A modular cis-regulatory system controls isoform-specific pitx expression in ascidian stomodæum

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Abstract

Pituitary homeobox (pitx) genes have been identified in vertebrates as critical molecular determinants of various craniofacial ontogenetic processes including pituitary organogenesis. Accordingly, a prominent conserved feature of pitx genes in chordates is their early expression in the anterior neural boundary (ANB) and oral ectoderm, also known as the stomodæum. Here we used the ascidian model species Ciona intestinalis to investigate pitx gene organization and cis-regulatory logic during early stages of oral development. Two distinct Ci-pitx mRNA variants were found to be expressed in mutually exclusive embryonic domains. Ci-pitx and vertebrate pitx2 genes display remarkably similar exon usage and organization, suggesting ancestry of the pitx transcriptional unit and regulation in chordates. We next combined phylogenetic footprinting, transient transgenesis, and confocal imaging methods to study the Ci-pitx cis-regulatory system, with special emphasis on the regulation of isoform-specific ANB/stomodæal expression. Among 10 conserved noncoding sequences (CNSs) interspersed in C. intestinalis and Ciona savignyi pitx loci, we identified two separate cis-regulatory modules (CRMs) that drive ANB/stomodæal expression in complementary spatiotemporal patterns. We discuss the developmental relevance of these data that provide an entry point to investigate the gene regulatory networks (GRNs) that position and shape oral structures in chordates.

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Introduction

In vertebrates, craniofacial development involves cells of diverse ontogenetic origins including anterior mesendoderm, cranial neural crest, neural tube, placodes, and epidermis (Le Douarin et al., 1997). In particular, olfactory and oral epithelia, as well as the adenohypophysis, derive from a morphogenetic unit known as the stomodæum, which eventually invaginates, fuses with endodermal pharyngeal epithelium, and forms the oral opening. Lineage studies showed that the stomodæum arises from an anterior ectodermal territory that encompasses the anterior neural boundary (ANB) and contacts anterior endoderm (Couly and Le Douarin, 1985; Kouki et al., 2001). Thus, stomodæal cell specification must integrate a dual epidermal/neural origin, a pivotal position in the body plan, and maintain diverse developmental potentialities (Wardle and Sive, 2003).

During organogenesis, field-specific selector genes are key components of the gene regulatory networks (GRNs) that specify homogenous morphogenetic fields through the integration of various developmental cues. These integrative properties are mostly hardwired in selector gene cis-regulatory systems, which exhibit transcriptional modularity, and a complex logic for each autonomous cis-regulatory module (CRM; Carroll et al., 2001; Davidson, 2001).

Pituitary homeobox (pitx) genes are conserved early markers of the stomodæal ectomere in chordates (Boorman and Shimeld, 2002b; Essner et al., 2000; Lancêlot et al.,...
1997; Schweickert et al., 2001b; Uchida et al., 2003). In humans, mutations of PITX2 in Axenfeld–Rieger patients cause severe facial defects (Semina et al., 1996). In addition, numerous functional studies have demonstrated that various aspects of craniofacial development require pitx genes (Kioussi et al., 2002; Schweickert et al., 2001a; Suh et al., 2002; Szeto et al., 1999), suggesting these latter are critical components of the selector gene battery specifying the ANB/stomodeum morphogenetic field. The above-mentioned considerations raise further questions about upstream mechanisms that trigger and precisely define pitx stomodeal expression and to which extent these mechanisms are conserved in chordates.

In ostechthyans, pitx gene regulation occurs at both transcriptional and posttranscriptional levels. For instance, the conserved pitx2 transcriptional unit uses distinct distal and proximal promoters to produce different isoforms with identical homeodomains and carboxy termini, but different amino termini (Arakawa et al., 1998; Essner et al., 2000; Schweickert et al., 2000).

In addition, pitx paralogues and pitx2 isoforms are expressed in both specific and common domains (Essner et al., 2000; Lancôt et al., 1997; Schweickert et al., 2001b). Interestingly, functional and comparative gene expression data suggest that a conserved genetic hierarchy controls isoform-specific pitx2 expression during the establishment of left–right asymmetry (Boorman and Shimeld, 2002a). Thus, key aspects of pitx pre- and posttranscriptional gene regulation rely on an ancestral genomic organization and probably emerged before the early radiation of vertebrates. Investigations in a broad range of chordate species are therefore required to address key issues regarding pitx cis-regulatory systems (Goodyer et al., 2003; Shiratori et al., 2001).

