FGF signaling establishes the anterior border of the Ciona neural tube

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SUMMARY
The Ciona tadpole is constructed from simple, well-defined cell lineages governed by provisional gene networks that have been defined via extensive gene disruption assays. Here, we examine the patterning of the anterior neural plate, which produces placodal derivatives such as the adhesive palps and stomodeum, as well as the sensory vesicle (simple brain) of the Ciona tadpole. Evidence is presented that the doublesex-related gene DMRT is expressed throughout the anterior neural plate of neurulating embryos. It leads to the activation of FoxC and ZicL in the palp placode and anterior neural tube, respectively. This differential expression depends on FGF signaling, which inhibits FoxC expression in the anterior neural tube. Inhibition of FGF signaling leads to expanded expression of FoxC, the loss of ZicL, and truncation of the anterior neural tube.

KEY WORDS: Ciona intestinalis, FGF/MAPK, FoxC, Neural boundary, Placode

INTRODUCTION
The generation of diverse cell types from a fertilized egg remains a fundamental problem for developmental biologists. The simple chordate Ciona intestinalis is well suited to address this question owing to its small cell number, detailed cell lineage characterization, experimental tractability and provisional gene regulatory networks (Passameaneck and Di Gregorio, 2005; Satou et al., 2009). Ciona, an ascidian, belongs to the urochordates, which represent the closest living relatives of the vertebrates (Delsuc et al., 2006). Ascidians are sessile, filter-feeding animals with incumbent and excurrent siphons that develop following the settlement and metamorphosis of a swimming tadpole larva. Ascidians diverged from vertebrates before the two rounds of genome duplication that created the large multigene families that complicate genetic analysis in vertebrates (Dehal and Boore, 2005).

The Ciona central nervous system (CNS) develops via neurulation of cells within the neural plate, forming a simple brain, which is also called the sensory vesicle, and a caudal nerve cord (Nicol and Meinertzhagen, 1988; Lemaire et al., 2002). Ciona also has neurogenic tissues that derive from the anterior neural plate, forming a simple brain, posterior nervous system. Less is known about the early stages of Ciona nervous system development. Much progress has been made in recent years toward uncovering the gene regulatory networks that control the development of the posterior nervous system. Less is known about the early patterning mechanisms in the anteriormost nervous system. Here, we examine the role of FGF signaling in a cell fate choice between the palp placode and the anterior CNS, which expresses FoxC and ZicL, respectively. We find that FGF-MAPK signaling is required to establish the posterior boundary of the FoxC expression domain, as abrogation of this pathway results in ectopic FoxC expression and loss of CNS markers. We identified an Ets binding site in a minimal FoxC enhancer that is required to repress FoxC in the CNS progenitors. Strikingly, we observe a truncated anterior neural tube in tailbud stage embryos that experience FGF-MAPK perturbation, as well as a posterior shift in the position of the neuroepore. Thus, FGF signaling delineates the boundary between the palp placode and the anterior neural tube.

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EMBRYO ELECTROPERATION

Ciona intestinalis adult animals were collected at Pillar Point Harbor in Half Moon Bay, California, or obtained from M-Rep (San Diego, CA, USA). Dechorionation, fertilization, and electroperoration of eggs were performed as described (Christiaen et al., 2009b). Each experiment was performed at least twice. Electroperoration was with 40-100 μg of each plasmid. Fluorescent images were obtained with a Zeiss Axiocam.2 upright fluorescence microscope or a Zeiss 700 laser-scanning confocal microscope.

MOLECULAR CLONING

Enhancers were amplified from Ciona intestinalis genomic DNA with DMRT -1k PstF (5'-GAAGTACTGCAG) and DMRT NR (5'-GAAGTACTGCAG) using the RNeasy Mini Kit (Qiagen). The following in situ hybridization probes were obtained from plasmids (Invitrogen). A total of 10 mM MOPS pH 7.4, 0.5 M NaCl, 1 mM EGTA, 2 mM MgSO4, 0.05% Tween 20 overnight at 4°C. Embryos were transferred to FASW containing U0126 or DMSO as control.

