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Ci-FoxA-a is the earliest zygotic determinant of the ascidian anterior ectoderm and directly activates *Ci-sFRP1/5*

Clement Lamy*, Ute Rothbächer, Danièle Caillol and Patrick Lemaire*

This work focuses on the anteroposterior patterning of the ectoderm in the invertebrate chordate *Ciona intestinalis*. Previous work indicated that, by the eight-cell stage, the anterior and posterior animal blastomeres have acquired different properties, including a differential responsiveness to inducing signals from the underlying mesendoderm. Here, we investigated the molecular basis of this distinction. For this, we studied the regulation of the earliest marker specific for the anterior ectoderm, *Ci-sFRP1/5*, which is activated at the 64-cell stage. We first found that the activation of this marker in the anterior ectoderm does not involve communication with other lineages. We then identified, by phylogenetic footprinting and deletion analysis, a short conserved minimal enhancer driving the onset of expression of *Ci-sFRP1/5*. We showed that this enhancer was a direct target of the *Ci-FoxA-a* gene, a *FoxA/HNF3* orthologue expressed in anterior ectodermal and mesendodermal lineages from the eight-cell stage. Gain- and loss-of-function experiments revealed that Ci-FoxA-a is necessary and sufficient within the ectoderm to impose an ectodermal anterior identity, and to repress the posterior programme. Thus, *Ci-FoxA-a* constitutes a major early zygotic anterior determinant for the ascidian ectoderm, acting autonomously in this territory, prior to the onset of vegetal inductions. Interestingly, while vertebrate *FoxA2* are also involved in the regionalization of the ectoderm, they are thought to act during gastrulation to control, in the mesendoderm, the expression of organizer signals. We discuss the evolution of chordate ectodermal patterning in light of our findings.

KEY WORDS: *Ciona intestinalis*, Anteroposterior patterning, Organizer, Ectoderm, Ascidian, Gene regulation, FoxA, Wnt signalling, sFRP

INTRODUCTION

In vertebrate embryos, the anteroposterior and dorsoventral polarities of the three embryonic layers are set up during gastrulation by the action of a signalling centre, the organizer. This organizer (known as the Spemann organizer in *Xenopus*, the node in mouse and chicken, the shield in zebrafish) emits factors to determine the polarity of the layers. These secreted factors antagonize the extracellular BMP, Nodal and Wnt ligands, which establish a stable gradient of intracellular signalling in the developing embryo during gastrulation movements (reviewed in Niehrs, 2004).

Vertebrates share the chordate body plan with two invertebrate chordate groups, the cephalochordates and the tunicates (Blair and Hedges, 2005; Delsuc et al., 2006). Among the tunicates, the ascidians have been proposed to be lacking an organizer (Dehal et al., 2002), a property that may represent an ancestral chordate state (Kourakis and Smith, 2005). The availability of two assembled *Ciona* genomes, of powerful molecular tools and of extensive gene expression atlases make *Ciona intestinalis* a seducing model to address how chordate anteroposterior patterning can be achieved in absence of an organizer.

Ciona intestinalis forms a typical chordate tadpole larva made of only around 2600 cells. The larval trunk/tail region contains the rostral sticky palps, the anterior neural sensory vesicle (thought homologous to the forebrain and midbrain of vertebrates), endoderm and mesenchymal tissue. The larval tail is organized with a dorsal nerve cord (thought homologous to the vertebrate spinal cord), a central notochord, ventral endodermal cells and flanking muscles (for reviews, see Nishida, 2005; Lemaire et al., 2002).

Institut de Biologie du Développement de Marseille, UMR 6216, CNRS/Université de la Méditerranée, Parc Scientifique de Luminy, Case 907, F-13288 Marseille Cedex 9, France.

*Authors for correspondence (e-mail: lamy@ibdm.univ-mrs.fr; lemaire@ibdm.univ-mrs.fr)

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Ascidian embryogenesis is well described and can be followed at the cellular level until the late gastrula stage. Cell cleavages are regular and the arising cell lineages are invariant. From the eight-cell stage, there is a segregation between vegetal blastomeres (A- and B-lines) that give rise to the endodermal and mesodermal tissues of the larva, and the animal blastomeres (a- and b-lines), which mainly develop into neural and epidermal tissues.

Ascidians were until recently considered as a classical model for mosaic development, based on the inheritance of localized maternal factors in the developing embryo. The identification of *Macho-1* as a maternal determinant of posterior vegetal fates (Nishida and Sawada, 2001; Kobayashi et al., 2003) gave cues to understand axial patterning of this chordate embryo for the mesendodermal fates. An anteroposterior difference also exists between the two ectodermal lineages (a- and b-lines) from the eight-cell stage (Rose, 1939; Reverberi and Minganti, 1947; Okado and Takahashi, 1990; Hudson and Lemaire, 2001). The anterior ectodermal a-line gives rise to the trunk epidermis and the sensory vesicle. The posterior ectodermal b-line gives rise to the tail epidermis and the dorsal row of the posterior nerve cord. Upon neural induction by endogenous FGF signals emitted by anterior vegetal cells or by treatment with recombinant FGFs, anterior a-line cells form pigment cells and express anterior neural markers (such as *Ci-Otx* and *Ci-gsx* genes) at tailbud stage. By contrast, posterior b-line cells rarely form pigment cells or express anterior neural markers in response to FGF neural inducers (Hudson and Lemaire, 2001).

In this study, we made use of the extensive available molecular tools in *Ciona* (Satoh and Levine, 2005) to address the molecular mechanism of the establishment of the anteroposterior difference in the animal hemisphere. We show that there is a global difference between the anterior and posterior ectodermal lineages set up autonomously from the eight-cell stage in the ascidian embryo. By analyzing the mechanisms of activation of the earliest anterior ectodermal specific gene, *Ci-sFRP1/5*, we show that Ci-FoxA-a, a

member of the FoxA family of transcription factors is a key zygotic activator of the anterior ectodermal program and a repressor of posterior ectodermal fates.

MATERIALS AND METHODS

Embryo culture and manipulation

Ciona intestinalis adults were collected at the Station Biologique de Roscoff, France. Fertilization, embryo culture, animal cells isolation, bFGF (100 mg/ml, Sigma) and U0126 (3 μM, Calbiochem) treatments were as described (Hudson et al., 2003; Hudson and Lemaire, 2001).

Microinjection and electroporation

Microinjection was performed as described (Bertrand et al., 2003) in unfertilized eggs. The sequence of the *Ci-FoxA-a* morpholino (MO-CiFoxA-a) targeted against the ATG region was: 5′-TTGACGGTGGAGACGACAACATCAT-3′ (Gene Tools, LLC). The sequence of the control morpholino (MO-Control) was: 5′-CCTCTTACCTCAGTTACAATTTATA-3′. The morpholinos were injected at a stock concentration of 0.7 mM.

DNA constructs (50 μg) were introduced by electroporation as in Bertrand et al. (Bertrand et al., 2003), except when analysing the effect of MO injection on the pSP1.72-sFRP-118::*NLSLacZ* and pSP1.72-pFOG::*Ci-mFoxA-a* constructs. In this case, morpholino (0.7 mM stock) and DNA constructs (20-30 ng/μl) were co-injected in unfertilized eggs. DNA electroporation or microinjection leads to the mosaic inheritance and expression of the electroporated plasmid in developing embryos, explaining that a proportion of embryos does not express the construct. Expression of the transgenes was always included within the endogenous expression of the corresponding gene.

Cis-regulatory analysis

The *Ci-sFRP1/5* regulatory region was analysed by phylogenetic footprinting using Vista (http://genome.lbl.gov/vista/) (Dubchak et al., 2000) and ClustalW (http://www.ch.embnet.org/) (Thompson et al., 1994) alignments. The search for transcription factor binding sites was performed using Transfac Professional 8.2 (http://www.gene-regulation.com/pub/databases.html) (Matys et al., 2003). Ci-sFRP1/5 cis-regulatory fragments were PCR amplified from *Ciona intestinalis* sperm genomic DNA using Accuprime Taq Polymerase (Invitrogen) and inserted in pSP1.72-RfB::*NLSLacZ* or pSP1.72-RfA-pBra::*NLSLacZ* Gateway (Invitrogen) destination vectors. These vectors were generated by insertion of an RfB or RfA cassette in pSP1.72 (Corbo et al., 1997) or in pBra pSP1.72 (Bertrand et al., 2003) vectors in *Bam*HI-*Not*I and *Hin*dIII sites, respectively. A precise map of these vectors is available upon request.

