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A combinatorial code of maternal GATA, Ets and β-catenin-TCF transcription factors specifies and patterns the early ascidian ectoderm

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Our understanding of the maternal factors that initiate early chordate development, and of their direct zygotic targets, is still fragmentary. A molecular cascade is emerging for the mesendoderm, but less is known about the ectoderm, giving rise to epidermis and nervous tissue. Our cis-regulatory analysis surprisingly places the maternal transcription factor Ci-GATAa (GATA4/5/6) at the top of the ectodermal regulatory network in ascidians. Initially distributed throughout the embryo, Ci-GATAa activity is progressively repressed in vegetal territories by accumulating maternal β-catenin. Once restricted to the animal hemisphere, Ci-GATAa directly activates two types of zygotic ectodermal genes. First, *Ci-fog* is activated from the 8-cell stage throughout the ectoderm, then *Ci-otx* is turned on from the 32-cell stage in neural precursors only. Whereas the enhancers of both genes contain critical and interchangeable GATA sites, their distinct patterns of activation stem from the additional presence of two Ets sites in the *Ci-otx* enhancer. Initially characterized as activating elements in the neural lineages, these Ets sites additionally act as repressors in non-neural lineages, and restrict GATA-mediated activation of *Ci-otx*. We thus identify a precise combinatorial code of maternal factors responsible for zygotic onset of a chordate ectodermal genetic program.

KEY WORDS: Ectoderm, *Ciona intestinalis*, Ascidian, GATA, Ets, β-catenin

INTRODUCTION

With the possible exceptions of mammals and polyembryonic wasps, most animal embryos studied are pre-patterned by the action of localized maternal determinants (Goldstein and Freeman, 1997). Following fertilization, inheritance of these factors initiates distinct developmental programs in embryonic cells. The subsequent transcriptional activation of combinations of zygotic genes relay, amplify and refine the maternal cues. This suite of events has been best described in *Drosophila* in which precisely localized maternal determinants shape the embryonic axes and determine the germline. The identification of axial maternal determinants and of how they combinatorially initiate tissue-specific zygotic gene expression has had a profound conceptual impact on developmental biology (Nüsslein-Volhard, 1995).

Several key vertebrate maternal axial determinants and a few of their direct zygotic targets have also been identified in *Xenopus* and fish (reviewed by Heasman, 2006a; Schier and Talbot, 2005), which has led to the initiation of the reconstruction of gene regulatory networks for the mesendoderm (Koide et al., 2005; Loose and Patient, 2004). These networks are at present very incomplete. First, some maternal determinants are most likely missing. Second, deciphering the precise cis-regulatory logic that enables combinations of maternal factors to activate their targets in distinct territories has proved difficult owing largely to the size and complexity of the vertebrate genomes. As an additional difficulty, the early genetic program can significantly differ between amphibians and fishes. For example, the zebrafish orthologue of the major *Xenopus* maternal determinant of vegetal territories, VegT, is not expressed in early fish embryos (Ruvinsky et al., 1998).

In this context, studies on ascidians, members of the chordate phylum, have recently emerged as particularly informative. First, ascidian embryos have an early gastrula fate map and tadpole structure remarkably similar to lower vertebrates (Kowalevsky, 1866; Kowalevsky, 1871; Kourakis and Smith, 2005), yet use a particular mode of development based on a fixed cell lineage (Conklin, 1905; Nishida and Satoh, 1985; Nishida, 1987a), extensive use of maternal determinants (Chabry 1887; Nishida, 1987b; Nishida and Sawada, 2001) and very local inductions (reviewed by Nishida, 2005; Tassy et al., 2006). Comparison of the ascidian and vertebrate strategies should therefore allow discrimination between crucial events expected to be shared, and adaptations to specific modes of developments. Second, studies on the ascidian *Ciona intestinalis* have progressed at an impressive pace since the release of the draft sequence of its genome (Dehal et al., 2002), culminating recently in the first whole embryo draft zygotic gene regulatory network in a metazoan (Imai et al., 2006). This progress was made possible by a low level of genetic redundancy (Dehal et al., 2002), the availability of efficient gene gain- and loss-of-function strategies (Corbo et al., 1997; Satou et al., 2001), and the ease of identifying the cis-regulatory modules that drive zygotic gene expression. These advances open the way to an understanding of the developmental genetic program at a similar resolution level to that in flies (e.g. Bertrand et al., 2003).

In ascidians, the third cleavage separates the ectoderm, which occupies the animal half of the embryo, from the mesendoderm, which occupies its vegetal half (reviewed by Nishida, 1997a). Imai and colleagues (Imai et al., 2000) showed that maternal β-catenin progressively accumulates in the vegetal nuclei of cleaving *Ciona* embryos where it acts as a key determinant of the vegetal territories.

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Their work also suggested candidate direct zygotic targets of this determinant (Imai, 2003). As in vertebrates, little is known about animal specification. Fusion of animal egg fragments to isolated vegetal blastomeres of eight-cell embryos drove epidermal development in the vegetal host cell, suggesting the existence of localized epidermal determinants (reviewed by Nishida, 1997b). These experiments also showed that rearrangements following fertilization redistribute and concentrate the epidermis determinants to the animal region. Extensive microarray analyses have, however, failed to detect maternal mRNAs enriched in early animal blastomeres, suggesting that the animal determinants are not encoded by localized mRNAs (Yamada et al., 2005).

In this study, we sought to identify animal determinants by following an alternate strategy, i.e. the analysis of the cis-regulatory logic of one of the earliest animal-specific zygotic genes, *Ci-fog*. We show that maternal Ci-GATAa (a vertebrate GATA4, 5 and 6 orthologue) is necessary and sufficient for animal gene expression from the 8-cell stage and for the subsequent differentiation of the two major ectodermal tissues, epidermis and anterior neural plate. We further show that the restriction of the activity to animal territories of this ubiquitous maternal protein is due to an antagonism by the β-catenin-TCF complex in vegetal territories. Finally, we determined the transcriptional logic that allows Ci-GATAa to sequentially activate pan-animal genes at the 8- to 16-cell stage and neural-specific genes in response to neural induction at the 32-cell stage.

MATERIALS AND METHODS

Cis-regulatory constructs

The pSP72-1.27 NLS-*lacZ* expression vector (Corbo et al., 1997) or the pSP1.72-RfB::*NLS-lacZ* Gateway destination vector (Lamy et al., 2006) (Invitrogen) were used for analysis of regulatory sequences. Computational analysis was performed using Vista, ClustalW and Transfac8.2 programs as described previously (Lamy et al., 2006).

