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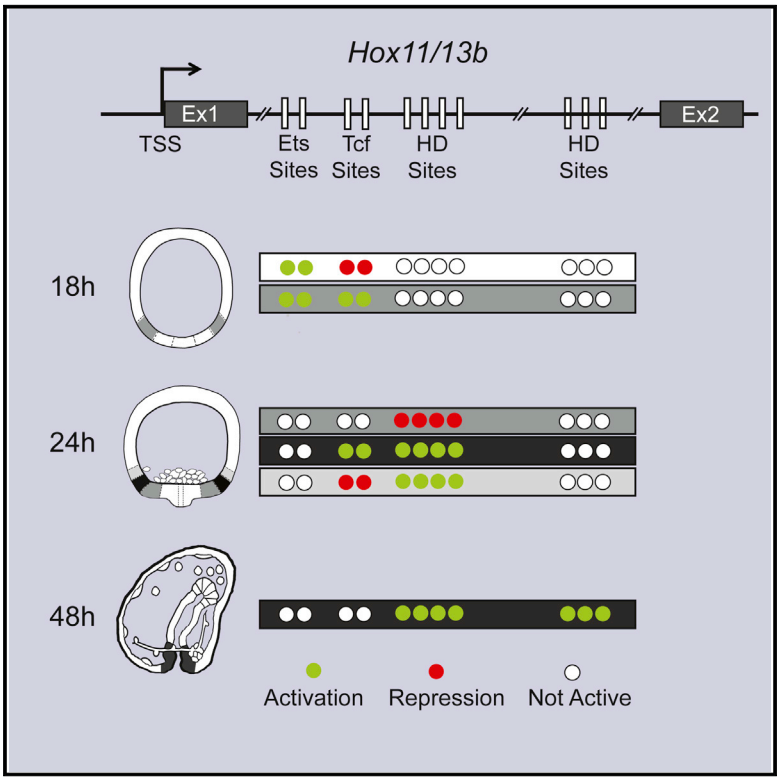
Sequential Response to Multiple Developmental Network Circuits Encoded in an Intronic *cis*-Regulatory Module of Sea Urchin *hox11/13b*

Graphical Abstract



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In Brief

Gene expression is controlled by developmentally changing regulatory states. Cui et al. now find that the dynamic patterns of *hox11/13b* expression in response to distinct regulatory states during sea urchin endoderm development are controlled by sequentially operating transcription factor binding sites within an intronic *cis*-regulatory module.

Highlights

- A *cis*-regulatory module controls *hox11/13b* expression throughout gut organogenesis
- This *cis*-regulatory module responds to developmentally changing regulatory inputs
- Alternative use of binding sites provides context-specific gene regulation
- Combination of two juxtaposed modules is necessary for expression in the hindgut

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Sequential Response to Multiple Developmental Network Circuits Encoded in an Intronic *cis*-Regulatory Module of Sea Urchin *hox11/13b*

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SUMMARY

Gene expression in different spatial domains is often controlled by separate *cis*-regulatory modules (CRMs), but regulatory states determining CRM activity are not only distinct in space, they also change continuously during developmental time. Here, we systematically analyzed the regulatory sequences controlling *hox11/13b* expression and identified a single CRM required throughout embryonic gut development. We show that within this CRM, distinct sets of binding sites recognizing Ets, Tcf, and homeodomain transcription factors control the dynamic spatial expression of *hox11/13b* in each developmental phase. Several binding sites execute multiple, sometimes contradictory, regulatory functions, depending on the temporal and spatial regulatory context. In addition, we identified a nearby second CRM operating in inter-modular AND logic with the first CRM to control *hox11/13b* expression in hindgut endoderm. Our results suggest a mechanism for continuous gene expression in response to changing developmental network functions that depends on sequential combinatorial regulation of individual CRMs.