The specific sequences of the different primers used for cloning *Ci-sFRP1/5* cis-regulatory elements were: sFRP-proximal-for, 5′-TGTATGCCGTACATATGCCAG-3′; sFRP-proximal-rev, 5′-TTCGGGGCTCTGAAAAAACATAG-3′; sFRP-1067 distal-for, 5′-TTACGCTCGATGGCGCAACG-3′; sFRP-1067 distal-rev, 5′-AACTAAACCGCAACTATAGTATG-3′; sFRP-640-for, 5′-CGCTGTATATACAACTTTGC-3′; sFRP-640-rev, 5′-TGTCACTATCAAAGCATGTG-3′; sFRP-262-for, 5′-CGGGTTCTCATAGAATCTAC-3′; sFRP-262-rev, 5′-TTGCTATCAAATTAGTGTAGC-3′; sFRP-172-for, 5′-CGGGTTCTCATAGAATCTAC-3′; sFRP-172-rev, 5′-CAACACCAACAGATTACAATG-3′; sFRP-118-for, 5′-GCAAACAAACGACTTGTTTAC-3′; sFRP-118-rev, 5′-GCACGTTTGTTTAAATTGGAG-3′; sFRP-150-for, 5′-CGGGTTCTCATAGAATCTAC-3′; sFRP-150-rev, 5′-TCAATCCTCTGGCACCAAC-3′; sFRP-126-for, 5′-CGGGTTCTCATAGAATCTAC-3′; sFRP-126-rev, 5′-TAATCTGTTAACGGGACAAG-3′; sFRP-95-for, 5′-GCAAACAAACGACTTGTTTAC-3′; sFRP-95-rev, 5′-CAACACCAACAGATTACAATG-3′; sFRP-92-for, 5′-TGTCCCGTTAACAGATTAGG-3′; sFRP-92-rev, 5′-GCACGTTTGTTTAAATTGGAG-3′; sFRP-67-for, 5′-TGTCCCGTTAACAGATTAGG-3′; sFRP-67-rev, 5′-CAACACCAACAGATTACAATG-3′.

The 6-Fox construct was obtained by cloning: 5′-TCAGCAAACAAACGACTTGTTTACTTTTCAGCAAACAAACGACTTGTTTACTTTTCAGCAAACAAACGACTTGTTTACTTT-3′ (Fox sites underlined) into the pSP1.72-RfA-pBra::*NLSLacZ* vector.

Ci-FoxA-a animal overexpression

The pSP1.72-pFOG::RfA destination vector for overexpression in animal cells was generated first by PCR amplification on genomic DNA of *Ci-FOG* cis-regulatory sequence that contains –1980 bp upstream of the ATG of *Ci-FOG* (Friend of GATA, ci0100149797). The amplified fragment was cloned into *Pst*I/*Bam*HI sites of a pSP1.72-based expression vector in which the *lacZ*-coding region was replaced by the Gateway RfA cassette.

The *Ci-FoxA-a* ORF was amplified from the cicl44j20 clone using T3/T7 primers containing the attB1/attB2 sequences: attB1-T3, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATTAACCCTCACTAAAGGGA-3′; T7-attB2, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTAATACGACTCACTATAGGG-3′). The PCR product was recombined into the p221 donor vector (Invitrogen) and the resulting entry clone was recombined into pSP1.72-FOG::RfA destination vector, giving rise to pSP1.72-FOG::*Ci-FoxA-a*.

To rescue the Morpholino phenotype, the pSP1.72-pFOG::*Ci*-m*FoxA-a* construct was generated as above except the ATG region recognized by the morpholino was altered by amplification of the ORF with attB1-*mFoxA-a* (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAAAAATGATGCTTAGCAGCCCTCCATCAAAGTACCAACCCTTCC-3′) and T7-attB2.

In situ hybridization and X-gal staining

In situ hybridization and X-gal staining were performed as described (Bertrand et al., 2003). DIG probes were synthesized from the following cDNAs: *Ci-sFRP1/5* (clone 12ZE09, GenBank AL666897), *Ci-Otx* (Hudson et al., 2003), *Ci-FoxA-a* (GR1:cicl044j20), *Ci-RORa* (GR1:cilv008g20), *Ci-FoxC* (GR1:cilv050a24), *Ci-Delta2* (GR1:cieg005o22) and *Ci-Msxb* (GR1:cign067l18).

Expression patterns were retrieved from the GHOST (http://hoya.zool.kyoto-u.ac.jp/otherfr.html) and Aniseed (http://aniseed-ibdm.univ-mrs.fr) databases. In situ hybridization results from this study were submitted to Aniseed.

RESULTS

Ci-sFRP1/5 expression reveals an autonomous specification of the anterior ectodermal a-line

Scanning the *C. intestinalis* gene expression database (Imai et al., 2004), we identified a gene specifically expressed in the whole anterior animal a-line. This gene, *Ci-sFRP1/5*, is orthologous to vertebrate Secreted Frizzled Related Protein (sFRP) genes 1 and 5. Its expression starts from the 64-cell stage in all blastomeres of the a-line (Fig. 1A), persists throughout its progeny up to neurulation and disappears from the ectoderm by the early tailbud stage (Imai et al., 2004) (and data not shown). This restricted a-line expression from the 64-cell stage makes it the earliest known animal specific anterior marker.

Wada and colleagues (Wada et al., 1999) showed that vegetal interactions are necessary at least until the 32-cell stage for anteroposterior patterning of the epidermis. To address whether *Ci-sFRP1/5* expression is autonomous to the a-line or induced by vegetal signals, we first inhibited the major vegetal inducer Ci-FGF9/16/20 (reviewed in Nishida, 2005) by treating the embryos with the U0126 MEK inhibitor from the eight-cell stage. This treatment abolishes neural tissue formation (Hudson et al., 2003) but the expression of *Ci-sFRP1/5* remained as wild type in all a-line cells (data not shown).

To further address the issue of the autonomous activation of *Ci-sFRP1/5* in animal cells, we isolated animal blastomeres at the eight-cell stage (when animal cells segregates from vegetal cells), cultured the explants until the 64-cell stage, and assayed *Ci-sFRP1/5* expression in explants by in situ hybridization. When a whole animal cap was explanted, half of the cells of most explants expressed strongly *Ci-sFRP1/5* at the 64-cell stage (80% of explants, *n*=75, Fig. 1B). The remaining explants (20%) expressed a weak, diffuse background-like staining. To assay whether this staining

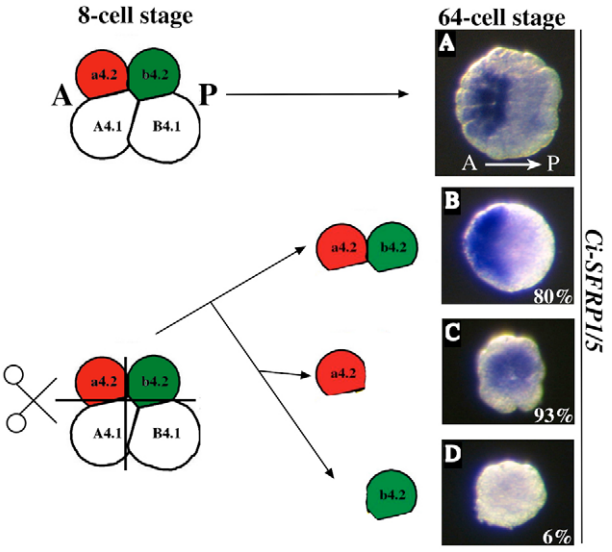

Fig. 1. *Ci-sFRP1/5* marks the difference between anterior and posterior ectoderm. In situ hybridization for *Ci-sFRP1/5* at the 64-cell stage on whole embryos (**A**, animal view, anterior towards the left), whole animal explants (**B**), anterior animal a-line explants (**C**) and posterior animal b-line explants (**D**) explanted at the eight-cell stage. The scheme to the left illustrates an eight-cell stage embryo in lateral view. a- and b-line cells are shown in red and green, respectively.

corresponded to anterior cells, we independently isolated anterior a-line or posterior b-line cells at the eight-cell stage. At the equivalent of the 64-cell stage, all 16 cells of a-line explants strongly expressed *Ci-sFRP1/5* gene (93% of explants, n=15, Fig. 1C) while no or very few cells of b-line explants expressed *Ci-sFRP1/5* (6% of explants showing any staining, n=18) (Fig. 1D).