fog (friend of GATA, ci0100149797) regulatory sequences covering 1981 bp upstream of the ATG (pfog-2000) were isolated by PCR from sperm genomic DNA with primers 5′P-pFOG aaaactgcagGCAACTATTGTAACACCACACG and 3′B-pFOG cgcggatccTATGTGTGTTATTTTTGTATAGACC (flanking *Pst*I or *Bam*HI sites, underlined) using AccuPrime Taq DNA Polymerase (Invitrogen). The pfog-566::*NLS-lacZ* construct contains sequences downstream of an endogenous *Ssp*I site at position –566 and includes the conserved block, as shown in Fig. 1. Construct pfog-300-bpbra*::NLS-lacZ* contains evolutionarily conserved FOG 5′ sequences between nucleotides –566 (*Ssp*I site) and –213 (*Hin*dIII site) cloned with *Sal*I-*Bam*HI upstream of the basal promoter of *Ci-Brachyury* (bpbra, –68/+26 fragment) (Yagi et al., 2004). Deletion constructs within this conserved region were obtained by Gateway technology (Invitrogen) using attB1 and attB2 flanked PCR primers. FOG-specific sequences at the 5′ end of constructs were: pfog-530: AGAAAACAACCTTGTTATTACTC; pfog-354: CAAGCAGTAACGAGAAAACAAG; pfog-314: CTGGAGAAGACCAAGATAAAG; pfog-m314: CTGGAGAAGACCAACATAAAGTATTTC (mutations in GATA binding sites, underlined), pfog-290: CTCAAAATTCAGGAAACGGTC, whereas the 3′ end was generated with a Gateway version of 3′B-pFOG. The dINT construct (positions –314 to –85) is as pfog-314 at the 5′ end and deletes intron 1 and downstream

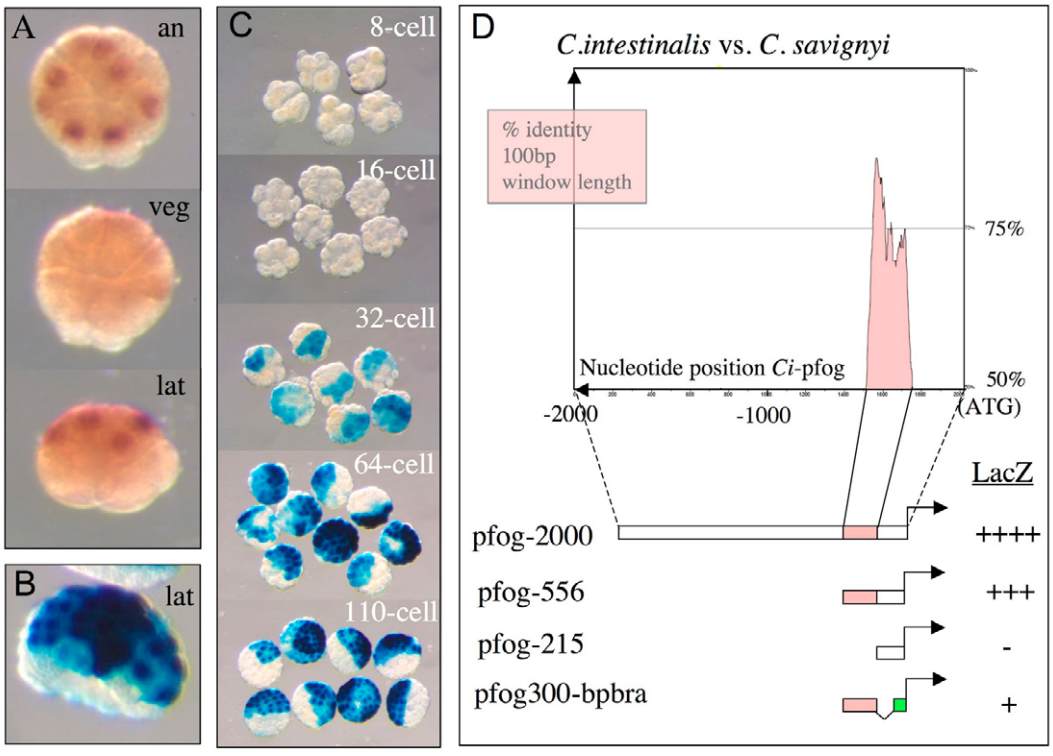

Fig. 1. Isolation of a cis-regulatory module driving early animal expression. (**A**) *Ci-fog* expression at the 16-cell stage. Anterior is to the top for animal (an) and vegetal (veg) views, to the left for the lateral (lat) view. (**B**) *lacZ* reporter activity (X-Gal) at the 110-cell stage following electroporation of the pfog-2000::*NLS-lacZ* construct. Lateral view, as in A. (**C**) Timing of *lacZ* reporter activation in animal blastomeres after electroporation of the pfog-2000 construct. Animal views of representative embryos. (**D**) Phylogenetic footprinting between *C. intestinalis* and *C. savignyi* sequences covering 2 kb upstream of the ATG of *Ci-fog*. The conserved region is shown in pink, the heterologous *Ci-bra* basal promoter in green. The level of *lacZ* reporter activity is indicated for each electroporated construct ($n \geq 80$), from very strong (++++) to absent (–); with more than half of embryos counted having all animal cells stained (++++), 50-90% of animal cells stained (+++), 25-50% (++) or 1-25% (+) of animal cells stained.

sequences at the 3′ end by using the pfog-specific reverse primer sequence: 5′ TGTGACCACCTGCCTTGTC 3′. 2GdINT is a modification of dINT where the two endogenous *Ci-fog* GATA sites were replaced by GATA sites from the *otx* neural a-element and contains the sequence CAATATCTAAGATAGGAAAATTCAGGAAACGGTCCAAG at its 5′ end (GATA sites underlined). The G-12 construct, under the control of bpbra, contained the following GATA site multimer *Xho*I and *Hin*dIII (inspired from the *otx* a-element) (Bertrand et al., 2003): CGTTATCTCTCAATATCTAAGATAGGACGTTATCTCTCAATATCTAAGATAGGACGTTATCTCTCAATATCTAAGATAGGACGTTATCTCTCAATATCTAAGATAGGAG.

The 12X TCF sequence was isolated from the dTF12 vector (N. Perrimon Lab) (DasGupta et al., 2005) using *Sca*I and *Sma*I sites and inserted into a *Hin*dIII-blunted site upstream pfog-215::*NLS-lacZ* (basal promoter with an activity slightly weaker than pfog-290).

To generate constructs with heterologous enhancers upstream of the *fog* basal promoter, the following enhancer sequences were inserted into *Xho*I and *Hin*dIII sites, upstream of pfog-290::*lacZ*: the reduced *otx* neural a-element (Bertrand et al., 2003), the Ets site mutated a-element CGTTATCTCTAACaGAAGTTTTCGAAAAaGAAATTGTTCAATATCTAAGATAGGA (GATA and Ets sites underlined, mutations in lower case letters), or the *Ci-bra* enhancer (eBra) covering position –470 to –62, using forward and reverse primers 5′CCGCTCGAGAACACACCCAACGTACAATAAAAC3′ and 5′CCCAAGCTTCTTCTTTTTGAAATTTTATGTTTG3′.

RACE PCR was performed with GeneRacer (version D; Invitrogen) according to the manufacturer's instructions from total RNA of 64-cell *Ciona intestinalis* embryos using nested reverse primers dINT and –143rev 5′attB2AAACTGCCTTCTTCTCTTGTC3′.

Embryo handling, electroporation and injection

Animal handling, electroporations and injections were as described previously (Bertrand et al., 2003). Embryos were treated with bFGF (100 ng/ml; Sigma), cytochalasin B (4 μg/ml; Sigma) or U0126 (5 μM; Calbiochem) as previously described (Hudson et al., 2003; Hudson and Lemaire, 2001), or 50-300 mM LiCl (Yoshida et al., 1998). For electroporations, volumes were scaled to half and for microinjections, 20-50 ng/μl DNA plasmid, 0.5 mM morpholino oligonucleotides (MO; Gene tools, LLC) and 0.3-0.5 μg/μl synthetic mRNA were used.

