y) sox9 in situ hybridization at stage 26. Bracket: cranial NC-free midline region. Arrow: normal extent of first arch cranial NC. Scale bars in (Q)–(T), 200 μm; scale bars in (U)–(Y), 400 μm. (Y–d) kng morphants showed reduced expression of slug at stage 22, whereas cpn and nNOS morphants and kng morphants co-injected with kng mRNA showed control slug levels. Arrow: specified neural crest. Dorsal view. Scale bars, 800 μm.

(e–m) Cell proliferation and death in cranial NC cells. (e–g) sox10 in situ hybridization at stage 22 in axial section. Control and cpn morpholino plus cpn mRNA embryos showed normal expression, whereas cpn morphants had reduced expression. Scale bar, 200 μm. (h) Schematic demonstrating axial section. (i–k) Ph3 staining of axial sections show increased positive cells in control (i) relative to cpn morphants (j). Embryos injected with cpn morpholino plus cpn mRNA (k) had more Ph3-positive cells than cpn morphants. Scale bar, 170 μm. (l and m) Quantification of Ph3 and TUNEL staining, with SD included; p value: one-way ANOVA with multiple comparisons.

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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) (Borgonio 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 used. (B) Experimental design. (C–H) Abnormal mouth phenotype after kng LOF prevented by 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) (Borgonio 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).
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 S5A–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 S5A–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–SSJ). 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 developed in isolated heads lacking a heart and cultured from prehatching stages 31 and 32, before heartbeat until stage 41 (swimming tadpole) (Figures 4S–4T).

If NO mediates craniofacial development, it should be detectable in developing facial regions and decrease after kng, cpn, and nNOS LOF. NO was measured by incubating late neurula (stage 20) embryos with DAF-FM diacetate, which emits green fluorescence after reacting with NO. Tail bud (stage 26) control embryos showed fluorescence in the outer epidermis (Figure 4U), where nNOS is strongly expressed. Diminished fluorescence was seen and quantified in kng, cpn, and nNOS LOF embryos (Figures 4V–4X and 4c). nNOS LOF was associated with the smallest reduction in NO production, perhaps due to other NOS isoforms. We predicted that xBdk peptides would increase NO production (Figure 1A), and this was confirmed by implanting xBdk-beads into the presumptive mouth region of kng morphants (Figures 4Y–4c). These data demonstrate production of NO in the EAD is dependent on Kinin-Kallikrein gene function, occurs during facial development, and is responsive to xBdk.

cpn Is Expressed in the EAD and Is Required Locally for Mouth Opening and Modulates Arginine Levels

Based on LOF phenotypes, we hypothesized that kng, cpn, and nNOS function in the EAD is locally required in the presumptive mouth and globally required for cranial NC development. This was tested by transplanting the EAD from kng, cpn, and nNOS LOF embryos at early tail bud (stage 22) into sibling controls (Figure 5A) (Jacox et al., 2014). Control transplants led to normal mouth opening, nostril formation, and pigmentation (Figures 5B and S5B, and quantified in Figure 5F). Strikingly, when cpn LOF EAD was transplanted into control embryos, open mouths or nostrils failed to form, and heads were narrow and lacked pigment, similar to global cpn LOF (Figures 5C and 5C'). In contrast, transplant of nNOS and kng LOF EAD into control embryos led to milder phenotypes (Figures 5D–5E'), consistent with the highly preferential expression of cpn in the EAD, and more widespread expression of kng and nNOS. We further showed that cpn expression in the EAD is required for cranial NC formation because sox9 expression at late tail bud is abnormal and reduced after EAD cpn LOF transplants (stage 28, Figures 5G–5H').

The activity of Cpn predicts it modulates levels of Arg (Figure 1A). To examine this, we used a quantitative assay where Arg is converted into urea whose levels can be measured (Figure 5I). As hypothesized, after cpn LOF, lower levels of urea relative to control embryos were present. Specificity was demonstrated as urea levels increased after injection of cpn mRNA into LOF embryos (Figure 5J). Together, these data indicate a requirement for Cpn activity localized in the EAD during mouth, cranial NC, and face development.

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 6B'), control NC transplanted into cpn morphants failed to segregate into branchial arches and did not migrate (Figures 6C and 6C'), 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'–6E'), embryos with a cpn LOF EAD showed reduced NC migration at late tail bud (stage 28) (Figures 6F'–6F'), 6J, and 6J'). In particular, first arch NC showed highly reduced migration anteriorly and medially (Figures 6J and 6J'), 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–6G') and a normal cartilaginous skeleton (Figures 6K and 6K'). However, cpn EAD LOF transplants failed to form normal mouths or faces (Figures 6H and 6H') and had substantially less NC-derived tissue (Figure 6H') with deformed Meckel’s and ceratohyal cartilages (Figures 6L and 6L'). 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-l peptide. Zebrafish kng is expressed during NC development and mouth opening.
**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 pathway and is necessary for mouth and neural crest (NC) development. Third, the extreme anterior domain (EAD) functions as a craniofacial organizer and facilitates migration of first arch cranial NC into the face via Kinin-Kallikrein signaling. These findings add insight into localized signaling essential for craniofacial development.

Epistatic relationships demonstrated for the adult pathway appear to be conserved in the embryo, such that loss of function in kng, cpn, and nNOS is overcome by application of the predicted peptide xBdk or by the downstream effector NO. Further, cpn activity and xBdk modulate levels of endogenous NO, connecting NO and Kinin-Kallikrein signaling. Consistent with a role in craniofacial signaling, pathway genes are expressed at the front of the embryo; however, their nonoverlapping expression domains suggest that initial processing of Kng to yield xBdk occurs distal to the site of xBdk processing and NO production. We did detect different sensitivity of the embryo for intact xBdk and xBdk after C-terminal Arg removal. Thus, with reduced cpn activity, an open mouth is formed in response only to the 8AA peptide, whereas overall face morphology is corrected by both peptides, suggesting that different downstream receptors or alternate forms of peptide processing may be available to the NC.