Therefore, the ability to express *Ci-sFRP1/5* at the 64-cell stage appears autonomous to the a-line.

Fox binding sites are necessary for anterior ectodermal expression of *Ci-sFRP1/5*

Ci-sFRP1/5 is expressed from the 64-cell stage, while the difference between the anterior and posterior ectodermal lineages is set up as early as the eight-cell stage. This suggests the existence of localized upstream regulators of *Ci-sFRP1/5* expression. To identify them, we analysed the cis-regulatory sequences of *Ci-sFRP1/5*. Phylogenetic footprinting between *Ciona intestinalis* and *Ciona savignyi* genomes identified three conserved blocks in the 2 kb upstream of the putative *sFRP1/5* transcription start site (Fig. 2A).

We cloned two fragments of this region upstream of a *NLSLacZ* reporter gene and revealed the expression of this reporter by X-gal staining at the 110-cell and neural plate stages. A proximal fragment (from the putative transcription start to –985 bp, Fig. 2A) containing two conserved blocks produced no specific staining (data not shown). By contrast, sFRP-1067, a distal 1 kb fragment (from –927 bp to –1993 bp, Fig. 2A), drove specific anterior animal expression from the 64-cell stage.

Deletion of 195 bp in the 3′ extremity of this fragment abolished the expression of the transgene. a-line-specific expression could, however, be restored by adding a minimal promoter (from *Ci-Bra* gene) (Bertrand et al., 2003), between the cloned fragment and the reporter gene, suggesting that the minimal promoter of *Ci-sFRP1/5* is located in this deleted region (data not shown).

Next, we generated 5′ and 3′ deletion constructs by PCR (see Materials and methods) and assayed whether they drove MEK-independent expression throughout the a-line from the 64-cell stage as expected from the analysis of endogenous *Ci-sFRP1/5* expression (Fig. 2B). The sFRP-67 construct had no activity (Fig. 2Bc). The sFRP-150, -126, -95 and -92 constructs drove a low frequency expression in only part of the a-line (Fig. 2Bb). Unlike *Ci-sFRP1/5*, this expression was MEK dependent (not shown). Finally, sFRP-640, -262, -172 and -118 drove pan-a-line (Fig. 2Ba), MEK-independent (Fig. 3A and not shown) expression that recapitulated endogenous *Ci-sFRP1/5* expression.

To identify candidate factors acting in trans on sFRP1/5, we searched in silico combinations of putative binding sites, conserved between *C. intestinalis* and *C. savignyi*, and present in all enhancers recapitulating *Ci-sFRP1/5* expression but not in the other fragments. We found a perfect correlation between the presence of at least three Fox-binding sites and the ability of the fragment to drive pan-a-line expression. To study the importance of these putative Fox-binding sites, we focused on the smallest fragment driving expression throughout the a-line, sFRP-118 (Fig. 2C). We point mutated the core of the Fox binding sequences (TGTTT→TGCTT) (Overdier et al., 1994). Mutation of the two 5′ binding sites resulted in a great reduction of the staining in a-line precursors (Fig. 3A). Most of the residual activity was MEK dependent and therefore distinct from the endogenous *Ci-sFRP1/5* regulation. Point mutations in the 3′ putative binding site abolished a-line staining. These experiments (n>60 embryos, and at least two independent experiments for each condition) suggest that putative Fox-binding sites are necessary for the expression of the transgene from the 64-cell stage.

We next investigated if these putative Fox-binding sites were sufficient for the expression in anterior animal cells from the 64-cell stage. We trimerized the 5′ region of the sFRP-118 element containing two putative FOX binding sites to build a synthetic fragment of 81 bp containing six identified Fox sites (6-Fox, Fig. 3B), which was placed in the pSP1.72-RfA-bpBra::*NLSLacZ* vector. This construct drove strong MEK-independent expression throughout the anterior animal a-cells from the 64-cell stage (Fig. 3A and not shown). Additional staining in the anterior vegetal A-line cells was also detected in a large proportion of electroporated embryos. This expression was weaker than a-line staining (Fig. 3A). Consistent with the idea that this anterior expression is due to the presence of the Fox-binding sites, similar results were observed in *Halocynthia roretzi* by Oda-Ishii and colleagues (Oda-Ishii et al., 2005), and repeated by us in *Ciona intestinalis* (not shown), with the Fox-BS construct. This artificial construct is made of four Fox-binding sites from the *Hr-Otx* cis-regulatory sequence and solely shares the Fox-binding sites with 6-Fox (not shown). The anterior vegetal expression observed with 6-Fox is not due to a generally higher level of activity of this construct compared with sFRP-118, as both elements drive similar levels of expression in animal cells. Thus, the restriction of the expression of the sFRP-118 element to the anterior animal region may involve additional trans-acting factors that could either be animal-specific activators or vegetal repressors.

Ci-sFRP1/5 is a likely direct target of Ci-FoxA-a

Next, we looked for a Fox factor expressed at the right place and time to be an activator of *Ci-sFRP1/5*. This Fox factor should be present in the anterior half of the embryo before the 64-cell stage. There are 27 putative *Fox* genes in *Ciona intestinalis* genome (Imai et al., 2004). Four of these are presumably not expressed in embryos, as they could not be detected by RT-PCR (Imai et al., 2004). The remaining 23 genes were analysed by in situ hybridization and only