GATAa mRNA, *EnR-GATAa* mRNA, GATAa-MO and Ci-β-catenin-MO were as described previously (Bertrand et al., 2003; Satou et al., 2001). The CiTCF-MO (against ci0100131330) was TCATCCGAGTTTAACTGAGGCATTC. The GATAb-MO (against ci0100136326) was GTTGCTCGCTACTTGTTGGCATCAT (ATGs underlined). The ATG for *GATAb* was determined according to the conservation to vertebrate GATA2 and GATA3 protein and *Ciona savignyi* cDNA sequences and upon verification by RT-PCR (details available upon request).

In situ hybridization and reporter assays

In situ hybridizations and β-galactosidase (β-gal) reporter assays (X-Gal stain) were performed as described previously (Bertrand et al., 2003) with digoxigenin-labeled probes generated from the following clones: *lacZ* (pSP1.72-lacZ) (Corbo et al., 1997), *Ci-fog* (GR1 cieg033m14), *Ci-GATAa* (Bertrand et al., 2003), *Ci-epiB* (GenBank AL666073) and *Ci-epi1* (Hudson et al., 2003). pfoxD is p(–3.6)Cs-FoxD/lacZ (Imai et al., 2002).

RESULTS

Isolation of an early pan-animal cis-regulatory region

To identify maternal factors that define the animal hemisphere, we searched for a gene with an early animal-specific expression pattern and studied its regulatory sequences. Analysis of the expression pattern of potential early transcriptional regulators revealed that the *Ciona friend of GATA* gene (*Ci-fog;* ci0100149797) perfectly matched this criterion. *Ci-fog* is specifically expressed in the animal region from the 16-cell stage to the beginning of gastrulation (Fig. 1A and data not shown). To identify the cis-regulatory regions responsible for this early expression pattern, we PCR amplified a 2 kb genomic fragment, pfog-2000, extending 5′ from the ATG of *Ci-fog* and placed it upstream of a nuclear β-galactosidase reporter gene (*NLS-lacZ*). This expression construct, pfog-2000*::NLS-lacZ*, was electroporated into *Ciona intestinalis* fertilized eggs and the reporter activity was assayed by X-Gal staining during cleavage stages. pfog-2000 contained all necessary information to drive animal-specific expression at the 110-cell stage (Fig. 1B). The earliest reporter expression was detected by X-Gal staining at the 32-cell stage (Fig. 1C). Taking into account that NLS-β-gal protein synthesis takes one cell cycle (Bertrand et al., 2003) (see also *lacZ* mRNA detection in Fig. 4A), this suggests that animal transcription is already active at the 16-cell stage, thus recapitulating endogenous *Ci-fog* (Fig. 1A).

Phylogenetic footprinting of pfog-2000 between *Ciona intestinalis* and *Ciona savignyi* pointed to the presence of a 294 bp highly conserved fragment (Fig. 1D). A 566 bp fragment including this region and extending up to the ATG of *Ci-fog*, pfog-566, had similar activity to pfog-2000, whereas deletion of the conserved region suppressed this activity (pfog-215). A construct, pfog300-bpbra::*NLS-lacZ*, in which this conserved fragment was placed upstream of the unrelated *Ci-bra* basal promoter (Erives et al., 1998) drove animal β-gal expression. The conserved fragment, therefore, contains all the information required to drive early animal expression.

Maternal GATAa is necessary and sufficient for animally restricted gene expression

Through 5′ deletion analysis of the 566 bp fragment, we narrowed down the animal cis-regulatory region to a minimal fragment. This region, pfog-314 (Fig. 2), contains two consensus binding sites for GATA transcription factors conserved between the genomes of *Ciona intestinalis* and *Ciona savignyi*. Deletion or point mutation of these sites abolished all activity (Fig. 2A). Thus, GATA binding sites are critically involved in animal gene expression. Injection of mRNA for a dominant negative form of GATA (fusion of the GATAa-DNA binding domain to the engrailed-repressor domain) (Bertrand et al., 2003) blocked endogenous *Ci-fog* expression (92% *Ci-fog*-negative embryos, *n*=12), confirming that GATA factors are required for its activation.

We next tested whether GATA-binding sites, placed in front of a basal promoter, are sufficient to drive early pan-animal expression. We previously reported that a synthetic construct carrying six GATA-binding sites in front of the *Ci-bra* basal promoter drove FGF-dependent expression in animal neural precursors only, starting from the 32-cell stage (Bertrand et al., 2003). To analyze whether an earlier, weaker, GATA activity existed, we constructed a more efficient sensor, G12-bpbra::*NLS-lacZ* (or G12 in short), in which 12 GATA-binding sites were placed in front of the *Ci-bra* basal promoter. This construct drove an early animal-wide expression, which strongly resembled the pfog-314::*NLS-lacZ* expression. By the late 32-cell stage, two levels of expression could be observed: a strong expression in the animal neural precursors (a6.5 and b6.5) as well as a weaker pan-animal activity (other a and b cells), which appeared stronger in posterior animal lineages (b cells) (Fig. 2D). This pan-animal activity was detected from the 16-cell stage onwards (Fig. 4A), prior to the activation of FGF signaling in the embryo, and suggesting that it was FGF signaling independent. Consistently, in embryos grown in the MEK inhibitor U0126, which blocks the FGF signaling cascade, G12 activity was unchanged in uninduced animal blastomeres (a6.6-6.8 and b6.6-6.8, yellow and light purple, respectively, in Fig. 2D), and reduced in FGF-induced neural precursors (a6.5 and b6.5, red and dark purple) to the level

