Encoding regulatory state boundaries in the pregastrular oral ectoderm of the sea urchin embryo

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By gastrulation the ectodermal territories of the sea urchin embryo have developed an unexpectedly complex spatial pattern of sharply bounded regulatory states, organized orthogonally with respect to the animal/vegetal and oral/aboral axes of the embryo. Although much is known of the gene regulatory network (GRN) linkages that generate these regulatory states, the principles by which the boundaries between them are positioned and maintained have remained undiscovered. Here we determine the encoded genomic logic responsible for the boundaries of the oral aspect of the embryo that separate endoderm from ectoderm and ectoderm from neurogenic apical plate and that delineate the several further subdivisions into which the oral ectoderm per se is partitioned. Comprehensive regulatory state maps, including all spatially expressed oral ectoderm regulatory genes, were established. The circuitry at each boundary deploys specific repressors of regulatory states across the boundary, identified in this work, plus activation by broadly expressed positive regulators. These network linkages are integrated with previously established interactions on the oral/aboral axis to generate a GRN model encompassing the 2D organization of the regulatory state pattern in the pregastrular oral ectoderm of the embryo.

results

Oral Ectoderm Regulatory States from Cleavage to Gastrulation. As shown in Fig. S1A, cohorts of regulatory genes are expressed in increasingly complex patterns as development proceeds. The process of regulatory state formation is summarized in diagrams (Fig. 1), which are based on single and double in situ hybridization from earlier studies of regulatory gene expression (5, 8–11, 14, 15) and from detailed additional observations that we reproduce in Fig. S1. Among 35 oral ectoderm and apical reg-

Significance

Regulatory state boundary formation is a general process in early development, in which embryonic territory is divided up into spatial domains that express distinct sets of regulatory genes. We establish the mechanistic principles by which multiple orthogonal boundaries of this kind are progressively formed on the oral side of the sea urchin embryo, according to an encoded genomic program. These boundaries separate prospective endoderm from ectoderm domains, neurogenic from non-neurogenic domains, and ciliated band from oral ectoderm domains and produce an orthogonal grid of regulatory states. Boundary formation invariably depends on spatial transcriptional repression superimposed on more widespread domains of transcriptional activation.

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ulatory genes previously reported, 12, generally the earliest markers of each individual regulatory state domain, were selected for further examination. These genes play critical roles in regulatory state formation and diversification. Their quantitative expression dynamics had been measured earlier (16), and the relevant kinetic data are reproduced in Fig. S2. The detailed sequence of expression patterns shown in Fig. S1 can be abstracted to provide the dynamically changing Boolean expression matrices shown in Fig. 2, where the contributions of the 12 genes to the regulatory state of each domain can be read horizontally (6, 7). These are the specific patterns of expression for which we seek causal explanation in the encoded architecture of the ectodermal GRNs.

The ectodermal boundary formation events in both time and space that are implied in the patterns of Fig. 1A are abstracted in the regulatory process diagram shown in Fig. 1B, where the orthogonal pairs of arrows mark the institution of regulatory state boundaries—red for boundaries in the oral/aboral axis and green for boundaries that arise along the animal/vegetal axis. These boundaries are the subject of the experimental work in this paper (except for one arising within the aboral ectoderm, the bottom-right image in Fig. 1A). An initial transient regulatory state boundary, already evident at 7 h, forms at the equatorial cleavage planes where it separates eve-expressing blastomeres that will give rise to endomesodermal constituents (macromere descendants) from foxq2-expressing blastomeres ancestral only to ectodermal and neurogenic components of the embryo (mesomere descendants) (Fig. S1, 7 and 9 h). A new regulatory state boundary is then established, which separates the nonapical ectoderm from the apical domain (10–15 h). A boundary between the transient regulatory states of all veg1 lineage cells and that of the overlying oral ectoderm forms at 12–18 h; at 15–18 h, another regulatory state boundary delimits the near-apical from the central-oral ectoderm regulatory states; and finally, at ~24 h, the
definitive regulatory state boundary separating veg1 posterior endoderm from veg1 ectoderm is established. Boundaries within the apical neurogenic domain have not been analyzed, whereas formation of boundaries within the endomesoderm was solved previously (5, 6). We adduce earlier evidence regarding oral ectoderm regulatory state boundaries that form along the oral/aboral axis and integrate it with the animal/vegetal boundary mechanisms below.