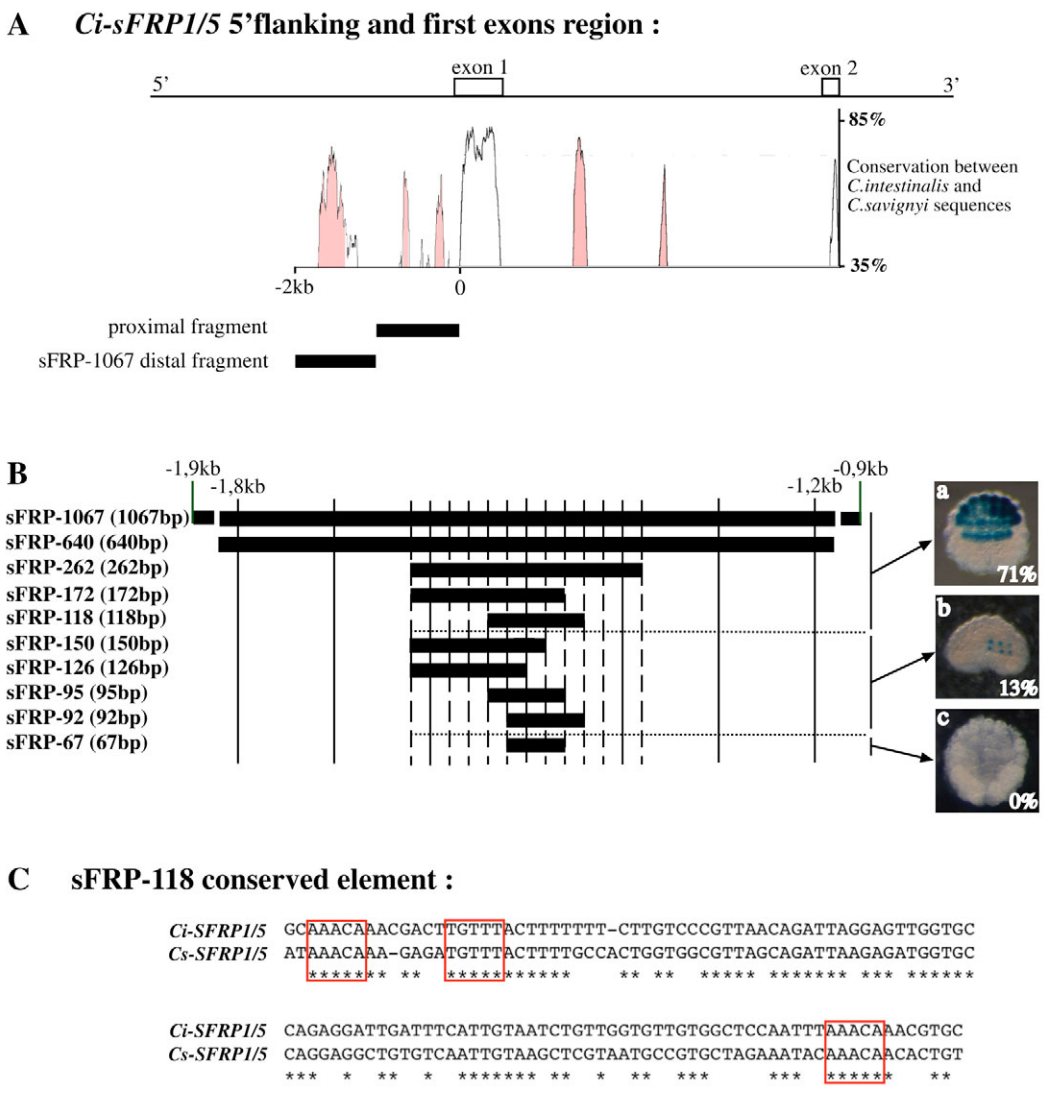

Fig. 2. Isolation of a minimum enhancer recapitulating *Ci-sFRP1/5* expression. (**A**) *Ci-sFRP1/5* locus in the *Ciona intestinalis* genome, showing the 5′ flanking region and the first two exons. Coding/non coding conserved regions between *C. intestinalis* and *C. savignyi* are shown in white/pink. The scheme shows the position of the distal sFRP-1067 and proximal fragments tested in vivo. (**B**) Schematic view of the regulatory elements (with their length in brackets) tested at the 110-cell stage. With the exception of sFRP-1067, which includes the endogenous minimal promoter, all constructs were placed in front of the *Ci-brachyury* minimal promoter. (a-c) Representative X-gal staining for embryos electroporated with the indicated DNA (percentages correspond to embryos electroporated with sFRP-640, sFRP-95 and sFRP-67 respectively). (a,c) Animal view, anterior towards the top. (b) Anterior view, animal to the top. (**C**) Alignment of the *C. intestinalis* and *C. savignyi* sequences of the sFRP-118 element, with putative Fox binding sites framed in red.

two of them detected in ectodermal cells before the 64-cell stage (Imai et al., 2004). *Ci-Orphan-Fox1* is ubiquitously expressed from the 16-cell stage, while *Ci-FoxA-a* expression was reported at the 16-cell stage in vegetal cells and in the a-line blastomeres.

We performed in situ hybridization to establish a kinetics of activation of the *Ci-FoxA-a* gene in the animal territories. At the eight-cell stage, its expression is visible in both anterior lineages, vegetal A4.1 and animal a4.2 pairs (Fig. 4A). This early expression is consistent with the expression of the *C. savignyi* orthologue (Shimauchi et al., 2001). Expression of *Ci-FoxA-a* in animal territories persists at the 16-cell stage in the anterior blastomeres (a5.3 and a5.4), and disappears from the ectodermal cells by the 32-cell stage (data not shown).

To test the function of *Ci-FoxA-a* in *Ciona* embryos, we first performed loss-of-function experiments. When we injected an antisense morpholino oligonucleotide (MO) against *Ci-FoxA-a* in *Ciona* eggs prior to fertilization, the activity of the co-injected sFRP-118::*NLSLacZ* construct was significantly decreased. Owing to the mosaic inheritance of the plasmid, *lacZ* was detected in 58% of MO-control injected embryos (n=60, Fig. 4B). This proportion was lowered to 13% in embryos co-injected with MO-Ci-FoxA-a (n=71, Fig. 4C). The number of positive cells in these embryos was reduced (not shown). MO-Ci-FoxA-a-injected embryos also showed a strong decrease in endogenous *Ci-sFRP1/5* expression, both at the 64-cell stage (MO-control, 87% positive embryos, n=47; MO-Ci-FoxA-a, 3% positive embryos, n=37). A similar effect was observed at the neural plate stage (Fig. 4B, n=30; Fig. 4C, n=33). Thus, Ci-FoxA-a is necessary for the activation of *Ci-sFRP1/5* via the sFRP-118 enhancer.

We next sought to overexpress *Ci-FoxA-a* throughout the animal hemisphere. For this, we placed a full ORF cDNA under the control of the *Ci-FOG* gene regulatory sequences. The *Ci-FOG* gene is expressed in all animal cells from the 16-cell stage and is downregulated after the 64-cell stage (U.R. and P.L., unpublished).

We first used this approach to rescue the decrease of *Ci-sFRP1/5* expression in MO-Ci-FoxA-a injected embryos. Co-injection of MO-Ci-FoxA-a and pSP1.72-pFOG::*Ci-mFoxA-a* construct, which drives a mutated version of *Ci-FoxA-a* lacking the morpholino target site, restored expression of *Ci-sFRP1/5* in ectodermal cells at the 64-cell stage, demonstrating the specificity of the effect of the morpholino (MO-Ci-FoxA-a, 20%, n=5; MO-Ci-FoxA-a + pSP1.72-pFOG::*Ci-mFoxA-a*, 86%, n=14; data not shown).

To assay whether *Ci-FoxA-a* expression is sufficient to direct *Ci-sFRP1/5* in the posterior b-line, we co-electroporated pSP1.72-pFOG::*Ci-FoxA-a* with pSP1.72-sFRP-118-bpBra::*NLSLacZ*. X-gal

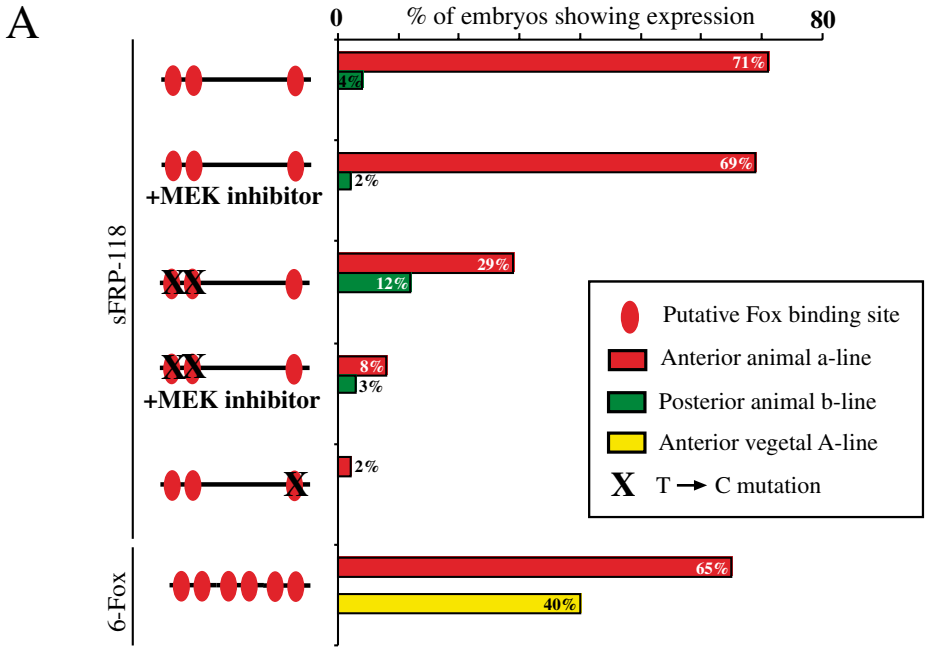

Fig. 3. Consensus Fox-binding sites are necessary and sufficient for anterior ectodermal expression. (**A**) Activity of various endogenous and synthetic constructs containing Fox sites (red circles). Left: scheme of the constructs tested. Right: histogram representing the percentage of embryos with X-gal staining in different lineages. (**B**) Sequence of the 6-Fox element artificial construct tested in A, with the Fox sites boxed.