Drug treatments

For antibody staining, embryos were fixed in MEM-FA (3.7% methanol-free formaldehyde, 0.1 M MOPS pH 7.4, 0.5 M NaCl, 2 mM MgSO4, 1 mM EGTA) for 30 minutes, rinsed several times in PBT (PBS with 0.1% Tween 20), and incubated with anti-dpERK antibody (Sigma M9692) at 1:1000 with 2% normal donkey serum (NDS) in PBT at 4°C overnight. Embryos were washed in PBT and then incubated with donkey anti-mouse secondary antibody (1:1000) to Alexa Fluor 555 (Molecular Probes) in PBT with 2% NDS for 2 hours at room temperature, then washed in PBT. For phalloidin staining, phalloidin (Molecular Probes) was diluted 1:500 in PBT, and embryos were incubated for 2-4 hours at room temperature, followed by PBT washes. For Hoechst staining, Hoechst (Molecular Probes) was diluted 1:1000 in PBT, and embryos were incubated for 0.5-2 hours. Then PBT was washed. All embryos were equilibrated and mounted in 50% glycerol in PBS containing 2% DABCO (Sigma).
vertebrates, the neural plate is defined on the basis of neurulation to form the CNS; the neurogenic tissues anterior to the plate are called placodes and their neurons contribute to the PNS. Thus, the common progenitor that expresses ZicL has isolated a ~1 kb enhancer upstream of the DMRT coding sequence and found it to recapitulate the native expression pattern in gastrula stage embryos (Fig. 1C). Posterior to the a8.18/a8.20 palp precursor cells are the a8.17/a8.19 cells, which contribute to anterior brain and stomodeum. The a8.17/a8.19 blastomeres express ZicL, but not FoxC. An enhancer for ZicL has been described (Shi and Levine, 2008). It drives expression in the a8.19/a8.17 cells, as well as in their lateral neighbor a8.25, and also in the vegetal neural progenitors (rows I/II) and mesodermal lineages (Fig. 1A-C). Thus, FoxC and ZicL and their enhancers are active in complementary patterns in the anterior neural plate and allow visualization of the boundary between the CNS and PNS.

The FoxC-expressing and ZicL-expressing cells derive from a common progenitor that expresses DMRT (Fig. 1B). Both FoxC and ZicL require DMRT for their activation (Imai et al., 2006; Tresser et al., 2010). DMRT is homologous to the Drosophila doublesex gene, which, in addition to a role in sex determination, also functions in the development of neurons controlling sex-specific behavior (Rideout et al., 2007; Rideout et al., 2010). In vertebrate embryos, DMRT genes mark anterior neural regions, including the nasal placode and presumptive forebrain (Winkler et al., 2004; Huang et al., 2011). In Ciona, DMRT is first expressed at the 64-cell stage in the a7.10, a7.9 and a7.13 blastomeres, which are progenitors of the anterior neural plate (a-line, rows III-VI) (Imai et al., 2006; Tresser et al., 2010). The medial two cells, a7.10 and a7.9, undergo a cell fate choice to produce daughters that express either ZicL posteriorly (a8.17/a8.19) or FoxC anteriorly (a8.18/a8.20) (see Fig. 1A,B). We have isolated a ~1 kb enhancer upstream of the DMRT gene and used this cis-regulatory DNA to perturb gene expression and fluorescently label cells of the anterior neural plate (rows III-VI).
Differential MAPK activity across the neural plate

What determines the outcome of the FoxC-ZicL cell fate choice? The FGF signaling pathway functions in multiple cell fate choice events during nervous system development in Ciona embryos (Hudson et al., 2007; Picco et al., 2007; Stolfi and Levine, 2011; Stolfi et al., 2011). The Map kinase (MAPK) signaling cascade can mediate signal transduction downstream of the FGF receptor, and active MAPK signaling can be detected with an antibody that recognizes a dual-phosphorylated (dp) form of the extracellular regulated kinase 1/2 (ERK1/2) (Yao et al., 2000; Hudson et al., 2003; Hudson et al., 2007). To determine the timing and levels of MAPK activation in developing neural tissue, we stained staged embryos with dpERK antibody. These embryos were electroporated with a FoxB reporter plasmid (driving H2B:CFP or GFP) to label the vegetal neural precursors that express FGF9/16/20 (A8.7, 8.8, 8.15, 8.16) (Imai et al., 2002; Tassy et al., 2010); this also assists in correct identification of the cells of interest.