We previously reported pitx gene expression in the anterior neural boundary and stomodeum during ascidian embryonic development (Christiaen et al., 2002). Ciona intestinalis and Ciona savignyi species are particularly well suited for the analysis of gene regulation (for recent review, see Satoh et al., 2003). Indeed, genomic information is readily available through interfaced data mining tools (Dehal et al., 2002), transient transformation of numerous embryos can be achieved by simple egg electroporation (Corbo et al., 1997), and reduced cell number throughout embryogenesis allows developmental gene activities to be studied at high resolution (Munro and Odell, 2002; Sardet et al., 2003). We therefore combined interspecific sequence comparison, transient transgenesis, and fluorescent imaging to study pitx locus organization and cis-regulation during development of the ascidian stomodeum (also referred to as “oral siphon primordium” or “primordial pharynx” in the literature). Our results suggest that the organization and usage of pitx exons are conserved in chordates. Moreover, we identified distinct cis-regulatory modules that govern isoform-specific ANB/stomodeal expression in complementary spatiotemporal patterns during embryogenesis.

Materials and methods

Animals and embryos

C. intestinalis adults were purchased at the Station Zoologique de Roscoff (Brittany, France). Embryos were obtained by in vitro cross-fertilization in artificial seawater as described (Hudson and Lemaire, 2001). Single cell zygotes were dechorionated according to Mita-Miyazawa et al. (1985), except that the protease was inactivated with glycine (1 mg/ml final in dechorionation solution), which substantially improves survival of the eggs. Electroporation and X-gal staining were performed as previously described (Bertrand et al., 2003; Corbo et al., 1997).

Sequence comparison and analysis

C. intestinalis and C. savignyi genomic sequences were obtained from JGI (Dehal et al., 2002, http://genome.jgi-psf.org/ciona/) and from the Broad Institute (http://www.broad.mit.edu/annotation/ciona/), respectively, and handled with the Vector NTI Suite (Informax). The VISTA algorithm (Mayor et al., 2000) was used for sequence alignment and comparison with the following parameters: 80 bp windows, 65% identity threshold. Conserved non-coding sequence (CNS) boundaries were refined by local alignment.

Molecular cloning and dissection of Ci-pitx genomic fragments

A mixture of 5' and 3' Ci-pitx RACE fragments was used as 32P-labeled probes to screen a C. intestinalis genomic cosmid library (Burgstorf et al., 1998). The distal fragment was PCR amplified from cosmid clone MPMGc11930337Q3 using vector-anchored forward primer and a reverse primer annealing in Ci-pitx exon1 (primer details in Supplementary Materials and methods) with the proofreading ThermusAec DNA polymerase (Invitrogen). The PCR fragment was inserted in pCRII-TOPO (Invitrogen), mapped by restriction analysis, and subcloned in pSP1.72-pPD27 expression vector (Corbo et al., 1997). Deleted constructs were obtained using native restriction sites and PCR-based constructs. The 8.5-kb proximal region was excised as an XhoI fragment from the cosmid DNA, subcloned into pSP1.72-pPD27, and recut with NotI. A first series of deleted constructs was made using native restriction sites. P1 to P3 were amplified using PfU DNA polymerase (Promega) and corresponding primer pairs (Supplementary Materials and Methods), digested and cloned into the pCES vector, which contains the Ci-fkh core promoter and first codons fused in frame with the lacZ coding sequence (Harafuji et al., 2002). The lacZ reporter gene was replaced in pSP1.72-pPD27 by the venus enhanced yellow fluorescent protein (EYFP; Nagai et al., 2002) coding sequence to create pSD1:venus. Distal and
proximal fragments were cloned into pSD1:venus as described above.

Whole mount in situ hybridization

The common 3’ probe was generated from a 3’ RACE clone as previously described (Christiaen et al., 2002). Isoform-specific fragments were amplified by PCR from the cosmid DNA, cloned into pCRII-TOPO, linearized, purified, and used for cRNA probe synthesis and digoxigenin labeling (Roche) by in vitro transcription with the Sp6 RNA-polymerase (Promega). In situ hybridization was carried out in an InSituPro automate (Intavis), following an established protocol (Moret et al., in press).