INTRODUCTION

Gene regulatory networks (GRNs) control cell fate specification by determining the combination of transcription factors expressed within each nucleus (Peter and Davidson, 2015). As a result of developmental GRN function, spatially and temporally distinct regulatory states are expressed throughout development. Although the expression of given genes in multiple spatial domains is frequently controlled by separate *cis*-regulatory modules (CRMs), the control of gene expression during different temporal phases of development is not as well understood. Many genes, in particular regulatory genes, are expressed over long periods of developmental time, and their *cis*-regulatory apparatus must cope with a continuously changing regulatory landscape and respond to multiple GRN circuitries active during development.

The GRNs determining endomesodermal specification in *Strongylocentrotus purpuratus* embryos are particularly well understood and provide an opportunity to study the organization of *cis*-regulatory sequences responding to known network circuits (Davidson et al., 2002; Peter et al., 2012). A system level perturbation analysis of the endodermal GRNs revealed that Hox11/13b is one of the earliest transcription factors required for specification of endodermal cell fates (Peter and Davidson, 2010, 2011). During pre-gastrular development, anterior and posterior endoderm become differentially specified (Peter and Davidson, 2010, 2011). Just prior to gastrulation, the anterior endoderm descending from endomesoderm precursors expresses regulatory genes such as *foxa* and *blimp1b*, whereas the posterior endoderm descending from endo-ectodermal progenitors expresses *hox11/13b*. After the onset of gastrulation, the anterior endoderm will give rise to foregut and midgut endoderm, whereas *hox11/13b* expression marks the progenitors of hindgut endoderm (Arenas-Mena et al., 2006; J. Valencia and I.S.P., unpublished results). Perturbation of Hox11/13b causes gut malformation, particularly affecting formation of the hindgut (Arenas-Mena et al., 2006). Hox11/13b is one of only two sea urchin Hox transcription factors expressed during embryonic development (Arenas-Mena et al., 2006; Cameron et al., 2006). Similar to *hox11/13b*, *hoxa13* and *hoxd13* are expressed in the posterior gut of vertebrates including mice, zebrafish, and chicken (de Santa Barbara and Roberts, 2002; Scotti et al., 2015; Warot et al., 1997; Zacchetti et al., 2007).

Previous GRN analyses identified several regulatory inputs controlling *hox11/13b* expression, including Wnt signaling, Even-skipped (Eve), and Hox11/13b itself, which mediates auto-repression (Figure 1C; Cui et al., 2014; Peter and Davidson, 2010, 2011). A Boolean computational model of the endomesodermal GRN demonstrated that these inputs are consistent with the spatial and temporal expression pattern of *hox11/13b* during pre-gastrular development (Peter et al., 2012). To identify the role of these and possibly additional regulatory inputs during gut development and to resolve the organization of the regulatory sequence responding to these inputs, we systematically analyzed the *cis*-regulatory control of *hox11/13b*. We used a