Shortly after the cell fate choice, at ~4.75 hpf, we detected dpERK staining in both the FoxC-expressing and ZicL-expressing cells, as well as in the mother cells on the right-hand side of the embryo, which have yet to divide (Fig. 2Aa,b). The level of staining appeared uniform in all nuclei. By contrast, when we looked a little later in the cell cycle, at 5.17 hpf, we noticed that the dpERK signal appeared graded (Fig. 2Ba,c,d). The strongest signal was seen in the vegetal A-line neural cells, in which it appeared to be both nuclear and cytoplasmic. In the a8.17/a8.19 cells, however, the dpERK signal was concentrated in the nuclei, whereas the anteriormost FoxC-expressing cells, a8.18 and a8.20, showed no detectable staining. The non-neural ectoderm adjacent to the FoxC-expressing cells showed elevated staining, comparable to that seen in the A-line neural cells. As a control, we stained embryos treated with the MAPK pathway inhibitor U0126, and found that the dpERK antibody signal was abolished (supplementary material Fig. S1). These results suggest that the level of MAPK activation is differentially regulated between the FoxC-expressing and ZicL-expressing cells, and might be an important factor in determining the downstream gene expression program.

FGF-MAPK signaling is required for differential FoxC and ZicL expression

The localized dpERK we observed in the ZicL-expressing cells is consistent with reports that FGF-MAPK signaling is important for its activation (Imai et al., 2006). We perturbed FGF-MAPK signaling by misexpressing a dominant-negative FGF receptor (DN-FGFR, which lacks the intracellular tyrosine kinase domain) using the DMRT 5’ enhancer and assayed the activity of the ZicL and FoxC enhancers. Although the cell fate choice is complete at the 112-cell stage, we assayed expression at mid-gastrula stage because the shape of the embryo makes it easier to visualize the cells of interest. DN-FGFR expression led to loss of ZicL in rows III/IV of the neural plate, although sometimes cells in the lateral-most columns escaped repression (compare Fig. 3Aa with 3Ab). Additionally, we observed ectopic expression of FoxC in rows III/IV of the neural plate, as compared with the control, in which FoxC is expressed only in rows V and VI (compare Fig. 3Ac with 3Ad). To confirm this result, we used the irreversable MAPK pathway inhibitor U0126 to block the MAPK signaling cascade downstream of the FGF receptor, and examined the expression of FoxC and ZicL transcripts. As with DN-FGFR, U0126 treatment led to loss of ZicL in rows III and IV (compare Fig. 3Ba with 3Bb), concomitant with ectopic FoxC expression (compare Fig. 3Bc with 3Bd). Expression of a constitutively active (CA) form of FGFR had no effect on the FoxC expression pattern. However, co-expression of CA-FGFR and DN-FGFR was able to rescue the ectopic FoxC pattern observed with DN-FGFR alone (supplementary material Fig. S2). We conclude that FGF-MAPK signaling is required for ZicL expression and for setting the posterior boundary of the FoxC expression domain.

FGF-MAPK signaling is required for patterning of the anterior neural plate

We next asked whether abrogation of MAPK signaling had a broader impact on neural plate patterning, beyond altered expression of FoxC and ZicL. Ephrin A-d, which encodes a membrane-localized signaling molecule, is co-expressed with FoxC in rows V and VI of the neural plate. Upon U0126 treatment,
however, the Ephrin A-d expression pattern expands to include rows III and IV, as does FoxC (Fig. 4A,B). RorA, which encodes an orphan receptor tyrosine kinase (RTK) that modulates non-canonical Wnt signaling, shows a graded pattern with strong expression in rows V/VI and weaker signal in rows III/IV (Fig. 4C). (A,C,D) Quantification of data from A. Error bars indicate s.d.