Fluorescent imaging

Electroporated embryos were fixed with 3.7% formaldehyde in calcium and magnesium-free artificial seawater (CMF-ASWH: 463 mM NaCl, 11 mM KCl, 25.5 mM Na2SO4, 2.15 mM NaHCO3, 10 mM HEPES, pH 8.1; recipe courtesy of Dr. P. Lemaire), permeabilized with PBTT1 (0.4% Triton X-100, 0.2% Tween 20 in phosphate buffer saline (PBS)), aldehyde autofluorescence was quenched with 50 mM NH4Cl in PBTT2 (0.2% Triton X-100, 0.1% Tween 20 in PBS) and counterstained with Alexafluor® 568-phalloidin (1/500) and DAPI (3 nM), diluted in PBTT2. Labeled embryos were equilibrated and mounted in glycerol/PBS (1:1, v/v), supplemented with 2% DABCO (w/v, Sigma D-2522). Images were captured within 2 days on a Leica DM RXA2 microscope equipped with a TCS SP2 confocal scanning system.

Results and discussion

Ci-pitx encodes distinct isoforms expressed in complementary domains

Previous studies independently reported cDNA cloning of a single C. intestinalis pitx ortholog (Boorman and Shimeld, 2002b; Christiaen et al., 2002). Detailed comparison with current genomic and EST data led us to hypothesize the existence of an alternative Ci-pitx isoform (Supplementary Fig. S1) and to refine the current gene model (Fig. 1A): the Ci-pitx transcriptional unit, which encompasses a 14-kb region, produces two different mRNA isoforms composed of exons3–5 and either exon1 or exon2. We called these isoforms Ci-pitxa/b and Ci-pitxc by analogy with vertebrates pitx2a/b and pitx2c, which use distal and proximal isoform-specific upstream exons, respectively (Fig. 1A; note that in addition to comparable linear organization, Ci-pitx falls within the size range 5–20 kb of vertebrate pitx genes).

Differential isoform expression was assessed in parallel in situ hybridization experiments using isoform-specific and common probes (see Fig. 1A for probe mapping).

At mid-tailbud stage, Ci-pitxa/b is only expressed in three cells of the anterior neural boundary (ANB, Figs. 1B and E, and Christiaen et al., 2002). In hatching larvae, the Ci-pitxa/b-specific probe labels tail muscles and a crown-shaped domain restricted to the deepest (ventral) part of the stomodeum (Figs. 1C, D, F, and G).

In mid-tailbud embryos, the Ci-pitxc-specific probe revealed an epidermal expression in an anterior cap and dorsolateral caudal stripe (Figs. 1B and H), which corresponds to the asymmetric epidermal expression reported in C. intestinalis (Boorman and Shimeld, 2002b) and Halocynthia roretzi (Morokuma et al., 2002). In addition, transient Ci-pitxc expression was observed in two cells of the posterior cerebral vesicle (Figs. 1B and H). At hatching, Ci-pitxc is expressed in photoreceptor cells, left visceral ganglion, and left posterior trunk endoderm (Figs. 1C, D, I, and J, and Supplementary Fig. S2). Interestingly, only Ci-pitxc is expressed asymmetrically, which further supports its homology with vertebrate pitx2c isoforms (e.g., Essner et al., 2000; Schweickert et al., 2000).

Strikingly, isoform-specific expression patterns are mutually exclusive and the pitx3’ probe labels every above-mentioned domain (Figs. 1B–D), showing that isoform-specific Ci-pitx expression profiles are complementary (Figs. 1K–M). In particular, ANB/stomod cells uniquely express Ci-pitxa/b from mid-tailbud stage till hatching.

Ci-pitx regulation involves separate basal promoters and cis-regulatory regions

To investigate Ci-pitx cis-regulation, the spatiotemporal transcriptional activity of two non-overlapping genomic fragments was assessed by transient transgenesis. These fragments map upstream from exons1 and 2 and will henceforth be referred to as distal and proximal, respectively (Fig. 2A).