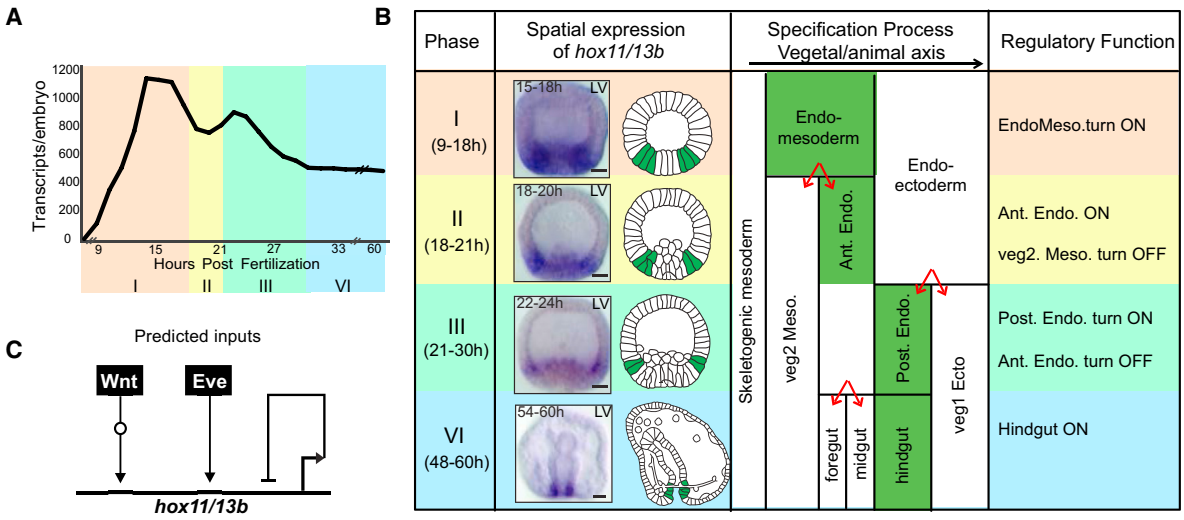


Figure 1. Developmental Expression of *hox11/13b*

(A) Time course of *hox11/13b* expression levels analyzed by qPCR.

(B) Schematic summary of developmental phases of *hox11/13b* spatial expression. The left panel shows images of embryos stained by WMISH detecting *hox11/13b* expression to the left, next to embryo schemes representing *hox11/13b* expression in green. In the middle panel, the specification of vegetal cell fates are shown schematically, with expression of *hox11/13b* (green) in the endomesoderm (phase I), anterior endoderm (phase II), posterior endoderm (phase III), and hindgut endoderm (phase IV). Red arrows indicate the specification of different cell fates descending from common precursors. The right panel indicates the regulatory functions that are encoded in the *hox11/13b cis*-regulatory sequence. Scale bar, 20 μm.

(C) The predicted regulatory inputs into *hox11/13b* (see text). Meso, mesoderm; Endo, endoderm; Ecto, ectoderm; Ant., anterior; Post., posterior; LV, lateral view.

high-throughput tag system to analyze 140 kb of genomic sequence at the *hox11/13b* locus for regulatory modules that function during gut organogenesis from 10 hr to 60 hr of embryonic development. We found that during this entire time period, the spatial expression of *hox11/13b* is controlled by an intronic CRM that responds sequentially to several regulatory network circuits. Different sets of transcription factor binding sites within this CRM are utilized alternatively to respond to the specific regulatory states expressed in each developmental phase. A second CRM, located 700 bp downstream of the first CRM, is required together with the first CRM for expression of *hox11/13b* in hindgut precursor cells after the onset of gastrulation. We identified binding sites in both CRMs that are simultaneously required for *hox11/13b* expression during late development, operating in AND logic despite a distance of about 1 kb. This alternative use of transcription factor binding sites within the *hox11/13b* CRM reveals a surprisingly simple and elegant mechanism to encode the response to developmentally changing regulatory states and diverse network circuits.

RESULTS

Identification of Active *cis*-Regulatory Sequences in the *hox11/13b* Locus

Throughout development of the gut in sea urchin embryos, *hox11/13b* is expressed specifically in the endoderm and its precursors. *Hox11/13b* expression in the endoderm lineage occurs in several phases, as summarized in Figure 1 (Peter and Davidson, 2010, 2011). First, *hox11/13b* is expressed in the endomesoderm at 9 hr, with peak levels at 15 hr (Figures 1A and 1B, phase I). Second, by 18 hr, expression of *hox11/13b* becomes restricted to the anterior endoderm and is cleared from the mesoderm (Figure 1B, phase II). However, a few hours later, at 21–24 hr, *hox11/13b* expression is activated in the posterior endoderm and turns off in the anterior endoderm (Figure 1B, phase III). After the onset of gastrulation (30 hr), *hox11/13b* continues to be expressed in posterior endoderm cells, giving rise to the hindgut (Figure 1B, phase IV).