**GRN Interactions Controlling the Apical Domain/Oral Ectoderm Boundary.** The mechanism leading to specification of the apical neurogenic domain begins with the zygotic expression of foxq2, which, as we have just seen, is activated during the fifth cleavage in all cells of the animal hemisphere (Figs. S1 and S2). A sharp upward retraction in the domain of foxq2 expression occurs between 9 and 12 h, however, and this process continues, so that by 15 h the spatial expression of foxq2 marks the apical plate region; thereafter, this gene is stably expressed in the central region of the apical plate domain (Fig. S1, 9–30 h). Our initial objective, in unraveling the gene interactions that set the boundary between the apical domain and the oral ectoderm, is to understand the cause(s) of retraction of the foxq2 expression domain until it arrives at and defines the apical plate boundary because it is the first apical-specific regulatory gene to be expressed. Some evidence indicated that the earliest 9- to 12 h phase of retraction could be mediated indirectly by Wnt8 signaling from the vegetal blastomeres, and indeed effects of Wnt signaling on the size of the apical domain were reported earlier (17). We show here, however, that after 12 h a known transcriptional repressor expressed in the oral ectoderm, not, which, however, is not a direct target of Wnt signaling, is specifically responsible for preventing foxq2 expression all the way down to the equator. This is demonstrated by the dramatic spatial effects of not morpholino antisense oligonucleotide (MASO) on foxq2 expression at 15 h, which, as seen in Fig. 3A, is then restored down to the oral equator. Fig. 3A also shows that this MASO effect cannot be seen 3 h earlier. The not gene, a direct early target of Nodal signaling (10), is expressed significantly in the equatorial oral ectoderm by 12 h and thereafter (Fig. S2).

Fig. S1 also shows that transcripts of the homeobox gene emx, the zinc-finger gene egr/z60 (18), and the Tgfβ gene univin abut the lower boundary of foxq2 expression at 15 and 18 h and that all three are transcribed in the regions from which foxq2 has earlier cleared. This is confirmed in the control emx/foxq2 double in situ hybridization shown in Fig. 3A, and in Fig. S3A the same is shown for univin and z60. The emx gene repressively controls the lower boundary of foxq2 expression after the midblastula stage, as shown by the spread of this domain into the near-apical oral ectoderm in emx MASO-treated embryos (Fig. 3A). Reciprocally, as Fig. 3A shows, foxq2 expression is required to prevent expression within the apical domain of emx (and also of univin and egr) (Fig. S3A). Thus, emx and foxq2 are linked in a mutual exclusion circuit (4, 19, 20), in which, within the normal domain of expression of each, expression of the other is prohibited. Thereby, the boundary separating foxq2 expression from the oral ectoderm regulatory state is locked down. The logical elegance of this boundary formation mechanism is underscored by the use of a common driver by both foxq2 and emx genes. This is the positively acting, pan-ectodermal regulator SoxB1 (21): as shown by the quantitative MASO perturbation experiments reproduced in Fig. S4, by 24 h emx and foxq2 expression are, respectively, about 90 and 97% depressed by knockdown of SoxB1 translation. Note that, later in blastulation, expression of emx, univin, and egr is cleared from the oral and/or aboral ectoderm (Fig. S1A) by repressors active in that domain, whereas their boundaries with foxq2 persist on the oral side of the embryo.