The distal fragment is active in tail muscles and posterior stomodeum at hatching, extending ectopically in dorso-posterior stomodeal cells (Fig. 2C). At mid-tailbud stage, its activity is restricted to the ANB (Fig. 2B). We obtained identical data with the Venus reporter (Figs. 2H and I) and by in situ hybridization (Supplementary Fig. S2), showing that the distal construct retains stomodeal activity during the whole embryonic period, while expression in tail muscles starts by the end of late tailbud stage (Figs. 2F and G). According to key temporal aspects of Ci-pitx ANB/stomodeal expression, the distal construct is not active before early tailbud stage (data not shown).

The proximal construct exhibits a more pleiotropic expression pattern. At mid-tailbud stage, endogenous Ci-pitx expression is recapitulated in the posterior cerebral vesicle, but neither in the ANB nor in the epidermis (Fig. 2A).
2D). In addition to widespread ectopic staining (compare Figs. 1D and 2E), the proximal construct labels endogenous Ci-pitx expression domains at hatching, including the anterior stomodaeum, ocellus, left visceral ganglion, and dorso-posterior cerebral vesicle (Figs. 2E and G; the latter is stained by β-galactosidase activity remaining from the tailbud stage, see Fig. 2D). Expression dynamics was assessed by in situ hybridization, showing that the proximal construct activity is restricted to the anterior stomodaeum, ocellus, and visceral ganglion at hatching (Fig. 2G and Supplementary Fig. S2).

The overlap between endogenous and reporter patterns fits the topological relationship between regulatory regions and isoform-specific exons (Figs. 1K–M and 2F and G), raising the simple straightforward conclusion that transcription of each isoform is driven by its own promoter and tissue-specific cis-elements. In particular, the distal regulatory region is sufficient for early Ci-pitxa/b expression in ANB (Figs. 2B and F).

However, each construct drives reporter gene expression in larval stomodaeum (Fig. 2G), which in contrast expresses only Ci-pitxa/b (Figs. 1F and G). Confocal imaging revealed that expression of the distal construct extends up to a flexure in the antero-ventral stomodaeal layer that faces an actin-rich area in endoderm (Figs. 2J and N). Interestingly, the proximal fragment drives reporter gene expression in an anterior crescent-shaped stomodaeal domain (Figs. 2M

Fig. 1. Ci-pitx isoforms are expressed in mutually exclusive domains. (A) Comparative maps of Ciona pitx, human and zebrafish pitx2 loci, and isoforms. Scale bar (1 kb) applies to all gene maps. (B–M) Ci-pitx isoforms expression patterns. (B–G) Ci-pitxa/b-specific expression in anterior neural boundary (anb, black arrows), stomodaeum (st, black arrows), and tail muscles (tm, out of focus in C and D, obscured by background in F and G). (B–D and H–J) Ci-pitxc-specific expression in posterior neural cells (pnc, black arrowheads in B and H), left epidermis (epi, black asterisks), ocellus (oc, white arrows), left visceral ganglion (vg, white arrowheads), and left posterior trunk endoderm (pte, white asterisks). (K–M) Summary of Ci-pitx isoforms expression. Scale bar: 25 μm.
Fig. 2. Distinct cis-regulatory regions control Ci-pitx expression in complementary stomodeal patterns. (A) VISTA plot (percent identity with Cs-pitx) and map of Ci-pitx locus showing distal and proximal constructs, exons (open and black boxes), and conserved noncoding sequences (CNSs, purple boxes). (B and C) Distal construct activity in ANB and posterior stomodaeum (black arrows, lateral views, inset: close-up, dorsal view). Note the ectopic expression in dorso-posterior stomodeal cells (squared bracket). (D and E) Proximal construct activity in posterior neural cells and derivatives (pnc, black arrowheads), anterior stomodaeum (ast, black arrow; inset: close-up, dorsal view), ocellus (oc, white arrows), and visceral ganglion (white arrowhead). Ectopic expression in mesenchyme (mes), tail tissues (tt), and ventral cerebral vesicle (vCV). (F and G) Summary of distal and proximal construct expression in endogenous Ci-pitx expression domains, lateral views. Note that asymmetric expression is recapitulated by none of the constructs, suggesting that it requires additional elements, possibly located in the downstream CNS (II-5) as it is the case in mice (Shiratori et al., 2001). (H–O) Detailed stomodeal activities of distal (H–K) and proximal (L–O) constructs using EYFP (green), lateral (H, J, L, and N) and dorsal (I, K, M, and O) views. Confocal sections (I, K, N, and O) show cell cortices (F-actin, red), nuclei (DAPI, blue); note the antero-ventral stomodeal flexure and endodermal actin-rich area (open arrowheads). Open arrows point to the tip of anterior-most expressing cells. Scale bar: 10 μm.
and O), which lays two cells above this stomodeal flexure (Fig. 2N) and is therefore adjacent to the distal expression domain. Taken together, these data show that distinct cis-regulatory elements control Ci-pitxa/b expression in complementary stomodeal territories at hatching.