To identify the regulatory sequences controlling *hox11/13b* expression during gut organogenesis, we used a recombinant *hox11/13b* BAC construct (sp4005C17), including 140 kb of the *hox11/13b* locus (Figure 2A). This BAC construct includes large parts of the intergenic region between *hox11/13b* and its neighboring genes *hox11/13a* and *hox11/13c* as well as two exons of *hox11/13c*. A GFP reporter gene cassette was inserted into the first exon of *hox11/13b*, and recombinant *hox11/13b* BACs were injected into sea urchin embryos to analyze spatial and quantitative reporter gene expression at several developmental stages (Figures 2B and 2C). Due to the mosaic incorporation of injected DNA, individual images may only represent partial expression patterns, and multiple embryos were scored to reveal the complete spatial expression pattern (Figure S1A). In this and all subsequent experiments determining the spatial expression of GFP, we used double in situ hybridizations, with probes detecting GFP as well as *foxa* transcripts, to mark the endomesoderm (up to 18 hr) and anterior endoderm (18–30 hr). Our results show that expression driven by the *hox11/13b* BAC largely recapitulates expression of endogenous *hox11/13b* (Figures 2B and 2C). Thus, the recombinant *hox11/13b* BAC encodes the complete set of *cis*-regulatory sequences controlling

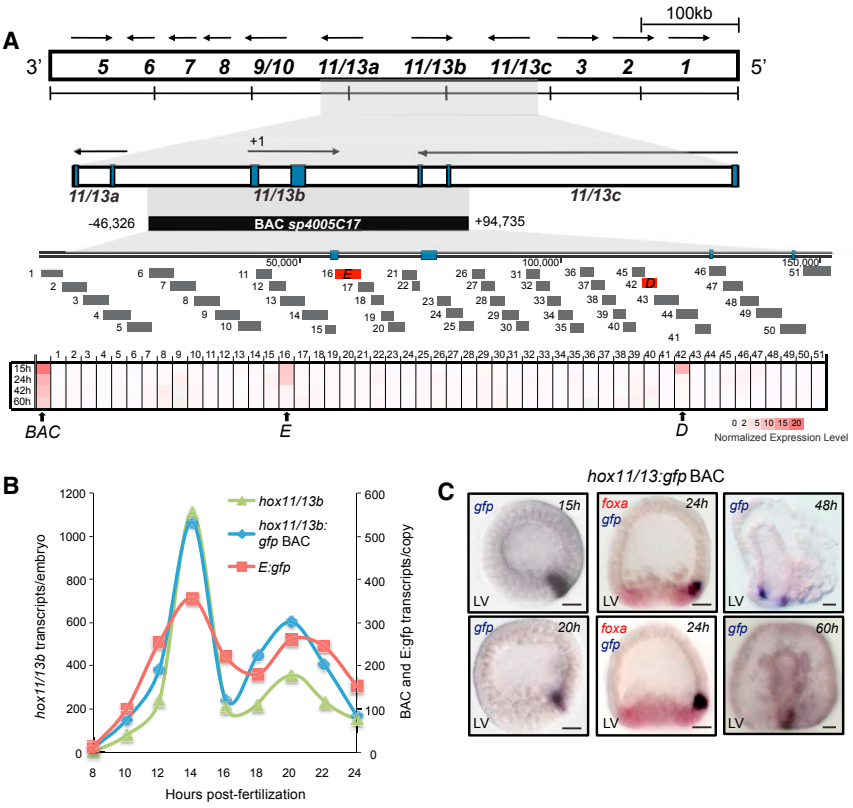


Figure 2. Identification of Regulatory Sequences Controlling Developmental Expression of *hox11/13b*

(A) From the top, a map showing the Hox locus, the region encoding *hox11/13b* and adjacent genes, the region included in the *hox11/13b* BAC, and the 51 fragments. The regulatory activity of each fragment as measured by qPCR at 15 hr, 24 hr, 42 hr, and 60 hr is shown on the bottom.