**FoxC can repress ZicL expression in the anterior neural plate**

Because inhibition of FGF-MAPK signaling led to ectopic FoxC and loss of ZicL expression, and because the lateral-most cells a9.49/a9.50 do not experience ectopic FoxC and sometimes escape repression of ZicL, we asked whether FoxC itself might repress ZicL. FoxC is known to act as a transcriptional repressor in various metazoans by virtue of a conserved Engrailed homology repressor domain (Yaklichkin et al., 2007). Interestingly, this conserved repressor motif is absent from the Ciona FoxC ortholog. Nonetheless, when full-length FoxC protein was misexpressed using the DMRT enhancer, ZicL was lost from rows III and IV (Fig. 5A). A truncated form of FoxC that lacks the C-terminal 293 amino acids downstream of the forkhead DNA-binding domain was also sufficient to repress ZicL expression (data not shown). These results suggest that, despite lacking an obvious transcriptional repression motif, FoxC can inhibit ZicL expression. Whether the repressive effect of FoxC on ZicL is direct or indirect will require further study.

We then asked whether ZicL could repress FoxC expression, reasoning that a mechanism of mutual repression could account for the establishment of the boundary between rows IV and V of the neural plate. However, misexpression of ZicL had no effect on the endogenous FoxC expression pattern (Fig. 5B). We verified this result by misexpressing ZicL with the Bmp2b enhancer, which is active much earlier, at the 16-cell stage, and should allow sufficient time to accumulate transgene expression (Christiaen et al., 2009b). Again, ectopic ZicL expression had no effect on FoxC (data not shown).
promoter (Rothbacher et al., 2007) (from the start codon, and cloned it upstream of a heterologous gene) to drive FOG expression in rows III/IV of the neural plate. (Fig. 6A). Deletion of the Ets site at –295 caused an otherwise normal FoxC-lacZ fusion gene to exhibit derepressed expression in rows III and IV of the neural plate (Fig. 6B,C). Deletion of the Ets site at –255 had no effect on reporter activity (Fig. 6D). This result suggests that an Ets family transcription factor might repress FoxC in rows III/IV of the neural plate.

The Ciona genome encodes ~15 Ets domain transcription factors. ELK was an intriguing candidate repressor of FoxC because it is expressed in rows III/IV of the neural plate in a MAPK-dependent manner (Fig. 4D). Furthermore, it has been documented that ELK can repress transcription in response to MAPK signaling (Usenko et al., 2002). However, when we misexpressed ELK with the DMRT enhancer, FoxC expression was unaffected (data not shown). Thus, although our data collectively suggest that an Ets family factor might function to repress FoxC expression in rows III/IV, the identity of such a factor remains unknown.

**FGF signaling is required for anterior brain development**

We next looked at tailbud stage embryos that developed under conditions of perturbed FGF signaling to examine the morphology of the sensory vesicle and palp placode. We predicted that the ectopic FoxC might lead to the specification of ectopic palps at the expense of anterior brain. In control embryos, cells of the anterior brain are marked by co-expression of DMRT and ZicL (pink cells in Fig. 7A), whereas cells expressing DMRT but not ZicL (row V/VI derivatives) label the anterior trunk ectoderm (palp placode and progeny of a9.51/a9.52; blue cells in Fig. 7A; see also Fig. 1B). In the presence of DN-FGFR, we observe a dramatic expansion of the anterior epidermis at the expense of anterior brain cells, as evidenced by a larger proportion of DMRT cells lacking ZicL expression (Fig. 7B). This was accompanied by an expansion of epidermis marker gene expression (supplementary material Fig. S3). The few remaining cells that co-express DMRT and ZicL derive from the lateral columns of the neural plate, a9.49/a9.50, which do not experience ectopic FoxC when FGF signaling is perturbed (Fig. 3Ad,Bd).

Phalloidin staining of DN-FGFR-expressing tailbud embryos reveals a smaller sensory vesicle, which is rounded rather than elliptical in shape (Fig. 7C,D). This truncated brain is entirely derived from vegetal neural plate cells, as evidenced by the lack of candidate sites that map to –255 bp and –295 bp relative to the native start codon and deleted them (Fig. 6A). Deletion of the Ets site at –295 caused an otherwise normal FoxC-lacZ fusion gene to exhibit derepressed expression in rows III and IV of the neural plate (Fig. 6B,C). Deletion of the Ets site at –255 had no effect on reporter activity (Fig. 6D). This result suggests that an Ets family transcription factor might repress FoxC in rows III/IV of the neural plate.