B

6-Fox element:

5'-TCAGCAAACAAACGACTTGTTTACTTTTCAGCAAACAAACGACTTGTTTACTTTTCAGCAAACAAACGACTTGTTTACTTT-3'

staining was detected at the 110-cell stage in both the anterior and posterior animal blastomeres (54% of embryos showed b-line expression, *n*=48) (Fig. 4E). The same qualitative result was obtained when assaying endogenous *Ci-sFRP1/5* expression at the 64-cell stage following pSP1.72-pFOG::*Ci-FoxA-a* electroporation (Fig. 4E). Ectopic b-line expression was also seen at neural plate stage (Fig. 4E). We conclude that in a normal embryonic context, animal *Ci-FoxA-a* expression is sufficient for activating *Ci-sFRP1/5* in all animal blastomeres. To rule out that this activation requires some relay via the vegetal cells, we electroporated in *Ciona* eggs *pSP1.72*-sFRP-118::*NLSLacZ* in the presence or absence of pSP1.72-pFOG::*Ci-FoxA-a*, explanted the animal blastomeres at the eight-cell stage, and analysed reporter activity in the explant at the 110-cell stage. In the absence of ectopic FoxA-a, a-line explants expressed *lacZ* at the neural plate stage (43% of positive explants, *n*=14, Fig. 5C), but b-line explants did not (8%, *n*=13, Fig. 5E). In pSP1.72-pFOG::*Ci-FoxA-a*-electroporated embryos, the expression of *Ci-sFRP1/5* reporter staining was observed at neural plate stage in both a-line (73% of positive explants, *n*=11, Fig. 5D) and b-line (69%, *n*=13, Fig. 5F) explants. This experiment shows that Ci-FoxA-a is sufficient for the autonomous expression of anterior ectodermal genes.

Taken together, these and the previous results suggest that Ci-FoxA-a activates *Ci-sFRP1/5* at the 64-cell stage via the direct regulation of the sFRP-118 enhancer.

Ci-FoxA-a is a global determinant of anterior ectoderm

We next tested the extent of the requirement of *Ci-FoxA-a* for ectodermal patterning. For this, we tested first the effect, at the neural plate stage, of interfering with the activity of this gene on several anterior ectodermal markers. *Ci-Otx* is expressed in the precursors of the sensory vesicle (a9.33, 34, 37, 38, 49, 50 cells) (Hudson and Lemaire, 2001), *Ci-FoxC* is expressed in the precursors of the anterodorsal epidermis and palps (a9.35, 36, 39, 40, 51, 52 cells) (Imai et al., 2004), and *Ci-Ror-a* is expressed throughout the a-line (Bertrand et al., 2003) (Fig. 5G,J,L).

When we downregulated Ci-FoxA-a activity by injection of MO-Ci-FoxA-a, expression was abolished for *Ci-Otx* (wt, 96%, *n*=47; MO-Ci-FoxA-a, 0%, *n*=29) (Fig. 5I) and *Ci-Ror-a* (wt, 90%, *n*=49; MO-Ci-FoxA-a, 0%, *n*=32) (Fig. 5N), suggesting that Ci-FoxA-a is generally required for the anterior ectodermal program.

This result is in keeping with two distinct scenarios for the establishment of the anterior identity in the *Ciona* ectoderm. The first one is an independent specification of the anterior and the posterior ectodermal territories, with *Ci-FoxA-a* promoting the anterior fate while other mechanisms specify the posterior ectoderm. The second one is the establishment of a posterior identity in the whole ectoderm in absence of anteriorizing signals, an identity repressed in the anterior region by the action of Ci-FoxA-a. To address this issue, we tested if Ci-FoxA-a participates to the inhibition of the posterior fate in the anterior ectoderm of the embryo.

When we overexpressed Ci-FoxA-a in all animal blastomeres by electroporating pSP1.72-pFOG::*Ci-FoxA-a*, we observed ectopic staining for anterior markers in b-line cells. *Ci-Otx* was expressed in the sensory vesicle precursors as in wild-type embryos, but also in lateral cells of the neural plate corresponding to the posterior neural cells (b9.33, 37, 38 cells, Fig. 5H). *Ci-FoxC* was expressed in anterodorsal epidermis and palp precursors, as well as in lateral cells of the embryo, adjacent to the posterior neural cells (b9.35, 36, 40, Fig. 5K). *Ci-Ror-a* expression was likewise extended throughout b-line cells (Fig. 5M). Thus, neural plate markers (*Ci-Otx* and *Ci-FoxC*) are ectopically expressed in the posterior neural lineage and the general anterior marker *Ci-Ror-a* is ectopically expressed more widely in the posterior ectoderm.

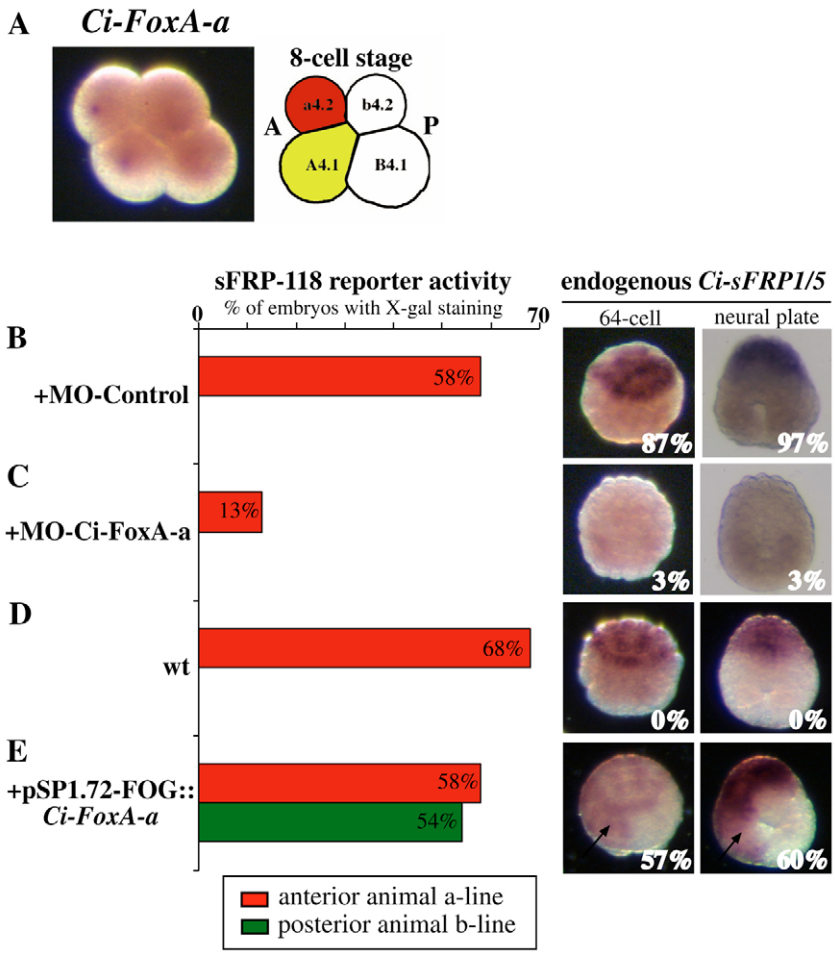

Fig. 4. *Ci-sFRP1/5* is a target of Ci-FoxA-a via the sFRP-118 element. (**A**) Weak expression of *Ci-FoxA-a* by whole-mount in situ hybridization at the eight-cell stage in the anterior animal (a4.2) and vegetal (A4.1) lineages. Lateral view, anterior towards the left, animal towards the top. The staining is detected in the nuclear region. (**B-E**) Effect of the down- or upregulation of *Ci-FoxA-a* on sFRP-118 activity at the 110-cell stage (histogram in centre is colour coded according to the lineage) and on endogenous *Ci-sFRP1/5* expression at the 64-cell and neurula stages (right). (B,C) Embryos microinjected with control or Ci-FoxA-a morpholinos. The percentage of embryos with a-line staining is indicated. (D) Control and (E) embryos electroporated with the indicated *Ci-FoxA-a* overexpression construct. The percentage of embryos with ectopic b-line staining is indicated. Black arrows indicate ectopic posterior ectodermal expression. Anterior is towards the top, animal views are shown at the 64-cell stage, vegetal views are shown at the neural plate stage.