**Phylogenetic footprinting of putative cis-regulatory modules in Ci-pitx locus**

Next, we sought to identify cis-elements regulating Ci-pitx with special emphasis on stomodeal expression. Conserved noncoding sequences (CNSs) have recently been emphasized as valuable predictive footprints of functional elements (e.g., Frazer et al., 2003), and actual transcription factor binding sites within Ci-otx early neural enhancer showed sequence conservation between C. intestinalis and C. savignyi (Bertrand et al., 2003). Aiming at uncovering regulatory modules, we extended interspecific sequence comparisons to the 20-kb pitx loci.

We found that pitx exons are conserved in both sequence and relative positions (Fig. 2A and data not shown). Moreover, conserved noncoding sequences allowed us to define putative cis-regulatory modules (pCRMs). We identified one (D1, 307 bp) and four (P1–4; 69, 470, 210, and 118 bp) pCRMs in the above-mentioned distal and proximal noncoding regions, respectively (Fig. 2A).

Noticeably, pCRMs could be readily identified in a global alignment, suggesting that the evolutionary distance between *Ciona* species is adequate to efficiently pinpoint functional elements (see below) by phylogenetic footprinting of large genomic data sets.

**D1 is an autonomous ANB/stomodeal enhancer**

Sequence conservation suggests that D1 may contribute to distal regulatory inputs in ANB/stomodeal cells. A first series of deleted constructs was generated and tested by transient transgenesis and X-gal staining at larval stage (Fig. 3A, #3–7). These data show that specific abolishment of stomodeal expression correlates with a deletion of the first 188 bp of D1.

We next generated internal deletions that remove D1 (Fig. 3A, #8, 9). These constructs were unable to drive reporter gene expression in the stomodeum, showing that the first 188 bp of D1 is absolutely required for stomodeal expression of the transgenes.

We then asked whether D1 could drive stomodeal expression in a minimal context (Fig. 3A, #10,11). Constructs #10 and #11 drive expression uniquely in the stomodeum, showing that partial D1 is sufficient to enhance transcription specifically in stomodeal cells. The complete and partial D1 fragments could also activate expression in tailbud ANB and larval stomodeum when

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**Fig. 3. D1 is necessary and sufficient for stomodeal expression.** (A) Molecular dissection of the distal construct. SL trans-splicing occurs during the maturation of the Ci-pitx/b primary transcript (Christiaen et al., 2002), distal promotor was roughly mapped between −1652 and −1328 bp (#2 and data not shown). Expression is represented as + or −, meaning that stomodeal expression is “all or nothing” (>85% or 0%), +/− means reduced expression relative to maximum (e.g., compare C and D for expression in tail muscles, and mesenchyme). Scale bar: 20 μm.
fused to the heterologous Ci-fkh basal promoter (pFkh in pCES construct, data not shown). Taken together, these data show that D1 is a bona fide stomodeal activation module that could be efficiently identified upon sequence conservation and accounts for ANB/stomodeal activity of the distal regulatory region during embryogenesis.

**P2 is a second major stomodeal activation module**

We next sought to identify proximal cis-regulatory elements driving Ci-pitx expression in the anterior crescent-shaped stomodeal territory. We generated a preliminary set of 5′ deleted constructs and focused on stomodeal expression. These deletions were designed to remove successively larger fragments containing the above-mentioned pCRMs, P1 to P4 (Fig. 4A).

The first deletion does not affect reporter gene pattern (Fig. 4B), suggesting that P4 is not required for stomodeal expression during embryogenesis.

The next deletion removes P3 and leads to a substantial reduction of stomodeal staining at hatching (Figs. 4A and C), but not at later stages (data not shown). In addition, P3 is unable to drive stomodeal expression when fused to a heterologous basal promoter (Fig. 4A, pCES:P3). Thus, P3 is likely to be a co-activation module, not sufficient for stomodeal expression on its own.