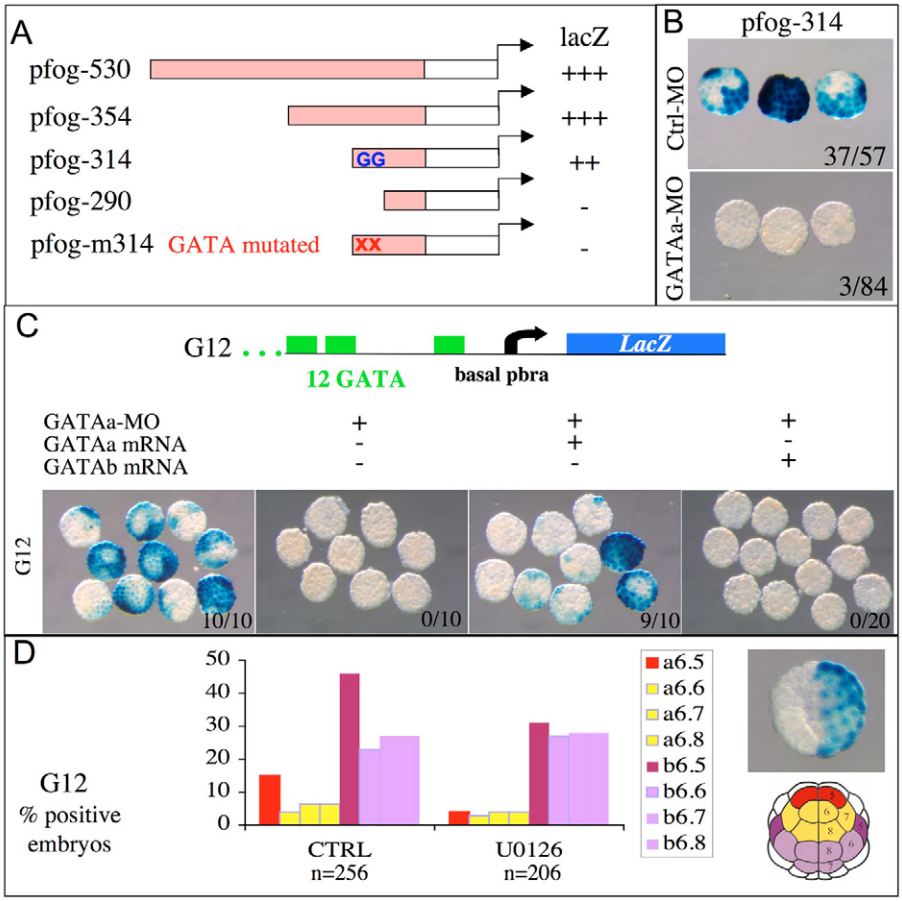

Fig. 2. GATAa is necessary and sufficient for FGF-independent animal-wide activity. (**A**) Deletion constructs of the conserved pfog region identify two GATA sites required for *lacZ* reporter expression. The level of *lacZ* reporter activity in animal blastomeres at the 110-cell stage is quantified as in Fig. 1; $n \geqslant 80$ for each construct. The pfog-m314 construct has a point mutation in each of the two GATA binding site (red crosses). (**B**) Animal view of 110-cell stage embryos co-injected with pfog-314 and either control-MO or GATAa-MO, stained with X-Gal. Numbers indicate the proportion of positive embryos. (**C**) *lacZ* reporter activity at the 110-cell stage in embryos co-injected with G12 and either water, GATAa-MO, GATAa-MO with *GATAa* mRNA or GATAa-MO with *GATAb* mRNA. Numbers indicate β-gal-positive embryos. (**D**) *lacZ* reporter activity at the 110-cell stage in embryos electroporated with G12 with or without U0126 treatment. The quantification of expression within the animal region is sorted by cell lineage derivatives of the 32-cell stage (bottom right corner). A representative untreated embryo is shown (110-cell stage, upper right corner, animal view, anterior is up).

found in the uninduced blastomeres (Fig. 2D, compare left and right panels). We conclude that, in addition to the previously identified FGF-dependent GATA activity in neural lineages, an earlier FGF-independent GATA activity exists throughout the animal hemisphere.

The maternal *Ciona* GATA factor, Ci-GATAa, was previously shown to mediate gene expression in neural progenitors in response to FGF (Bertrand et al., 2003). To test whether GATAa is also involved in the earlier animal-wide expression of *Ci-fog*, we blocked translation of GATAa with a morpholino (MO). Co-injection of GATAa-MO and the minimal pfog-314::*NLS-lacZ* construct, or G12-bpbra::*NLS-lacZ*, led to a dramatic reduction in *lacZ* expression (Fig. 2B,C). By contrast, injection of morpholino against the only other, more weakly maternally expressed, *Ciona* GATA factor, GATAb, had no effect on G12-bpbra::*NLS-lacZ* activity (75% of GATAb-MO injected embryos, n=28, were X-Gal-positive at the 110-cell stage compared to 61% in control MO injections, n=44; data not shown). To further test the relative contributions of GATAa or GATAb in the activation of G12, we blocked G12 with GATAa-MO and attempted to rescue G12 activity by injecting mRNA encoding either GATAa or GATAb. G12 activity could be rescued by *GATAa* (mutated for its MO binding site) (Bertrand et al., 2003) but not *GATAb* mRNA (Fig. 2C) supporting the idea that GATAa but not GATAb is the essential GATA factor involved in this early process.

Taken together, these results indicate that GATAa is a maternal factor that is necessary and sufficient to drive animal gene expression before, and independently of, neural induction. GATAa has thus two distinct roles in the embryo, an early FGF-independent role throughout the entire animal region, including *Ci-fog* activation, and a later, FGF-dependent function in the neural precursors, leading to *Ci-otx* expression.

GATAa is required for overall ectoderm differentiation

We next tested whether GATAa is required for the specification of the whole ectoderm. As GATAa activity is known to be required for neural tissue formation in the animal hemisphere (Bertrand et al., 2003), we focused on the role of GATAa in the formation of the other major ectodermal derivative, the epidermis. We injected a repressor version of *GATAa* (*EnR-GATAa*) into oocytes and analyzed the resulting embryos for epidermal marker expression at neurula stages. To better identify neural and epidermal precursors in these embryos, we arrested cleavage at the 64-cell stage using cytochalasin and tested for *epiB* or *epi1* epidermal marker expression. Uninjected control embryos showed strong *epiB* and *epi1* expression in epidermal precursors at the equivalent of the neurula stage (Fig. 3A). By contrast, *EnR-GATAa*-injected embryos had severely reduced expression of both epidermal markers (Fig. 3A). A similar result was obtained in normally cleaving, *EnR-GATAa*-injected neurula (not shown, 0/23 embryos expressed *epiB*, and 15/25 embryos weakly expressed *epi1*). At the tailbud stage, the *epi1* residual expression was not maintained in normally cleaving, *EnR-GATAa*-injected embryos (Fig. 3A), indicating that GATA function is strictly required for epidermis formation.

To assess whether GATAa and GATAb can both contribute to epidermis formation we inactivated GATA function with either GATAa-MO, GATAb-MO or a combination of both (Fig. 3B).

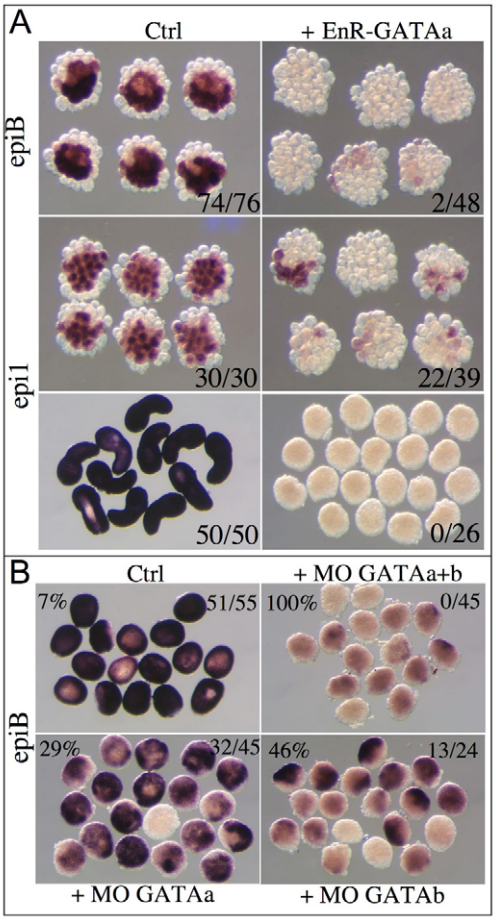

Fig. 3. GATAa specifies epidermis. (**A**) Expression of the epidermis markers epiB and epi1 in control and *EnR-GATAa* mRNA-injected embryos. Embryos shown in the top and middle panels were cleavage-arrested at the 64-cell stage with cytochalasin and stained at the equivalent of the neurula stage for the presence of *epi1* or *epiB* transcripts (animal view, anterior to the top). Embryos shown in the bottom panels were left to cleave normally and stained at the early tailbud stage for *epi1* transcripts. (**B**) Expression of *epiB* at late gastrula stage in control and GATAa-MO, GATAb-MO or double-injected embryos. Numbers indicate the proportion of positive embryos. Percentages refer to embryos with strongly reduced expression.