When FGF signaling is perturbed, we observe a dramatic expansion of the anterior epidermis at the expense of anterior brain cells, as evidenced by a larger proportion of DMRT cells lacking ZicL expression (Fig. 7B). This was accompanied by an expansion of epidermis marker gene expression (supplementary material Fig. S3). The few remaining cells that co-express DMRT and ZicL derive from the lateral columns of the neural plate, a9.49/a9.50, which do not experience ectopic FoxC when FGF signaling is perturbed (Fig. 3Ad,Bd).

**FoxC cis-regulatory analysis**

How does MAPK signaling keep FoxC repressed in rows III/IV of the neural plate? Ets family transcription factors are known effectors of FGF-MAPK signaling and can act as transcriptional repressors in various contexts (Oikawa and Yamada, 2003; Hollenhorst et al., 2011). We identified a ‘minimal’ FoxC enhancer by making 5’ and 3’ deletions of the original 2 kb fragment, to facilitate analysis of potential transcription factor binding sites. We isolated a 325 bp fragment that maps from –375 to –50 bp relative to the start codon, and cloned it upstream of a heterologous promoter (Rothbacher et al., 2007) (from the FOG gene) to drive lacZ expression. This minimal FoxC enhancer is conserved in the related species C. savignyi and was sufficient to drive a wild-type FoxC expression pattern (Fig. 6B).

We then searched for Ets sites in the minimal FoxC enhancer. Ets family transcription factors recognize a well-defined core consensus sequence: GGAATA (Hollenhorst et al., 2011). We found two
posteriorly induces a similar posterior shift of the palp placode. Shifting the domain of the CNS corresponds to the boundary between the palp placode and the presumptive stomodeum, and suggest that, normally, the boundary between the palp placode and the presumptive stomodeum, and suggest that FoxC expression might function in part to position the anterior neuropore (see below).

DISCUSSION

We have presented evidence that FGF-MAPK signaling is required to specify anterior CNS fates (ZicL) in rows III/IV of the neural plate, and that disruption of this pathway causes derepression of FoxC, which is normally restricted to rows V/VI (Fig. 3). Inhibition of FGF-MAPK signaling also causes the loss of other determinants of the anterior neural tube, including Six3/6, which marks the presumptive stomodeum (Fig. 4) (Mazet and Shimeld, 2005; Christiaen et al., 2007). A similar situation is seen in the posterior neural plate comprising rows I/II, which derive from the vegetal A-line and contribute to the posterior CNS, including the motor ganglion and caudal nerve cord. Hudson et al. showed that MAPK signaling is required for the expression of row I markers such as Mnx, Cdx and Ephrin A-B; the loss of row I markers in U0126-treated embryos was accompanied by the posterior expansion of row II markers into row I (Hudson et al., 2007). Thus, FGF-MAPK signaling broadly promotes posterior cell fates over anterior cell fates across the developing nervous system in Ciona. This trend is also seen in vertebrates, where neural induction generally promotes nervous tissue of anterior rather than CNS cell fate. The FoxC-misexpressing cells, labeled with the DMRT reporter, remain in a columnar epithelium on the dorso-anterior surface of the embryo. Notably, we found no evidence of anterior neuropore formation. The DMRT enhancer drives FoxC expression across all six columns of the anterior neural plate, whereas the DN-FGFR perturbation leads to ectopic FoxC only in the medial four columns. This suggests that proper specification of the lateral-most columns of the neural plate, in part through exclusion of FoxC, is necessary for neuropore formation (Nicol and Meinertzhagen, 1988).