We then looked at posterior ectodermal markers. At the 110-cell stage, expression of *Ci-Msxb* and *Ci-Delta2* is restricted to the b-line neural precursors. When we decreased Ci-FoxA-a activity by injection of MO-Ci-FoxA-a, we observed a strong ectopic activation of *Ci-Delta2* at the 64-cell stage in the anterior neural blastomeres (a7.9 and a7.10 cells) (wt, 0% of embryos with ectopic expression, *n*=14; MO-Ci-FoxA-a, 89%, *n*=9, Fig. 6B). Conversely, when we overexpressed *Ci-FoxA-a* throughout the animal hemisphere, we observed a strong reduction of the expression at the 110-cell stage of *Ci-Msxb* (wt, 95%, *n*=41; pSP1.72-pFOG::*Ci-FoxA-a*, 14%, *n*=60) (Fig. 6F) and *Ci-Delta2* (wt, 96%, *n*=152; pSP1.72-pFOG::*Ci-FoxA-a*, 23%, *n*=116) (Fig. 6D), suggesting that Ci-FoxA-a represses the activation of these genes.

Taken together, these results indicate that Ci-FoxA-a is an early global activator of the anterior genetic program in the ectoderm of *Ciona intestinalis* embryos. It actively promotes anterior identity and represses the expression of posterior ectodermal genes in anterior ectoderm. Moreover, in the absence of *Ci-FoxA-a* expression the anterior region of the ectoderm adopts a posterior fate. Thus, the most likely scenario is the establishment of an initial posterior identity throughout the ectoderm, antagonized in the a-line by the action of *Ci-FoxA-a*.

DISCUSSION

Our study uncovers the earliest event taking place in the ectoderm revealing that anteroposterior patterning occurs before inductions by endo-mesodermal cells. We dissected the cis-regulatory region of *Ci-sFRP1/5*, an ectodermal gene specifically expressed in the anterior region of *Ciona* embryos. We found that the anterior identity is autonomous to the a-line, and dominant over posterior fate. The *Ci-sFRP1/5* regulatory region is activated in vivo, via Fox-binding sites, by Ci-FoxA-a, the earliest zygotic anterior determinant. Although we have not carried out in vivo binding assays, *Ci-FoxA-a* is the only *Fox* gene in the *Ciona* genome expressed in the ectoderm in the a-line but not the b-line, strongly suggesting a direct regulatory interaction between Ci-FoxA-a and the *sFRP1/5* regulatory regions.

In this section, we discuss the emerging anterior cascade in ascidians and its evolutionary relevance.

Ci-FoxA-a is a conserved early zygotic determinant of anterior fate in the ascidian ectoderm

Ci-FoxA-a expression appears as the first step of differentiation in the *C. intestinalis* ectoderm (Fig. 7). Therefore, it provides a molecular mechanism for the acquisition of different anteroposterior competence to respond to neural induction revealed by previous studies (Rose, 1939; Reverberi and Minganti, 1947; Okado and Takahashi, 1990; Hudson and Lemaire, 2001). However, the action of *Ci-FoxA-a* is not restricted to the definition of the type of response to neural induction. Rather, it appears as a major regulator of anterior ectodermal identity. Its absence leads to the acquisition of posterior identity, while its ectopic expression in posterior territories is sufficient to give them an anterior character. This role as a major anterior ectodermal determinant is likely conserved in other ascidians as *Ci-FoxA-a* orthologues are also expressed in the

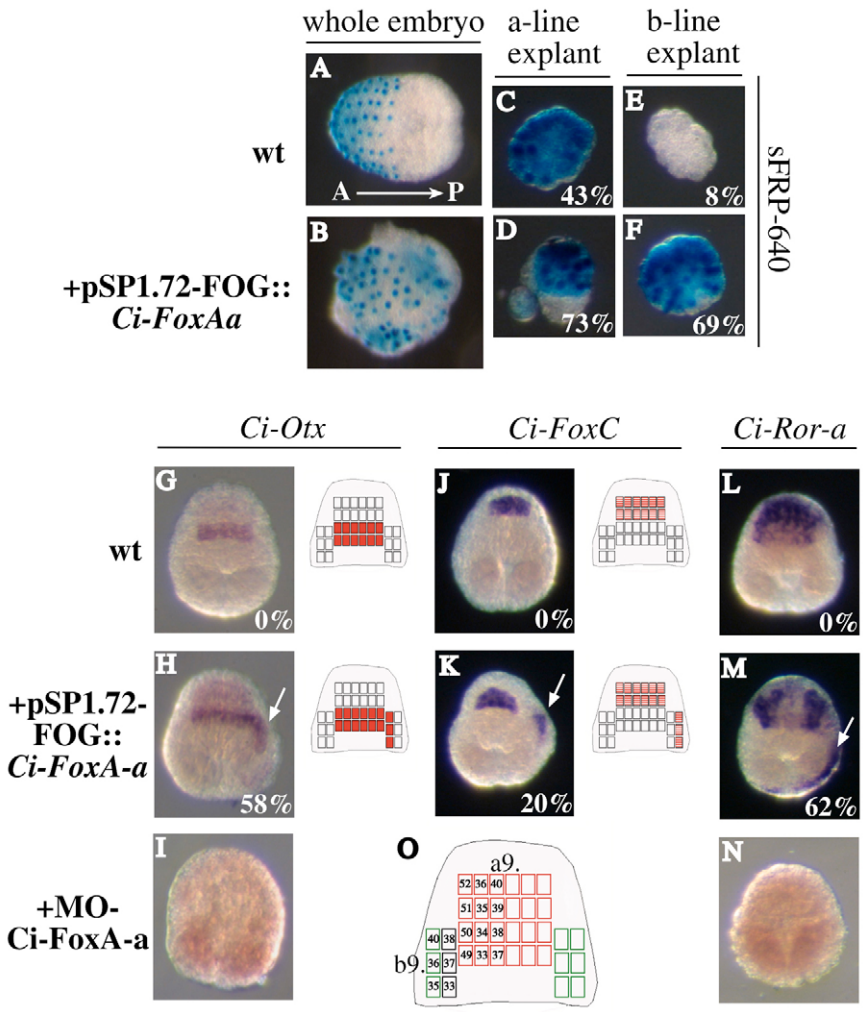

Fig. 5. Ci-Fox-A-a activates anterior ectodermal markers. (**A-F**) Effect of pan-animal *Ci-FoxA-a* overexpression on the neural plate-stage activity of the sFRP-118 enhancer, visualized by X-gal staining. (A,B) Whole embryos; (C,D) anterior ectodermal explants cut at the eight-cell stage and cultured in isolation; (E,F) posterior ectodermal explants. The percentage indicates the proportion of stained explants. Whole embryos are shown in animal views at the neural plate stage. (**G-O**) Effect of pan-animal overexpression or knock down of Ci-FoxA-a on *Ci-Otx* (G-I), *Ci-FoxC* (J,K) and *Ci-Ror-a* (L-N). All embryos are shown in vegetal view at the neural plate stage, anterior towards the top. The percentages of embryos with ectopic expression in b-line cells are indicated. (O) Schematic vegetal view of the neural plate showing the position of anterior (a9.) and posterior (b9.) ectodermal lineages at neural plate stage. Anterior is towards the top.

anterior, but not the posterior, animal blastomeres in *Ciona savignyi* from the eight-cell stage (Schimauchi et al., 2001) and in *Halocynthia roretzi* (a distantly related species) at the 16-cell stage (Shimauchi et al., 1997).