Individual MO injection reduced *epiB* expression, with a strong reduction in 29% of the injected embryos for GATAa-MO and 46% for GATAb-MO. Co-injection of both GATA MOs synergistically blocked the epidermal marker in 100% of embryos. Thus, GATAa and GATAb are both required for epidermal differentiation in the animal region of ascidians.

GATAa is blocked in the vegetal region by β-catenin-TCF

Maternal *GATAa* mRNA is ubiquitous and is ubiquitously translated at the 16-cell stage (Bertrand et al., 2003). The animal restriction of GATAa transcriptional activity at this stage is thus likely to be due to post-translational regulation and may involve either a vegetal repressor, or an animal co-activator. To understand when this animal restriction occurs, we first carefully mapped in time and space the onset of activity of pfog-2000, pfog-314 and G12 by more sensitive *lacZ* mRNA detection. All three constructs displayed the same activity. As predicted from Fig. 1C, strong levels of animally restricted *lacZ* mRNAs were detected at the 16-cell stage (Fig. 4A). By the 8-cell stage, when animal and vegetal blastomeres are first defined, GATAa activity was weaker and imperfectly localized to the animal cells as demonstrated by a quantification of the fraction of embryos showing preferentially animal, vegetal or ubiquitous expression (Fig. 4B). At even earlier stages (2- and 4-cell stages), a weaker transcriptional GATA activity was detected at low frequency throughout all blastomeres (18% n=66 at the 2-cell, 20% n=215 at the 4-cell and 39% n=44 at the 8-cell stage, analyzed for G12). This indicates that in *Ciona* zygotic transcription is initiated very early and that the initial GATA activity occurs throughout the embryo. Thus, as development proceeds, GATAa transcriptional response becomes progressively restricted to the animal hemisphere, a process completed at the 16-cell stage.

The gradual exclusion of GATAa activity from the vegetal hemisphere parallels the progressive accumulation of the vegetal determinant β-catenin in the vegetal nuclei (Imai et al., 2000). Analysis of the onset of activity of the regulatory sequences of the direct β-catenin target *foxD* (Imai et al., 2002), or of a 12-mer of DNA binding sites for the β-catenin co-factor TCF (DasGupta et al., 2005) placed in front of the *Ci-bra* basal promoter (12xTCF; Fig. 4A, right panel) revealed that the progressive restriction of GATAa activity to the animal hemisphere had the same kinetics as the increase in β-catenin transcriptional activity in the vegetal hemisphere (Fig. 4A,B). This perfect temporal and spatial correlation suggests that GATAa and β-catenin may cross regulate each other.

We first tested whether accumulating β-catenin could block GATAa in the vegetal region, thereby restricting its activity to the animal hemisphere. For this, we injected a morpholino against β-catenin into eggs and monitored GATAa activity using the G12-bpbra::*NLS-lacZ* reporter. This construct, normally restricted to the animal hemisphere, became ectopically expressed in the vegetal region when β-catenin translation was blocked from before fertilization, showing that β-catenin normally represses GATA activity in the vegetal hemisphere (Fig. 5A,B). Bringing further support for this notion, a morpholino targeted against the β-catenin cofactor TCF, also derepressed G12 in the vegetal region (Fig. 5A,B). Finally, activation of the β-catenin pathway by lithium treatment led to the suppression of G12 activity (Fig. 5C) indicating that ectopic activation of the β-catenin pathway is sufficient to block GATAa activity.

Conversely, we also tested whether GATAa could antagonize β-catenin transcriptional activity (Fig. 5D). For this, we lowered GATAa levels in the embryo with GATAa-MO or raised them by *GATAa* mRNA injection and monitored β-catenin-TCF activity with pfoxD::*lacZ*. Modified GATAa levels had, however, no significant influence on β-catenin-mediated gene expression.

Thus, early animal-vegetal patterning results from the progressive definition of a vegetal domain of activity of β-catenin, which restricts the activity of the initially ubiquitous GATAa to the animal territory, where it activates the animal program.

Mechanism of differential readout of GATAa activity in pan-animal versus neural territories

Finally, we addressed the molecular mechanism by which GATAa is able to sequentially activate *Ci-fog* and *Ci-otx* in nested territories. We were particularly puzzled by the lack of early pan animal activation of the *Ci-otx* neural element (a-element), in spite of its containing three GATA sites.

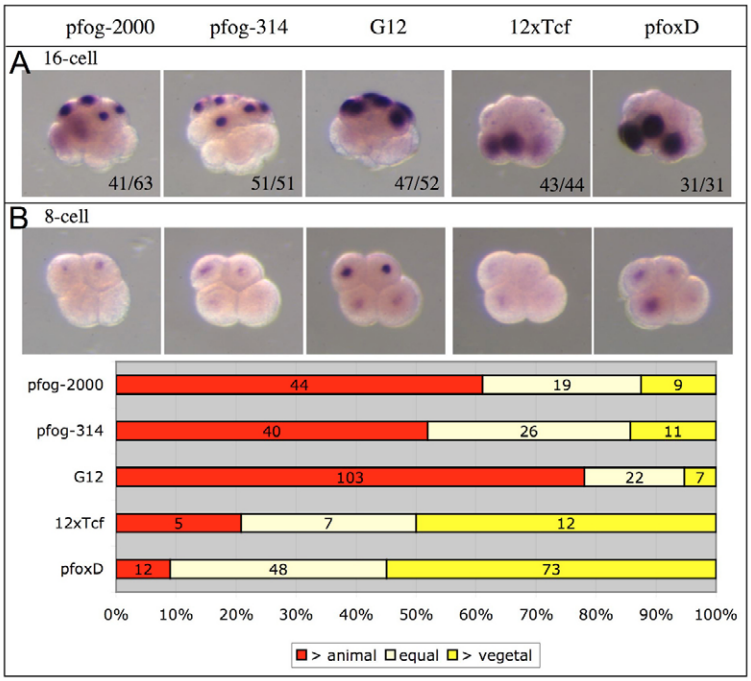

Fig. 4. Timing of animal restriction. (**A**) Animal and vegetal reporter expression at the 16-cell stage as visualized by *lacZ* mRNA detection. Lateral views, anterior to the left, numbers indicate the proportion of positive embryos with restricted expression. (**B**) At the 8-cell stage, the mRNA expression of animal and vegetal markers is partially restricted. Representative embryos are shown across the top; diagram of experimental results at the bottom. Red indicates the percentage of embryos with stronger animal expression; white indicates equal animal and vegetal expression; yellow indicates stronger vegetal expression. Numbers of embryos counted are indicated on the colored bars.

The simplest explanation would be a qualitative difference in the GATA binding sites present in the *Ci-otx* and *Ci-fog* regulatory sequences. We tested this by replacing 17 bp containing the two GATA sites and flanking sequence of pfog-314 (nucleotides –303 to –286) with a 17 bp fragment harboring two GATA sites and flanking sequence from the neural element of *Ci-otx* (nucleotides –1434 to –1418 of *potx*) (Bertrand et al., 2003). This permutation maintained early-animal-wide expression (Fig. 6B), suggesting that differences in the GATA sites present in the two genes cannot explain their differential activation.