The next deletion removes P2 and abolishes stomodeal expression, opening the possibility that this latter requires P2 (Fig. 4A). We next found that a P2:pFkh fusion drives reporter gene expression in anterior stomodeal cells (Figs. 4A and D). P2 thus appears as an additional autonomous stomodeal enhancer.

Given the topology of the Ci-pitx locus, Ci-pitxa/b expression implies that transcription is initiated from the distal core promoter in stomodeal cells (Figs. 1A and L). Therefore, we assessed the ability of P2 to drive stomodeal expression through the distal promoter (Figs. 4A and E, P2:pdist). This expression was enhanced by P3, which appears again as a co-activation module in stomodeal cells (Fig. 4A, P2P3:pdist).

The lack of detectable Ci-pitx expression suggests that the proximal core promoter is isolated from the P2 input in stomodeal cells. The mechanism of P2 promoter selectivity remains elusive. However, close examination of core promoter sequences revealed the presence of a TATA-box in the distal core promoter, while the proximal promoter only possesses a putative downstream promoter element (Kadonaga, 2002; see supplementary Fig. S3). Such a difference in basal promoter sequence could account for P2 promoter preference, as reported for AE1 and IAB5 elements in Drosophila (Ohtsuki et al., 1998), thus leading to correct isoform expression in stomodeal cells.

These observations led us to propose that P2 is a second major stomodeal enhancer, its input on the distal core promoter being eventually reinforced by the P3 co-activation module (Fig. 5D). The transcriptional outcome

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![Fig. 4. P2 is a major stomodeal enhancer. (A) Molecular dissection of the proximal construct. Expression is scored both qualitatively (+, +/-, -) and semiquantitatively (%exp: percentage of expression, n: number of embryos). (B–E) Characteristic embryos and corresponding constructs. Anterior stomodeal expression in B, D, and E (black arrow). Note the ectopic epidermal staining in C. Mosaic embryos (such as D and E) were scored positive for stomodeal expression.](image-url)
of such combinatory interplay of individual cis-regulatory modules would result in accurate Ci-pitx/b expression in the anterior crescent-shaped stomodeal territory (Figs. 5B and C), which is complementary to the D1 activation pattern in posterior stomodeal cells (Figs. 5B, C, and E) and ANB.

This current model, together with confocal imaging of reporter gene expression, lets us envision elements of stomodaeum patterning in ascidians (Figs. 5A–C). In particular, distinct superficial and deep stomodeal compartments can be defined upon restricted Ci-pitx/b expression in ventral/deep stomodeal cells. Lineage studies suggest that these superficial and deep stomodeal compartments originate from a/a6.7 and a/a6.5, sixth generation blastomeres, respectively (Nishida and Satoh, 1985). Moreover, neural induction overrides a-line epidermal default fate in a/a6.5 blastomeres (Hudson and Lemaire, 2001), suggesting that distinct stomodeal compartments in ascidians may reflect dual ontogenetic origin of the stomodeum in chordates.

Though clonal restriction of cell fate is of special importance in ascidians (reviewed in Satoh, 1994), it does not fully explain stomodeal cell specification. In fact, deep stomodeal cells originate from a/a8.19 and a/a8.17 blastomeres (descendants of a/a6.5), which share posterior position after the eighth cleavage, while their respective a/a8.20 and a/a8.18 anterior sister cells give birth to the palps (Nishida, 1987). In addition, the anterior cerebral vesicle also arises from a/a8.19 and a/a8.17 (Nishida, 1987). Taken together, these data suggest that stomodeal fate is established after the eighth cleavage, possibly by position-dependent induction events that overcome differences in clonal origins. Indeed, the stomodeal presumptive territory encompasses neural and nonneural ectoderm and contacts anterior endoderm. The complexity of pitx cis-regulation in ascidians might therefore witness combined developmental cues that position the ancestral chordate mouth and associated structures.

This study provides us with an entry point to investigate the genomic hardwiring of GRNs underlying oral development in chordates, with the hope that the Ciona system could lead the way toward a detailed comprehension of stomodeal GRN in the more complex vertebrate species.

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Appendix A. Supplementary data


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