Ectopic expression of FoxC via inhibition of FGF signaling does not cause transformation of the anterior brain into supernumerary palps. On the contrary, palp development is impaired (Fig. 7F). This is in agreement with a previous study showing that MAPK signaling is required at least through neurula stages for proper palp development (Hudson et al., 2003). Thus, although FoxC is a marker of the palp lineage, its expression alone does not suffice to direct morphogenesis of the palps. Our results show that abrogation of MAPK signaling causes a posterior shift in the boundary between the palp placode and the presumptive stomodeum, and suggest that FoxC expression might function in part to position the anterior neuropore (see below).

**Fig. 7. FGF signaling delineates the boundary between the anterior neural tube and the palp placode.** (A–B) Tailbud stage Ciona embryos electroporated with plasmids DMRT>lacZ and ZicL>mCherry, in the presence of DMRT>DN-FGFR or DMRT>lacZ as control. Anterior brain cells are marked by co-expression of ZicL and DMRT reporters (pink cells); cells expressing only the DMRT reporter derive from rows V/VI of the neural plate. DN-FGFR expression leads to loss of cells in the anterior brain, as compared with control. (C–D) Tailbud embryos were electroporated with DMRT>H2B:mCherry and stained with phalloidin. The brain shows an anterior truncation in the presence of DN-FGFR, but the neuropore still forms, albeit shifted posteriorly. (E) Tailbud embryo expressing DMRT>FoxC transgene shows truncation of anterior neural tube, similar to D. (F) Larvae expressing DMRT>DNFGFR were compared with controls expressing DMRT>lacZ and the number of palps was counted. The brain vesicle is outlined; asterisk indicates the neuropore.
The palps derive from an embryonic territory sandwiched between the non-neural ectoderm and progenitors of the CNS (stomodeum and anterior brain). The corresponding region in vertebrate embryos forms the anterior placodes, which give rise to the lens, olfactory epithelium and adenohypophysis (anterior pituitary) (Streit, 2008; Schlosser, 2010). In chick embryos, FoxC is expressed in the lens placode, and in mammals it functions in the development of the anterior eye (Saleem et al., 2003; Bailey et al., 2006; Berry et al., 2006). FoxC is also expressed in the developing Xenopus eye, although it is not clear whether this expression is in the lens placode and/or the surrounding tissue (Bowes et al., 2010). It is noteworthy that FGF signaling functions in the chick to repress lens fate and promote olfactory placode development (Bailey et al., 2006). A parallel situation is seen in the Ciona palp placode, which expresses FoxC and β-crystallin, another lens marker (Shimeld et al., 2005), and is repressed by FGF signaling.

We do not suggest homology of the Ciona palp placode with the vertebrate lens placode. Ciona has a light-sensing apparatus that consists of a pigmented ocellus and numerous photoreceptor cells, and these are located in the anterior brain (Imai and Meinertzhagen, 2007a; Horie et al., 2008). The palp placode also expresses olfactory placode markers such as DMRT, Coe and Dlx genes (Caracciolo et al., 2000; Winkler et al., 2004; Huang et al., 2005; Mazet et al., 2005; Veith et al., 2006; Wen et al., 2009). Thus, we refer to the Ciona palp primordium simply as an anterior placode, which might be derived from an ancestral structure that evolved into separate, specialized anterior placodes in the vertebrate lineage.

In summary, the boundary between rows IV and V of the Ciona neural plate is important because it delineates the neural tube as distinct from the anterior placode region. We have shown that FGF-MAPK signaling is required to establish this boundary and that it does so by repressing FoxC expression in the presumptive brain. Disruption of FGF signaling leads to a truncated anterior brain, reminiscent of the phenotype of triple FGF knockout mice, which lack the telencephalon (Paek et al., 2009). Ciona embryos develop from defined cell lineages, and the gene expression patterns that underlie important embryological boundaries form relatively early in development. In vertebrates, neural territories are composed of mixed fate cell populations, which segregate and form boundaries according to fate at comparatively later developmental stages in response to diverse signals, including FGFs (Puelles et al., 2005; Toro and Varga, 2007; Cajal et al., 2012; Sanchez-Arrones et al., 2012). Despite these differences, a common requirement for FGF signaling highlights the relevance of Ciona embryos to the study of patterning and the morphogenetic mechanisms underlying vertebrate development.

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The authors declare no competing financial interests.

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