**Gene Interactions Controlling the Boundary Between Animal and Vegetal Ectoderm.** All cells deriving from the animal half of the sea urchin embryo give rise to ectoderm, but, in addition, a few cells of the vegetal half located immediately beneath the equator also assume ectodermal cell fates. These cells are descendants of the veg1 lineage, which also gives rise to posterior endoderm. In the ancestors of veg1 ectodermal cells, eve expression initiates at
7 h, representing the first regulatory distinction from animal ectodermal cells (5, 6, 22). Until about 12 h, eve remains broadly expressed in the vegetal half, but by 15 h its expression is confined to the veg1 lineage, and transcript levels accumulate (Fig. S2) (5). Expression of eve precedes expression of the genes constituting the regulatory state of the adjacent nonapical ectoderm such as emx and lim1 (Figs. S1A and S2). When emx is activated, and until after 15 h, it is expressed all over the nonapical ectoderm, and in addition its lower boundary of expression overlaps with that of eve. The univin gene is expressed in exactly the same way (Fig. S1A). After 18 h, however, transcripts of both genes have cleared from the veg1 eve domain (emx is then expressed in the lateral and aboral ectoderm). This behavior suggests that eve is acting as a repressor of genes that define the animal ectoderm regulatory states on both the oral and the aboral side of the embryo.

The experiments of Fig. 3B demonstrate this eve function. In the first column, we see double in situ hybridizations showing eve and emx and eve and univin. Expression of univin still partly overlaps

Fig. 3. MASO perturbation and expression analysis to identify the roles of regulatory genes in establishing boundaries along the primary axis. Perturbation assays were performed to knock down expression of foxq2, not, or emx (A), eve (B), six3 and/or foxq2 (C), hox11/13b (D), orbmp2/4 (E). Expression pattern changes were investigated using WMISH. The apical region of endogenous foxq2 expression is marked with green arrowheads or dotted circles (A, C, and E). Red and green dashed arcs in B indicate the veg1 and veg2 regions, respectively. In D, the red arrowheads mark hox11/13b expression; the blue arrowheads indicate the gap between the veg1 ectoderm and the veg2 endoderm, and the black arrowheads show expansion of lim1/vegf3 expression into the veg1 endoderm in the hox11/13b morphants. A broader survey of more regulatory genes in response to MASO perturbation was shown in Fig. S3. lv, lateral view; vv, vegetal view; av, apical view; ov, oral ectodermal view.
that of eve at 18 h, but its expression and that of enm have become exclusive with respect to eve on either side of the vegl/animal ectoderm boundary by 24 h. Two additional genes expressed in the oral ectoderm GRN, gsc and foxG (9, 10), are also expressed right down to the boundary with the vegl eve expression domain at 24 h. The second column of Fig. 3B shows expression of these same four ectoderm genes together with that of the veg2 regulatory gene foxa: a gap of three to four rows of cells deep, which corresponds to the vegl domain, is clearly visible for all four genes. The third column demonstrates for each of these genes that, if eve expression is blocked by introduction of MASO, their expression extends right down to the veg2 cells, and the vegl gap no longer exists. Therefore, eve, which defines vegl regulatory identity and initiates the vegl GRN (6), also functions to exclude expression of these ectoderm genes from the vegl domain. Thereby, the vegl lineage boundary is maintained as a regulatory state boundary, i.e., until the following boundary transition in this region, which subdivides vegl into posterior endoderm and ectoderm.

Transcriptional Repressions Further Partitioning the Oral Ectoderm. The lim1 regulatory gene is activated by ~10 h, 2-3 h after foxa2 and six3 (Fig. S2). Its expression is also driven by the pan-ectodermal activator SoxB1 (Fig. S4). A priori this gene should therefore be able to express throughout the ectoderm, but, instead, from the outset its expression appears as a band extending around the embryo from the vegl domain below the equator up to about halfway into the animal oral ectoderm (Fig. S1B, 12–21 h). Its upper boundary of expression implies repression of lim1 in the apical and near-apical animal ectoderm. At 15 and 18 h, the expression pattern of six3 (15, 23) appears perfectly reciprocal to that of lim1. Thus, as seen in lateral view, six3 transcripts occupy the apical domain plus the near-apical animal ectodermal domains (Fig. S1B). An experiment shown in Fig. S3C demonstrates that six3 and foxa2 are indeed responsible for excluding lim1 expression from the apical and near-apical ectoderm because foxa2 + six3 MASOs cause lim1 expression to extend to the whole animal half of the embryo. The central animal ectoderm and the near-apical animal ectoderm regulatory states are therefore separated by the lim1/six3 boundary. This boundary soon pertains only to the oral side, where it persists, as lim1 expression is lost from the aboral ectoderm between 21 and 24 h of gastrulation (Fig. S1B). Further lateral extension of the spatial repression mediated by foxa2 predicts six3 expression in the central apical domain (Fig. 3C). Thus, the relations foxa2 repressing six3; foxa2 + six3 repressing lim1 produce a central disk of foxa2 expression; a surrounding torus of six3 expression, the lower boundary of which bisects the nonapical animal ectoderm; and an abutting lower torus of lim1 expression. Following the confinement of lim1 and six3 to the oral side of the embryo by aboral ectoderm repression (see below), these boundaries persist in the oral ectoderm and oral apical domains (Fig. S1B).