An alternative explanation would be the presence of regulatory sequences in the cis-elements of *Ci-fog*, but absent in those of *Ci-otx*, that could synergize with GATAa. To test this, we first analyzed the transcriptional activity of pfog-290, obtained from pfog-314 by

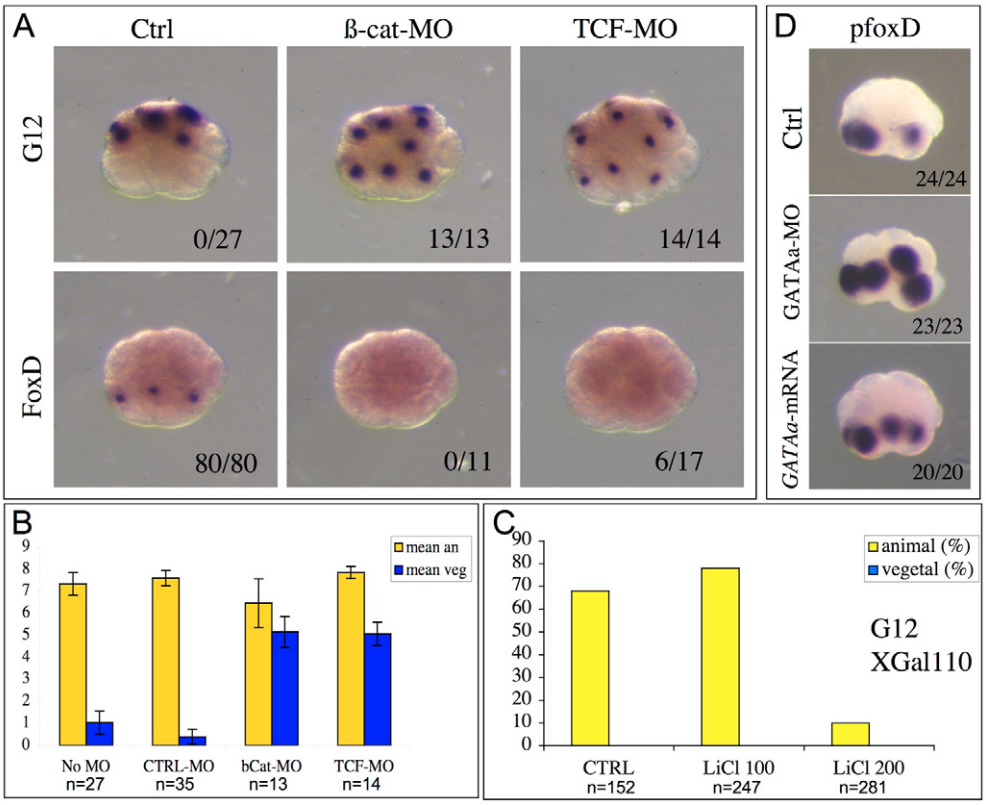

Fig. 5. GATAa activity is blocked in the vegetal region by β-catenin-TCF. (**A**) GATA transcriptional activity (G12 reporter mRNA, upper panel) and β-catenin-TCF activity (*Ci-foxD* mRNA, lower panel) was visualized at the 16-cell stage in control, β-catenin-MO- and TCF-MO-injected embryos. Lateral views, anterior to the left. Numbers indicate embryos with vegetal expression. (**B**) Bar chart of the mean number of positive cells per embryo in each condition in A (yellow is animal, blue is vegetal expression; error bars indicate confidence limits for P=0.05). (**C**) G12 reporter activity at the 110-cell stage in control, 100 mM and 200 mM LiCl-treated embryos. The *x*-axis represents the percentage of positive embryos; the *y*-axis indicates values at increasing concentrations of LiCl. The total numbers of embryos counted (*n*) are indicated at the bottom for each experimental condition. (**D**) Effect of GATAa-MO or *Ci-GATAa* mRNA injection on pFoxD activity at the 16-cell stage. Animal to the top, anterior to the left, numbers indicate the proportion of embryos with restricted vegetal expression.

deleting the two GATA sites. This element displayed no significant transcriptional activity on its own (Fig. 2A). Next, we placed the early *Ci-bra* notochord enhancer (Yagi et al., 2004) (ebra) upstream of pfog-290. Electroporation of this construct (ebra-pfog-290::*NLS-lacZ*) led to robust notochord expression (Fig. 6C), demonstrating that the pfog-290 sequences did not introduce an animal bias in the response to an enhancer. Lastly, if sequences in pfog-290 could synergize with GATAa, placing the *Ci-otx* a-element (which contains three GATA sites) upstream of pfog-290 should lead to early animal-wide expression. However, this construct, a-pfog-290::*NLS-lacZ*, drove strong expression in neural precursors (as in its endogenous *Ci-otx* genomic context or when placed upstream of the *Ci-bra* basal promoter) (see Bertrand et al., 2003), but only weak sporadic expression in uninduced animal cells (Fig. 6D). Taken together, these experiments suggest that pfog-290 behaves as a basal promoter. It may harbor sequences weakly synergizing with GATAa, but these are not sufficient to override the transcriptional logic of the a-element and efficiently activate it in uninduced animal cells.

A major difference between the two elements is the presence of two Ets sites in the *Ci-otx* a-element. To test whether these Ets sites could have a repressive activity in uninduced animal blastomeres, we generated point mutations in the two Ets sites of the *Ci-otx* enhancer in a-pfog-290 (amEts-pfog-290::*NLS-lacZ*). This led to a strong animal-wide expression similar to that obtained with G12bpbra::*NLS-lacZ* or pfog-314::*NLS-lacZ* (Fig. 6E).

Taken together, these results indicate that a major determinant of the differential responsiveness of *Ci-fog* and *Ci-otx* to GATAa is the presence of Ets binding sites in the a-element. Before the onset of neural induction and in uninduced animal cells, these sites cause active repression of the a-element. In response to the neural inducing FGF signal, Ets binding sites become activating cis-elements and synergize with GATAa sites to turn on *Ci-otx* in a6.5 and b6.5 neural precursors (Bertrand et al., 2003).

DISCUSSION

Here, we showed that Ci-GATAa, a maternal *Ciona* orthologue of the vertebrate GATA4, 5 and 6 transcription factors, is required for the formation of ectodermal territories in the ascidian *Ciona intestinalis*. Restriction of the activity of this protein to the animal hemisphere results from the progressive repression of its activity by β-catenin in vegetal territories between the 8- and 16-cell stages. Once GATAa activity is restricted to the animal hemisphere, it acts at successive stages of the ectodermal genetic regulatory network. From the 8- to 16-cell stage, it activates *Ci-fog* throughout the animal hemisphere. At the 32-cell stage, it additionally turns on *Ci-otx* in FGF-induced neural precursors. Cis-regulatory analysis allowed us to decipher the logic underlying GATAa target gene choice in these two territories. This work thus provides a mechanistic framework for the early patterning of the whole animal vegetal axis in a chordate and initiates the reconstruction of the early ectodermal gene regulatory network.