Although *Ci-FoxA-a* is both necessary and sufficient for a proper anterior ectodermal patterning, we cannot rule out the possibility that it may collaborate with other genes for the specification of the anterior region. Other genes have been indeed reported to be expressed in anterior lineages at the eight-cell stage in another ascidian *Halocynthia roretzi* (Miya and Nishida, 2003; Makabe et al., 2001).

A mechanism for the anterior activation of *Ci-FoxA-a*

Our findings suggest that, at the eight-cell stage, the animal territories have a posterior default identity, which is counteracted by the activity of *Ci-FoxA* in the a-line. This apparently contrasts with the current model for AP regionalization in the vegetal territories in which posterior determinants, found in the vegetal-posterior cortical region of the egg after fertilization, are thought to define the anteroposterior axis of endo-mesodermal fates by repressing a default anterior identity (for a review, see Nishida, 2005). As a result, the patterning of endo-mesodermal tissues is currently explained without the help of an anterior determinant. At first sight, this argues that independent mechanisms pattern the AP axis in the ectoderm and endomesoderm. Consistent with this proposal, *Ci-FoxA-a* expression is not sensitive to the knock-down of the major vegetal posterior determinant identified to date, Macho-1 (Yagi et al., 2004).

This apparent paradox may, however, principally stem from our lack of understanding of the maternal factors, which activate *Ci-FoxA-a* at the eight-cell stage. Two, non exclusive, scenarios are possible for restricting *Ci-FoxA-a* anterior expression. A maternal activator may be specifically active in the anterior region of the zygote. Such an activator is unlikely to be encoded by a maternal messenger localized in anterior territories as a large scale microarray analysis did not succeed in identifying such localized maternal mRNA (Yamada et al., 2005). Alternatively, the activation of *Ci-FoxA-a* could result from the combination of a ubiquitous activator, and a repressor, distinct from Ci-Macho1, present in the posterior region. In the latter case, the activation of *Ci-FoxA-a* would be a 'default' property of the embryo, repressed in the posterior blastomeres, a situation similar to that observed in the vegetal hemisphere. The identification of an enhancer recapitulating the activation of *Ci-FoxA-a* in anterior territories during the cleavage stages (Di Gregorio et al., 2001) (C.L., unpublished) opens the way to a better understanding of this important issue.

An overall conservation of ectodermal patterning in chordates

The results presented here, combined to previous observations, give an overall view of ectodermal patterning in ascidians, which appears to be a multistep process. First, anterior and posterior identities are

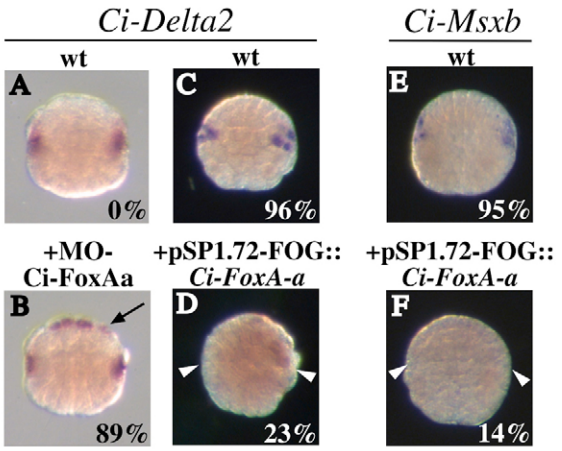

Fig. 6. Ci-FoxA-a suppresses posterior fates. Effect of morpholino knock-down (**A**,**B**) or pan-animal *Ci-FoxA-a* overexpression (**C-F**) on the expression of *Ci-Delta2* (A,B, 64-cell stage; C,D, 110-cell stage) and *Ci-Msxb* (E,F, 110-cell stage). Expression is detected in the b7.9, 10 (*Ci-Delta2*) and b8.17, 18, 19, 20 (*Ci-Delta2, Ci-Msxb*) b-line neural precursors in wild-type embryos (A,C,E). The black arrow in B indicates ectopic expression in the anterior neural precursors (a7.9, 10) at the 64-cell stage. Percentages indicate the proportion of embryos with staining in the anterior (A,B) or posterior (C-F) ectoderm. All embryos are shown in vegetal view, anterior towards the top.

established autonomously from the eight-cell stage as a result of *Ci-FoxA-a* activation. Secondary signals emanating from the vegetal hemisphere then act to refine this initial ectodermal patterning (Wada et al., 1999; Darras and Nishida, 2001; Hudson et al., 2003; Hudson and Yasuo, 2005). At the 32-cell stage, Ci-FGF9/16/20 induces both anterior (Bertrand et al., 2003) and posterior (Bertrand et al., 2003; Hudson and Yasuo, 2005) neural tissue within the *Ciona* ectoderm. Interestingly, while Ci-FoxA-a specifies the anterior or posterior identity of ectodermal cells in response to this induction, it does not affect which blastomeres are induced by FGF. Upon *Ci-FoxA-a* overexpression, a new anterior neural plate thus forms in the presumptive posterior neural territory, with sensory vesicle markers expressed closest to the vegetal-inducing cells, palp marker expressed in a more lateral region and anterior epidermis marker expressed in the posterior non-induced cells. This result is in keeping with the proposal that the selection of the induced animal cells relies mainly on the extent of the contact between inducing and competent cells (Tassy et al., 2006). Subsequent, yet uncharacterized, vegetal signals are then required to pattern the neural plate along the AP axis at the beginning of gastrulation, discriminating the anterior palp identity from the more posterior rostral CNS (Nishida and Satoh, 1989).

A conceptually similar cascade is at work in vertebrates. In *Xenopus*, the ectoderm autonomously acquires an early pregastrula patterning prior to the action of vegetal signals. This early pattern specifies the type of tissues induced by these signals (Sokol and Melton, 1991; Otte and Moon, 1992; Baker et al., 1999; Kodjabachian and Lemaire, 2001) and is marked by the autonomous ectodermal activation of specific dorsal markers (Kodjabachian and Lemaire, 2001; Kuroda et al., 2004). Finally, explants experiments carried out in zebrafish embryos identified a pre-existing anteroposterior patterning in the ectodermal region of the zebrafish embryo before gastrulation (Koshida et al., 1998). Following this initial phase, vegetal signals induce neural tissue, and pattern it along the anteroposterior axis during gastrulation. Two major models for neurectodermal patterning during gastrulation have been put forward. The 'two-inducer' model postulates that different signals induce the ectoderm to form anterior and posterior neural tissues (Niehrs et al., 2001). The 'two-step' model postulates an initial induction of anterior neural fate throughout the neural plate, followed by the posteriorization of part of this tissue by signals emitted by the underlying mesendodermal organizer, including FGF, Wnt and Nodal inhibitors, and retinoic acid (reviewed in Stern, 2001).

It is currently unclear how deep the similarity between ascidians and vertebrates extends. Only part of the ascidian neural tissue is induced (reviewed by Lemaire et al., 2002), but a single inducer, FGF9/16/20, is at work, differentially interpreted by anterior and posterior cells. This superficially appears different from the 'two steps' model as the anterior and posterior identity of the induced tissue are specified at the time of induction, prior to gastrulation. It should, however, be stressed that the precise timing of action of the two vertebrate signals has not been determined. Furthermore, our finding that *Ci-sFRP1/5* and *Ci-Ror-a*, two antagonists of the canonical Wnt pathway (Dennis at al., 1999; Forrester et al., 2004) are specifically expressed in anterior territories, under control of *Ci-FoxA-a* and after the induction process, is in keeping with a two-step model. However, Ci-sFRP1/5 morpholino knock-down experiments have no apparent effect (C.L., unpublished), indicating that in a wild-type situation this secreted antagonist of Wnt ligands may play a less crucial role in the *Ciona* ectoderm than it does in zebrafish (Houart et al., 2002). A rigorous assessment of the role of Wnt signalling and other extracellular signals in ascidian ectodermal patterning, coupled to the deciphering of the regulatory networks at work in both ascidians and vertebrates, will be required to estimate the level of conservation of this event in chordates. However, at this stage, we can already point to the overall conservation of a logic combining an early autonomous subdivision of the ectoderm, which affects its competence to respond to a subsequent cascade of vegetal signals.