Exclusion of the Oral Ectoderm GRN from the Apical Domain. Repression by foxa2 plus six3 has a further spatially specific effect, the ultimate significance of which expands as early development proceeds. This is the repression of nodal expression. Although the repressive role of foxa2 was previously proposed (14), the regulatory circuit governing the dynamic nodal expression is more complicated and involves synergetic repression. As summarized in Fig. 2, the nodal gene is expressed strongly in the central oral ectoderm and more weakly in the near apical oral ectoderm (see foxa2/nodal double in situ hybridizations in Fig. S1C). In the absence of [foxq2 + six3] expression, nodal transcription spreads upward over the whole oral apical domain (Fig. 3C), although treatment with either foxa2 or six3 MASO alone has only minor effects (Fig. S3). This observation, at 21 h, suggests that persisting Six3 protein is responsible together with Foxq2 protein for apical nodal repression (by 21 h, six3 is no longer being transcribed in the apical domain; Fig. 3C). Transcriptional target genes of Nodal signaling such as lefty are, as expected, affected by [foxq2 + six3] MASOs in exactly the same way as is nodal expression (Fig. 3C). Partial repression by Six3 probably accounts for the relatively weak nodal expression in the near-apical oral ectoderm. Expression of the nodal gene is the primary transcriptional response to the redox polarization that in causal terms initially generates the future oral/aboral axis (24–28). Therefore, because much of the oral ectoderm-specific GRN is wired downstream of Nodal response genes (9–11, 29, 30), Foxq2 repression of nodal hierarchically confines the whole oral ectoderm GRN to the region below the foxq2 expression boundary, that is, the “apical/near apical” boundary of Fig. 1A and B.

Transcriptional Repression Defining the Boundary Between Ectoderm and Endoderm. The last of the pregastrular boundaries formulated on the animal/vegetal axis to be considered here is that separating all ectoderm fates from endodermal fates. This boundary forms within the vegl cell lineage, which gives rise to posterior endoderm and to ectodermal cells located just below the equator (31, 32). Directly or indirectly, Wnt5 signaling is involved in initial vegl specification (33). Further separation of the vegl regulatory state, and ultimately of embryonic fate, occurs after 24 h in the late mesenchyme blastula stage (Fig. 1B). By the end of gastrulation, vegl-derived endoderm has constituted the hindgut and part of the midgut, whereas vegl-derived ectoderm has formed the wall of the embryo surrounding the anus. The first spatial regulatory state changes denoting formation of this boundary are separation of the expression domains of lim1 and veg3 from that of hox11/13b (Fig. 2). Up to 24 h, lim1 and veg3 are transcribed in all vegl cells, including the future endoderm precursors; thus the expression domains of these genes abut that of the veg2 gene foxa as can be seen in the double in situ hybridizations of Fig. S1B (foxalim1) and Fig. S1C (foxalim1). By 24 h, hox11/13b expression is initiated in the lower rings of vegl cells, defining those destined for endodermal fate (6). Thereafter, lim1 and veg3 cease to be expressed in these presumptive endoderm cells, and, by 30 h, the vegl endoderm below the newly formed endoderm/ectoderm boundary expresses hox11/13b and not lim1 or veg3, whereas the vegl oral ectoderm cells do not express hox11/13b but do express lim1, and those oral cells that express lim1 but not veg1 become endoderm and express veg1. Put more generally, vegl cells expressing hox11/13b become endoderm and vegl cells not expressing hox11/13b become ectoderm. Because the lim1 and veg3 genes are expressed only a few hours earlier coincidently with hox11/13b, a reasonable prediction is that, when hox11/13b is transcribed in the lower vegl cells, this gene establishes the endoderm/ectoderm boundary by repressing ectoderm-specific genes within its domain of expression. Fig. 3D shows that, if hox11/13b expression is blocked by MASO treatment, clearance of neither lim1 nor veg3 expression from prospective posterior endoderm cells fails to occur. This is demonstrated by the extension of the domains of expression of these genes right to the vegl boundary of foxa expression. Thus, hox11/13b acts as a critical domain-specific repressor on the endoderm side of the boundary separating endodermal from ectodermal cell fate in the sea urchin embryo.