On the existence of a maternal determinant for *Ciona* ectoderm

We found that the early animal-specific gene *Ci-fog* is directly activated by maternal Ci-GATAa, that a multimer of GATA sites is sufficient to drive animal-specific expression, and is selectively activated by GATAa but not GATAb, whereas inhibition of GATAa activity represses both epidermal and neural fates. The mRNA for this factor is, however, ubiquitously distributed and we have no indication of differential translation of this message by the 16-cell

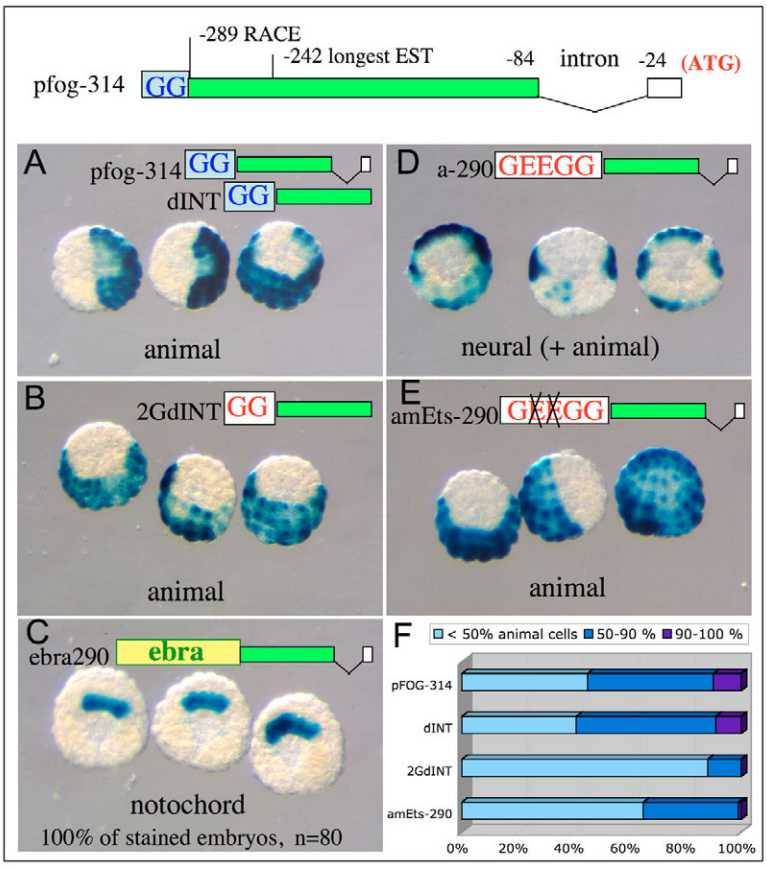

Fig. 6. Mechanism of differential readout of GATAa activity in pan-animal versus neural territories. Top: Schematic of the pfog-314 construct, showing the nucleotide position of the longest available EST, RACE-PCR fragments (results not shown) and position of an intron; green indicates sequences with basal promoter activity (–290). The putative GATA-binding sites of pfog are indicated in blue. Representative embryos electroporated with the indicated construct were stained for *lacZ* reporter activity at the 110-cell stage. A,B,D,E are animal views and C is a vegetal view; anterior to the top; $n \geqslant 35$. (**A**) Eliminating the intron maintains animal expression. (**B**) GATA sites from the *otx* neural a-element (red) maintain animal-wide expression. (**C**) –290 sequences in association with the *ebra* enhancer drive expression in notochord precursors and (**D**) in association with the *otx* neural a-element show neural and sporadic animal expression. (**E**) Ets site deletion can derepress animal-wide expression. (**F**) Quantification of constructs showing animal-wide expression. 90-100% indicates the percentage of embryos with all animal cells stained; 50-90% indicates the percentage of embryos with more than half of animal cells stained; <50% indicates the percentage of embryos with fewer than half of animal cells stained.

stage when its target gene *Ci-fog* is specifically activated in animal territories (Bertrand et al., 2003). In addition, GATAa activity appears permissive rather than instructive as interference with GATAa function has no effect on the early vegetal program as measured by the activation of the β-catenin target gene *Ci-foxD*. Notably, ectopic GATAa does not impose the ectodermal program on vegetal cells. Ci-GATAa can thus be considered a permissive maternal activator of the ectodermal program.

Our results also suggest the existence of a GATA network within the ectoderm to form epidermis with a likely scenario where maternal GATAa signaling is complemented by mostly zygotic GATAb signaling. This is consistent with the strictly maternal expression of GATAa, while GATAb is weakly maternal, then zygotically expressed from the 64-cell stage in the ectoderm (Imai et al., 2004). Furthermore, the two proteins appear to have distinct activities as GATAb overexpression is unable to make up for the early ectodermal GATAa function to activate pan-animal expression (Fig. 2C). Understanding the role of FOG will need additional work. In contrast to GATAa and GATAb, FOG-MO knockdown did not interfere with EpiB or Otx neural element expression (not shown) suggesting that this gene may either act redundantly with other factors or contribute to more subtle aspects of epidermal differentiation.

The permissive nature of GATA signaling is in keeping with the finding that the down regulation of β-catenin leads to the formation of ectopic epidermis in the vegetal territories (Imai et al., 2000). However, our results are surprising in light of embryological experiments carried out in another ascidian, *Halocynthia roretzi*, which suggested that an instructive animal maternal determinant was distributed as a gradient along the animal vegetal hemisphere of fertilized eggs (Nishida, 1997b). Although we cannot exclude that additional localized animal determinants may act in ascidians, or that the *Ciona* and *Halocynthia* logics may differ, our discovery that vegetally localized maternal β-catenin represses GATAa activity may be sufficient to solve this apparent paradox. By analogy to the *Xenopus*, fish and sea urchin situation, β-catenin is probably also activated in ascidians (Yoshida et al., 1998) as a result of the action of cortically localized factors such as dishevelled (Miller et al., 1999; Rothbächer et al., 2000; Weitzel et al., 2004). The *Halocynthia* experiments were carried out by fusing egg fragments to vegetal blastomeres, and therefore led to the local replacement of the vegetal cortex by cortex of a more animal nature. This may have led to the local lack of activation of β-catenin, and thus to the local derepression in the vegetal cells of GATAa.

Overall, our study suggests that ascidian ectoderm is established permissively and that an instructive maternal animal determinant may not exist in ascidians. It also highlights that ectoderm is a ground state that needs to be repressed by vegetal β-catenin for other germlayers to form.

A direct repression of GATAa transcriptional activity by β-catenin-TCF in the vegetal hemisphere?

In a previous study (Bertrand et al., 2003) we found that ectopic GATAa-GFP was transiently restricted to the animal hemisphere during the late 32-cell stage, at the time of neural induction. This protein was, however, ubiquitously distributed up to the 16-cell stage, at the time when *Ci-fog* expression becomes restricted to the animal hemisphere. Regulation of protein stability is thus unlikely to be crucial for the early definition of the animal hemisphere. Instead, our results suggest an interference with the transcriptional activity of GATAa. We do not know at present whether this is achieved at the level of its ability to bind DNA or to activate transcription.

The β-catenin-TCF complex usually functions in transcriptional activation and the simplest mechanism would be that it activates a zygotic repressor of GATAa activity in the vegetal territory. This would, however, result in a restriction of GATAa activity occurring later than the activation of the primary β-catenin-TCF transcriptional targets. In contrast, we found that the animal restriction of GATAa activity, measured by G12 and *Ci-fog* activation, is simultaneous to the activation of two direct targets of β-catenin, 12xTCF and *Ci-foxD*. This goes against a transcriptional relay mechanism and suggests that the β-catenin-TCF complex may directly repress GATAa activity. It will be interesting to test, in the future, whether these proteins are found in a single complex.