NO has not previously been appreciated as critical for craniofacial development. In Xenopus, it was proposed that NO suppresses cell proliferation and promotes convergent extension, 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 (Kurihara et al., 1994).

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**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 b-catenin (E–H) and laminin immunolabeling (I–L). b-catenin: green. Laminin: green, with nuclear propidium iodide: red. qc, cement gland. (E–H) b-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, 10 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) Frontal view at stage 40. (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) Extirpated 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) Frontal 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 9AAscr 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).

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).

(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 longstanding 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

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**Figure 7. Function of *kng* in Craniofacial Development Is Conserved in Zebrafish**

(A and 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 mRNA 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 (99% 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 coinjected 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 40 hpf, first PA has condensed into Meckel’s cartilage in control embryos. (O and O’) At 60 hpf, first PA remains disorganized in morphants and does not condense. (P and P’) At 72 hpf, Meckel’s cartilage is prominent in control embryos. (Q and Q’) 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, cpm 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 cpm (BC059995), kng (BC083002), nNOS (Peunova et al., 2007), sox9 (AY035397), sox10 (Aoki et al., 2003), xant1 (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 included one start site MO targeting Sive et al. (2000), without proteinase K treatment. Double-staining protocol was used for reverse transcription (RT) and qPCR to measure nNOS level relative to control.

Morpholinos and RNA Rescues
Xenopus antitense morpho-modified oligonucleotides (morpholinos [MOs]) included one start site MO targeting kng and nNOS, and a standard control MO. Sequences are as follows: cpm MO 5'–ACCAACATCCGACATCTCCTCCT–3', kng MO 5'–TGTATCGGAAACACAGGACACCTG–3', nNOS MO 5'TGCTAAGAAAGCAAGACATC–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 S2E confirms a reduction in normal kng mRNA transcript following MO treatment. Dario morpholinos include a start site and a splice site MO targeting kng1. Sequences are kng1 MO (5'–CAAGCTCTTGTCCACGCGCATGGT–3') and kng1 MO (5'–AGCGTGGAAAGACACACACACACACACACATC–3'). The splice site kng1 morpholino binds the terminal 22 bp of intron 2 and the first 3 bp of exon 3.

kng1 MO (5'–ATTGTCTCTTACCTGTC–3') according to Carmona-Fontaine (2011). For rescues, beads resuspended in agarose beads (50–100 mesh, Bio-Rad) loaded with peptides were prepared. RNA (0.2 mg/ml peptide solution were implanted in the side of the head or presumptive mouth region at stage 22 and scored at stage 40. For NC assays, beads resuspended in 0.1 mg/ml peptide solution were implanted in the presumptive mouth region of embryos at tail bud (stage 26) for hybridization analysis. For rescue assays, partial LOF morphants 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.

Nitric Oxide Staining and Quantification
Embryos were incubated in NO indicator 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate (1:150). (DAF-FM diacetate; Invitrogen; Leplier et al., 2007) for 2-3 hr at 26°C. Embryos were fixed in 4% paraformaldehyde overnight, embedded in 4% agarose, vibratome sectioned (100 um), counterstained with DAPI, and imaged on a Zeiss LSM 700 Laser Scanning Confocal. For NO quantification, 120 embryos per condition were decapitated, washed, dounced, and spun (10 min, 1,300 rpm). The clear fraction was divided in triplicate and loaded on a microplate (Gorning 3995- half-area, flat bottom, black), and fluorescence was measured using a Teica microplate reader. Untreated head solution was used to measure background fluorescence.

Urea Assay
A bovine Arginase solution (2 mg/ml lyophilized bovine Arginase [Sigma # A2333] 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 90 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 detected using the Abcam Urea Assay Kit (Abcam #AB83362). Absorbance was read on a Teica “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-jl–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 Zeiss LSM 700 and 710 Laser Scanning Confocal microscopes. Images were analyzed using Imars (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 Mancilla 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.

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–S3), and in situ hybridization experiments (Figures 2Q–2g, 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 morphants were employed to maximize viability.

NO donor, S-Nitroso-N-acetyl-DL-penicillamine (SNAP) (Sigma) was diluted to 100 mM in a 50% DMSO solution. For early rescue, 1 nM SNAP solution was co-injected with 17 ng of morpholinio into one-cell stage embryos. For late rescues (stage 20), 2–3 nL of SNAP was injected into the presumptive mouth region. The nNOS inhibitor, TRIM (Sigma, T7313), was diluted to 1 μM concentration in DMSO and applied to late neurula (stage 20) embryos. Embryos were collected at tail bud (stage 26) for sox9 in situ hybridization and at swimming tadpole (stage 40) for craniofacial morphology.

Nitric Oxide Staining and Quantification
Embryos were incubated in NO indicator 4-aminomethylamine-2',7'-difluorofluorescesin diacetate (1:150). (DAF-FM diacetate; Invitrogen; Leplier et al., 2007) for 2-3 hr at 26°C. Embryos were fixed in 4% paraformaldehyde overnight, embedded in 4% agarose, vibratome sectioned (100 um), counterstained with DAPI, and imaged on a Zeiss LSM 700 Laser Scanning Confocal. For NO quantification, 120 embryos per condition were decapitated, washed, dounced, and spun (10 min, 1,300 rpm). The clear fraction was divided in triplicate and loaded on a microplate (Gorning 3995- half-area, flat bottom, black), and fluorescence was measured using a Teica microplate reader. Untreated head solution was used to measure background fluorescence.

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REFERENCES