Lateral Boundaries. Fig. 4A illustrates two bilateral boundaries on the oral face of the 30-h embryo that separate regulatory states along the oral/aboral axis. These are the boundaries on each side between the animal lateral ectoderm and the medial ectoderm territories, i.e., the near-apical and central-oral ectoderm and, within the vegl ectoderm, the boundaries on each side that separate the lateral vegl ectoderm from the central vegl oral ectoderm (in addition, there are the boundaries of the future stomodaenum, which are not treated here). It is to be noted that appearances can be deceiving, so to speak, in that the regulatory state map of the
The embryo is significantly more complex than the morphological map of the oral side of the embryo would suggest. Thus, as shown by comparing the 30-h lateral and oral views of Fig. 1A, the ciliated band actually consists of the two bilateral ectoderm regulatory states—an oral apical regulatory state and the central and lateral vegl regulatory states—although the regulatory gene one-cut (hnf6) is expressed early all around the future ciliated band.

The regulatory states of the aboral ectoderm domains (11, 12) differ from those of the oral ectoderm domains, although some key genes contributing to each are at first expressed in both oral and aboral ectoderm. We have already encountered two examples, six3 and lim1 (Fig. S1B). Resolution of their expression patterns to the oral side again depends on repression. Many genes of the aboral ectoderm require a positive boost from Bmp signals emitted from the oral ectoderm, as confirmed at the cis-regulatory level (12).

Because of the extensive feedbacks within the aboral ectoderm GRN, Bmp MASO essentially down-regulates the whole of this GRN. As shown in Fig. 3E, use of Bmp MASO demonstrates that the aboral ectoderm GRN includes repressor(s) that function to abolish transcription of six3 and lim1 in the aboral ectoderm. Returning to the two pairs of oral/aboral boundaries within the oral face, much has been learned about the specific repressions responsible for the boundaries between the medial and lateral ectodermal territories. Two known genes, not and gsc, encode spatial repressors that are expressed in the oral ectoderm, both activated by Nodal signaling. The not gene is expressed in the near apical, central, and vegl oral ectoderm by 15 h, and gsc is expressed in the same domains except for the vegl oral ectoderm. Repression by not silences multiple genes of the lateral oral ectoderm regulatory state in the medial oral ectoderm domains, leaving them to be expressed across the boundary with the lateral oral ectoderm (9, 10). For genes initially expressed across the vegl ectoderm such as vegfl, not repressor confines expression to the regions across the boundary with the vegl lateral ectoderm (10). Repression by gsc silences the onecut gene in the near apical and central oral ectoderm, confining its expression to the ciliated band domain across the boundaries with the lateral ectoderm on the sides, the apical domain above, and the vegl ectoderm that constitutes the oral/vegetal arm of the ciliated band (34). Aboral repressors such as irx restrict the expression of all of the genes of the lateral oral and vegl oral ectoderm to confined bands of cells, also obliterating their expression in the aboral ectoderm (34).

Thus, an essentially orthogonal, bilateral pattern of bounded oral ectodermal regulatory states is established by the time of gastrulation (Fig. 1A, 30 h). The observations that we summarize in this paper, taken together with those obtained earlier, show that the mechanism by which this complex pattern is established is mainly sequential, spatially confined, transcriptional repression, occurring along both axes of the embryo.