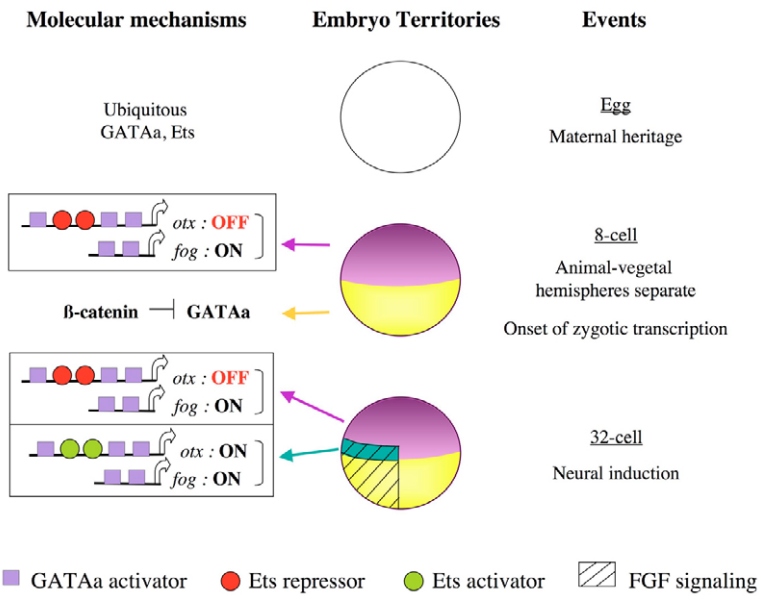

Fig. 7. Model for early animal patterning in ascidians. Schematic views of the different events taking place in the early *Ciona* egg, 8-cell and 32-cell embryo and the corresponding molecular mechanisms defining the ectodermal territories.

Transcriptional logic underlying successive target selection by Ci-GATAa

In addition to turning on target genes such as *Ci-otx* in the FGF-induced a6.5 and b6.5 neural progenitors at the 32-cell stage (Bertrand et al., 2003), we found that Ci-GATAa has earlier pan animal targets such as *Ci-fog*. Our analysis further allowed us to shed light on the transcriptional logic for the selection of different target genes in nested territories at successive developmental time points (Fig. 7).

Our results point to a major role for the two Ets sites in this process. In the absence of FGF signaling, Ets sites act as repressive elements for the *Ci-otx* neural enhancer. Our previous analysis (Bertrand et al., 2003) indicated that upon reception of an FGF signal, they act as positive transcriptional elements and synergize with the neighboring GATA sites. Thus, conversion of Ets sites from ongoing repressive elements in uninduced cells to activating elements in individually induced cells is a key determinant of the activation of *Ci-otx*. Conversion of Ets factors from repressor to activator upon MAPK signaling has been described in vertebrates (Maki et al., 2004) and insects (Rebay and Rubin, 1995). It will therefore be interesting to examine in the future whether Ci-Ets1/2, which mediates the activation of *Ci-otx* in FGF-induced cells, also acts as a repressor in uninduced cells or whether the repressive function is achieved by another *Ciona* Ets factor.

Interestingly, there does not seem to be a sharp transition between the maternal and zygotic programs, rather, distinct combinations of maternal factors are sufficient to collaborate with overlapping early zygotic signals to directly turn on distinct zygotic genes at successive developmental stages in nested territories of the ascidian ectoderm. This situation is somewhat reminiscent of the feed forward loops at work in the early regulatory network that sets the *Drosophila* anterior posterior axis, in which combinations of maternal factors collaborate with their first broadly expressed zygotic targets, the gap genes, to activate pair rule and segment polarity genes in smaller territories.

Evolutionary considerations

It is interesting to compare the ascidian ectodermal logic with that of other bilaterians. Our data point to a GATA network for epidermis formation in ascidians, where a maternal role for GATAa (GATA4/5/6) is complemented by probable zygotic ectodermal GATAb (GATA1/2/3). A large body of evidence is arising to give support to the idea that GATAb orthologues are implicated at various levels in epidermis formation across bilaterians (Gillis et al., 2007). The function of GATAb can be therefore considered a conserved feature. The novel role of maternal GATAa in the initiation of the ectodermal program, and its restriction/repression by β-catenin, has not been demonstrated before.

Ascidians are phylogenetically close to vertebrates, a relatedness apparent in the shared early gastrula fate map and larval body plan (reviewed by Nishida, 2005). Yet, the vertebrate situation appears to differ significantly from the ascidian one. The transition between maternal information and the zygotic program has been best studied in lower vertebrates, and in particular in *Xenopus* and zebrafish. Although maternal vegetal determinants may differ between these two systems, they converge at the level of the activation of nodal signaling, which is the major pathway inducing both mesoderm and endoderm (reviewed by Schier and Talbot, 2005; Heasman, 2006a). Analysis of the animal region of *Xenopus* or fish embryos identified several maternal mRNAs, such as *zic2*, the *soxB1* family gene *sox3* and *ectodermin* functioning in maternal inhibition of nodal signaling (Houston and Wylie, 2005; Zhang et al., 2004; Dupont et al., 2005). They act via different strategies and are crucial to set the position of the boundary between mesendoderm and ectoderm in the animal hemisphere. These results suggest that maternal repressors of nodal signaling are necessary for the vertebrate ectoderm to form. In ascidians, Nodal is not a major inducer of endoderm and mesoderm (Hudson and Yasuo, 2006), which may explain why such a strategy has not been conserved between ascidians and vertebrates. It should be noted, however, that in sea urchin embryos maternal SoxB1, repressed by vegetal β-catenin is probably required for ectodermal development (Angerer et al., 2005), although nodal has so far only been shown to be involved in oral ectoderm rather than mesendodermal induction (Duboc et al., 2004).

As no regulatory sequences driving early vertebrate ectodermal gene expression have been reported, it remains unclear whether inhibition of Nodal signaling is sufficient for the definition of the ectoderm in vertebrates, or if additional molecules are involved. At first sight, the GATAa orthologues GATA4, 5 and 6, seem unlikely candidates, as they have been shown to be important for the formation of the endoderm rather than the ectoderm (reviewed by Patient and McGhee, 2002; Loose and Patient, 2004). This is, however, a zygotic function of these proteins, which is probably an ancestral deuterostome feature (Gillis et al., 2007) as ascidian *Ci-GATAa*, sea urchin and starfish *gatae* are zygotically expressed throughout the endoderm (Imai et al., 2004; Hinman et al., 2003) and sea urchin and starfish GATAe function is crucial for endoderm formation (Hinman et al., 2003). In addition to this conserved zygotic function, however, *Xenopus GATA5* is expressed maternally (Partington et al., 1997). It will be important to carefully test the effect of the early loss of function of GATA4, 5 and 6 proteins on the vertebrate ectodermal program.

Conclusion

Our work constitutes a unique example of direct regulatory interactions linking maternal factors and the cis-regulatory elements of genes transcribed in the ectoderm of a chordate. Together with the recently published zygotic *Ciona* whole embryo gene regulatory network (Imai et al., 2006), it constitutes a base to reconstruct the ectodermal gene regulatory network. Comparison of ascidian and vertebrate networks should permit the identification of crucial conserved features, and also an assessment of the extent of deviation that is compatible with the adoption of a common body plan. Comparison with the urchin networks may point to deuterostome-specific traits.

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