Conservation and divergences in the function of chordate *FoxA* genes in anterior specification

We found that *Ci-FoxA-a* is a crucial determinant of the ascidian anterior identity. This appears to be a shared property of ascidians and vertebrates. In vertebrates, *FoxA* orthologues are also expressed before gastrulation (Ruiz i Altaba and Jessell, 1992; Sasaki and Hogan, 1993; Perea-Gomez et al., 1999; Strahle et al., 1993) and important for the formation of anterior ectodermal territories (Suri et al., 2004; Perea-Gomez et al., 1999). Furthermore, ascidian and vertebrate embryos share conserved molecular events downstream of FoxA genes. In ascidians, *Ci-FoxA-a* is necessary for the activation of the *Ci-Otx* gene, that has conserved functions among bilaterians to specify the anterior ectoderm (Hirth and Reichert, 1999). Likewise, in mouse and *Xenopus* embryos, FoxA2/HNF3-β are involved in the regulation of Otx2 (Perea-Gomez et al., 2001; Suri et al., 2004). Thus, among chordates, Otx is a conserved downstream target of FoxA necessary for the formation of anterior ectoderm.

Likewise, *Ci-FoxA-a* could play a role in the protection of the ascidian anterior ectoderm from posteriorizing signals known to caudalize the neurectoderm (Hudson et al., 2003), as proposed in mouse (Perea-Gomez et al., 1999) and in *Xenopus* in the 'two-step' model. This protection could occur through the activation of Ci-sFRP1/5 and Ci-Ror-a, two antagonists of the canonical Wnt pathway (Dennis et al., 1999; Forrester et al., 2004).

These observations suggest that *Ci-FoxA-a* and vertebrates *FoxA2* may trigger similar molecular pathways to pattern the ectoderm along the anteroposterior axis, although the relative individual

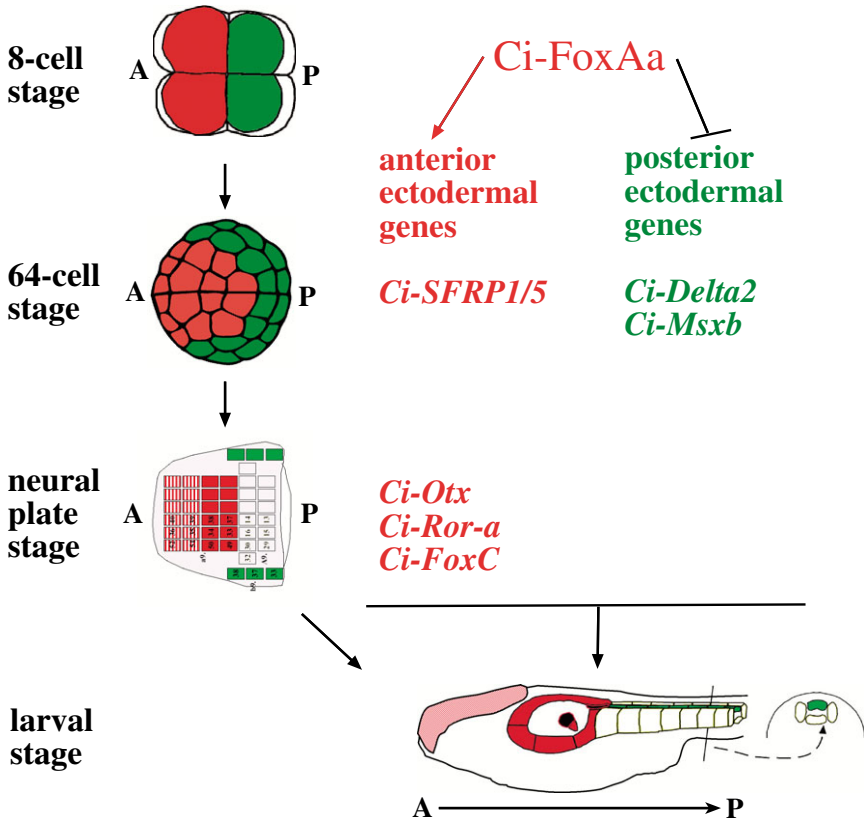

Fig. 7. A model of anterior fate determination in the ascidian ectoderm at the eight-cell, 64-cell and neural plate stages. The anterior ectodermal lineage (red) gives rise to the trunk epidermis (not coloured), the palps (striped motif) and the sensory vesicle (red), while the posterior ectodermal lineage (green) gives rise to the tail epidermis (not coloured) and the dorsal row of the nerve cord (green) at the larval stage. At the eight-cell stage, *Ci-FoxA-a* expression is activated in the anterior animal cells. Presence of this factor leads to the activation of anterior ectodermal genes at the 64-cell and neural plate stages, and to the repression of posterior ectodermal genes. Vegetal expression of *Ci-FoxA-a* is not represented in this model.

importance of these pathways may vary. It will be interesting to identify and compare additional targets of FoxA genes in ascidians and vertebrates.

The above similarities are, however, accompanied by major differences in the mode of action, but also in the site of expression and action of FoxA genes in ascidians and vertebrates. First, in contrast to what we see in ascidians, *Xenopus FoxA2* overexpression is not sufficient to induce anterior fate in the ectoderm (Ruiz i Altaba and Jessell, 1992). Second, although our experiments point to an autonomous role of FoxA-a in the ascidian ectoderm, vertebrate FoxA2 transcripts have only been detected in non-ectodermal embryonic territories, such as dorsal mesendodermal cells in *Xenopus* and *Danio*, and extra-embryonic AVE cells in mouse (Ruiz i Altaba and Jessell, 1992; Strahle et al., 1993; Sasaki and Hogan, 1993). As this was initially the case for several organizer genes that have since been shown to be also expressed and active in the presumptive ectoderm (Kodjabachian and Lemaire, 2001; Kuroda et al., 2004), it will be important to revisit the expression pattern of FoxA genes at early stages of vertebrate development. Yet, the fact that FoxA2 in mice is required only in the AVE and not in the epiblast suggests a different mode of action in vertebrates and ascidians (Perea-Gomez et al., 1999).

A possible scenario for the acquisition of different roles by FoxA while retaining a general function in anteroposterior patterning, may be an ancestral expression and function in both animal and vegetal anterior territories, followed in the vertebrate lineage by a gradual amplification of its role in the vegetal territories at the expense of its animal role. Dorsoanterior vegetal territories in amniotes correspond to the organizer, a structure that has been proposed to constitute a vertebrate innovation whose function is to maintain during gastrulation an initial coarse pattern (Kourakis and Smith, 2005). In support to this model, an organizer-independent activation of Hox genes (markers of posterior tissues) is seen in *Xenopus* in the mesoderm from the beginning of gastrulation (Wacker et al., 2004). Wacker and colleagues proposed that, during gastrulation movements, the organizer activity stabilizes and synchronizes the anteroposterior boundaries of Hox expression between adjacent ectoderm and mesoderm. We can speculate that, in vertebrates, the coordination of two different tissues streaming past one another requires constant mesodermal signals to adjust and maintain the boundaries of an already patterned ectoderm. By contrast, in ascidians the restricted gastrulation movements, which do not involve change in cell neighbourhood relationships (Munro et al., 2006), may not require this control mechanism. The acquisition of organizing activity by the vertebrate dorsoanterior mesendoderm may be linked to a shift of the major function of FoxA in ectodermal patterning from the animal to the vegetal territories. Alternatively, *FoxA* may have been specifically recruited in the tunicate ectoderm to form autonomously anterior structures.

Note added in proof

The importance of Ci-Foxa in anterior specification has been independently reported (Imai et al., 2006).

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