**Discussion**

**Principles of Boundary Formation in the Pregastrular Sea Urchin Embryo Ectoderm.** The 2D oral grid of ectodermal (and future neurogenic) regulatory states is established in this embryonic region in the complete absence of cell migration. The regulatory states are imposed on the single-cell-thick ectodermal wall of the embryo, each cell inheriting from its parent the output of the immediately preceding spatial gene expression pattern. Careful attention to the temporal sequence of spatial expression of the regulatory genes constituting each boundary reveals some simple commonalities: the process is invariably asynchronous, and the boundaries are formed by mechanisms that depend directly on the order of regional gene expression. Therefore, the premise noted at the outset works: i.e., the first expressed gene in a given domain always executes a key role in formation of the eventual boundaries of the domain that this gene characterizes. The system therefore operates in a determinate way. Never do we encounter simultaneously expressed “bi-stable states” expressed within the same cells and mediated by dueling mutual repressors. However, once the regulatory state domains are formed, the canonical circuits enforcing them are exclusion circuits: the output of each regulatory state includes specific repressors of the regulatory state across the boundary. Each of the boundaries considered here illustrates these principles.

In the formation of the apical neurogenic/oral ectoderm boundary, the first player to be expressed is the foxa2 repressor, responding to a maternal and zygotic pan-ectodermal activator, SoxB1. At the same time, nodal is expressed, driven by the same activator, by a redox-sensitive transcription factor that causes its expression to occur exclusively on the oral side of the embryo, and by feedback from its own signal transduction system (24, 35). One regulatory step later, Nodal signaling turns on not, which, after it is transcribed and its mRNA translated, expresses foxa2 in the oral ectoderm where it is expressed. The apical neurogenic/oral ectoderm boundary is formed with the aid of a second widely expressed soxB1 target gene, the repressor emx, required in the near-apical oral ectoderm where nodal and not expression are weaker. These repressions confine foxa2 expression to the apical domain. Then the exclusion functions kick in: foxa2 represses emx, and foxa2 plus six3 repress nodal and consequently the whole oral ectoderm GRN within the apical domain. The neurogenic foxa2 region is thus permanently segregated.

Within the oral ectoderm, another boundary forms—that separating the near-apical from the central oral ectoderm. Here another early regulatory player is six3, activated almost as early as foxa2 in the animal hemisphere, and by 18 h is expressed in the upper half thereof. The repressive target of six3 plus foxa2 is lim1, which because it is also driven by SoxB1 could express throughout the ectoderm, but because six3 and foxa2 are expressed first, lim1 can be transcribed only up to the lower boundary of the six3 expression domain, defining a central oral ectoderm region, whereas the six3-expressing region between the foxa2 and lim1 boundaries becomes the near-apical ectoderm. Thereafter, a further exclusion is instituted: foxa2 excludes six3 expression from the central apical plate and confines its expression domain to the surrounding near-apical ectoderm.

At the lower boundary of the ectoderm the regulatory state domains are formed in two successive steps. Here the first regulatory gene to be expressed in vegl is eve. Initially, several ectodermal regulatory gene expression domains overlap that of eve, but eve repression cancels their transcription, forming the boundary between vegl and the overlying oral ectodermal regulatory states. Later the first regulatory gene to be expressed in the portion of vegl to become endoderm is foxf1/13b. Transcription of the ectodermal lim1 regulatory gene is extinguished by foxf1/13b repression, setting the boundary between vegl endoderm and vegl ectoderm, in which lim1 continues to be expressed. The aboral/oral axial boundaries between the medial oral ectoderm and lateral ectodermal fates are also set sequentially: they depend on the build-up of the dominant Not repressor, which extinguishes transcription of a set of previously broadly expressed oral ectoderm genes.

To summarize, whereas a common activator is used to initiate the expression of most ectodermal genes, the complex patterns of gene expression are determined by the minuet of sequential repressions and thus by the encoded targeting of given genes by given repressors. These features cause the patterning process to be determinate and invariant.

**Enhanced Territorial GRN Model.** Fig. 4 incorporates the findings summarized here with our previously assembled GRN model for the oral ectodermal domain. However, the BioTapestry model accurately represents the spatial transactions within the oral ectoderm, as organized into the complex regulatory state domains shown in Fig. 1A for the 30-h embryo. The GRN model as portrayed indicates the linkages that are active or inactive in each domain. Over 60 regulatory linkages among about 30 transcription factor and signaling genes are included, the evidence for each of which is summarized in abbreviated form in Table S1. The Bio-
Tapestry model presents the predicted topology of the oral ectoderm regulatory system, displaying its modular circuit features (4), such as double-negative gates, community effect circuits, exclusion circuits, feedbacks, etc. Space does not permit discussion of these individual features and the logic operations that they execute; suffice it to say, the pregastrular oral ectoderm GRN models will soon support a global logic analysis similar to that recently applied to the endomesoderm GRN model (7). Fig. 4 is incomplete in that it does not include the networks functioning within the aboral ectoderm, the lateral ectoderm, and the other ciliated band domains or the stomodeal domain, all to be presented elsewhere. However, Fig. 4 does encompass the network of regional cross-repressive exclusion functions and the repressions of repressor genes that underlie boundary formation in the oral ectoderm, the newly discovered outcome of this work.

Materials and Methods

Gene Cloning and Constructs. *hox11/13b* and *lim1* were previously described (11, 36). *univin*, *z60* (*egr*), *emx*, *eve*, and *foxq2* were PCR-cloned. The primer sets used for gene amplification are listed in Table S2. Gene models generated from sea urchin transcriptome analysis were used as a reference for primer design (37). cDNA prepared from various development al stages was used as template for PCR. PCR products were purified and ligated into GEM-T EZ constructs. Cloned genes were PCR-amplified using the primer flanking the inert region, and PCR products were used to synthesize mRNA for microinjection or RNA probes for in situ.

Whole-Mount In Situ Hybridization. The protocol for whole-mount in situ hybridization (WMISH) to map gene expression has been described previously (38). Briefly, sea urchin embryos were fixed in glutaraldehyde solution. The fixed embryos were incubated in the hybridization buffer [50% (vol/vol) formamide, 5× SSC, 1× Denhardt’s, 1 mg/mL yeast tRNA, 50 ng/mL heparin, and 0.1% tween-20] with 0.5 ng/μL digoxigenin- and fluorescein-labeled RNA probe(s) at 60 °C for 18 h. Posthybridization washes were hybridization buffer, 2× SSC, 0.2× SSC, and 0.1× SSC each 30 min at 60 °C. Subsequently, the antibody incubations were performed out at room temperature with 1:1,000 diluted anti-DIG Fab (Roche). The embryos were extensively washed before staining reaction, including six times with MABT buffer (0.1 M maleic acid, 0.15 M NaCl, and 0.1% tween-20), twice with AP buffer [100 mM Tris·Cl (pH 9.5), 100 mM NaCl, 50 mM MgCl2, and 1 mM levamisole]. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium were used for staining. In the double in situ hybridization, embryos were treated with glycine stop solution [0.1 M glycine (pH 2.2), 0.1% tween-20] after the first color reaction and then directly followed by the second antibody incubation [1:1,000 diluted anti-fluorescin antibody (Roche)]. 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride/BCIP were used to stain the embryo.

Microinjection and Expression Analysis. MASO sequences of *eve*, *foxQ2*, *six3*, and *hox11/13b* were previously described (5, 14, 15, 36). The *emx* MASO sequence was 5′-ATTGTCTCTTTTCAACCCTGTTTCT-3′. Concentrations of MASOs used for microinjection were 300 or 150 μM each in double MASO injection. Approximately 3 pl of MASO solution was injected into each fertilized sea urchin egg. The injection solution included 120 mM KCl. A total of 200 MASO-injected embryos were collected at different time points. RNA was prepared using Qiagen RNAeasy Micro Kit. Total RNA was reverse-transcribed with Bio-Rad Script Kit.

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Fig. 4. The GRN model illustrating the genomic control of 2D expression pattern formation in the sea urchin ectoderm. This model is a BioTapestry presentation of all interactions among regulatory genes governing ectoderm regulatory state diversification up to the onset of gastrulation. The circuits show that domain-specific repressors are commonly used to define the boundaries along both embryonic axes. Evidence and references supporting the linkages shown in the network are summarized in Table S1.