(B) Time course of *hox11/13b* and GFP expression levels in embryos injected with *hox11/13b* BAC or module E-GFP analyzed by qPCR.

(C) Spatial expression of GFP transcripts in embryos injected with *hox11/13b* BAC. Scoring results shown in Figure S1. *Foxa* expression marks anterior endoderm at 24 hr. LV, lateral view. Scale bar, 20 μm.

endoderm-specific expression of *hox11/13b* throughout gut development. However, although expression of endogenous *hox11/13b* is cleared from the anterior endoderm by 24 hr, expression of GFP reporter is still detectable in this domain at 24 hr and 30 hr in embryos injected with the *hox11/13b* BAC, although at decreasing levels (Figure S1A). This difference is most likely due to distinct RNA degradation rates of GFP and *hox11/13b* transcripts.

To identify the individual CRMs driving *hox11/13b* expression in each developmental phase, 51 fragments were generated, covering the 140 kb of the *hox11/13b* BAC and excluding the two *hox11/13b* exons (Figure 2A). Each fragment was 3–5 kb, including 500 bp of overlapping sequence between adjacent fragments. The regulatory activity of these fragments was determined using a high-throughput "barcode" reporter system (Nam and Davidson, 2012; Nam et al., 2010). Thus, individual fragments were placed upstream of the *hox11/13b* basal promoter (BP), the GFP reporter gene, and a unique sequence tag used to distinguish GFP transcripts generated under control of different *cis*-regulatory fragments. The *hox11/13b* BP used in these constructs was identified by testing the transcriptional activity of sequences proximal to the transcription start site and includes the 279 bp upstream of the transcription start site (not shown). Reporter constructs were pooled and injected into fertilized sea urchin eggs, and tag-specific GFP expression levels were analyzed by qPCR at 15 hr, 24 hr, 42 hr, and 60 hr (Figure 2A). The results were compared to the activity of the *hox11/13* BAC. Surprisingly, out of 51 fragments covering almost 140 kb of potential regulatory sequence, only two fragments were found to activate reporter gene expression: module E and module D. Module E is located within the single *hox11/13b* intron and activated reporter gene expression at 15 hr and 24 hr. Module D is located about 62 kb downstream of the transcription start site of *hox11/13b* and activated expression at 15 hr only. None of the fragments drove expression at 42 hr or 60 hr, although the regulatory sequences responsible for *hox11/13b* expression during these later phases of development are included in the BAC.

Identification of a CRM Controlling *hox11/13b* during Pre-gastrular Development

The regulatory activity of modules E and D was tested by injection of individual reporter constructs into sea urchin embryos. Reporter gene constructs containing either module E or module D showed a temporal expression profile similar to *hox11/13b*, with no activity at 6 hr and peak expression levels at 15 hr (Figures 2B and 3A). After 15 hr, the activity of module D dropped sharply and was barely detectable at 21 hr and 30 hr, whereas embryos injected with module E continued to express the reporter gene, although at lower levels. When the spatial expression of GFP was analyzed, embryos injected with module D constructs showed ubiquitous GFP expression at 18 hr, whereas embryos injected with module E constructs showed specific expression in the endomesoderm at 18 hr and in anterior and posterior endoderm at 20 hr and 24 hr (Figures 3B, S1B, and S1C). Thus, module E encodes sufficient regulatory information to recapitulate the spatial and temporal expression pattern of *hox11/13b* during pregastrular development (Figure 2B).

Module E includes the first 5 kb of the *hox11/13b* intron. To identify active CRMs within module E, we tested successive deletion fragments of module E for transcriptional activity at 15 hr (Figures S2A and S2B). We identified module ME (597 bp), which, upon injection into sea urchin embryos, recapitulated spatially and temporally the expression pattern of module E and *hox11/13b* during

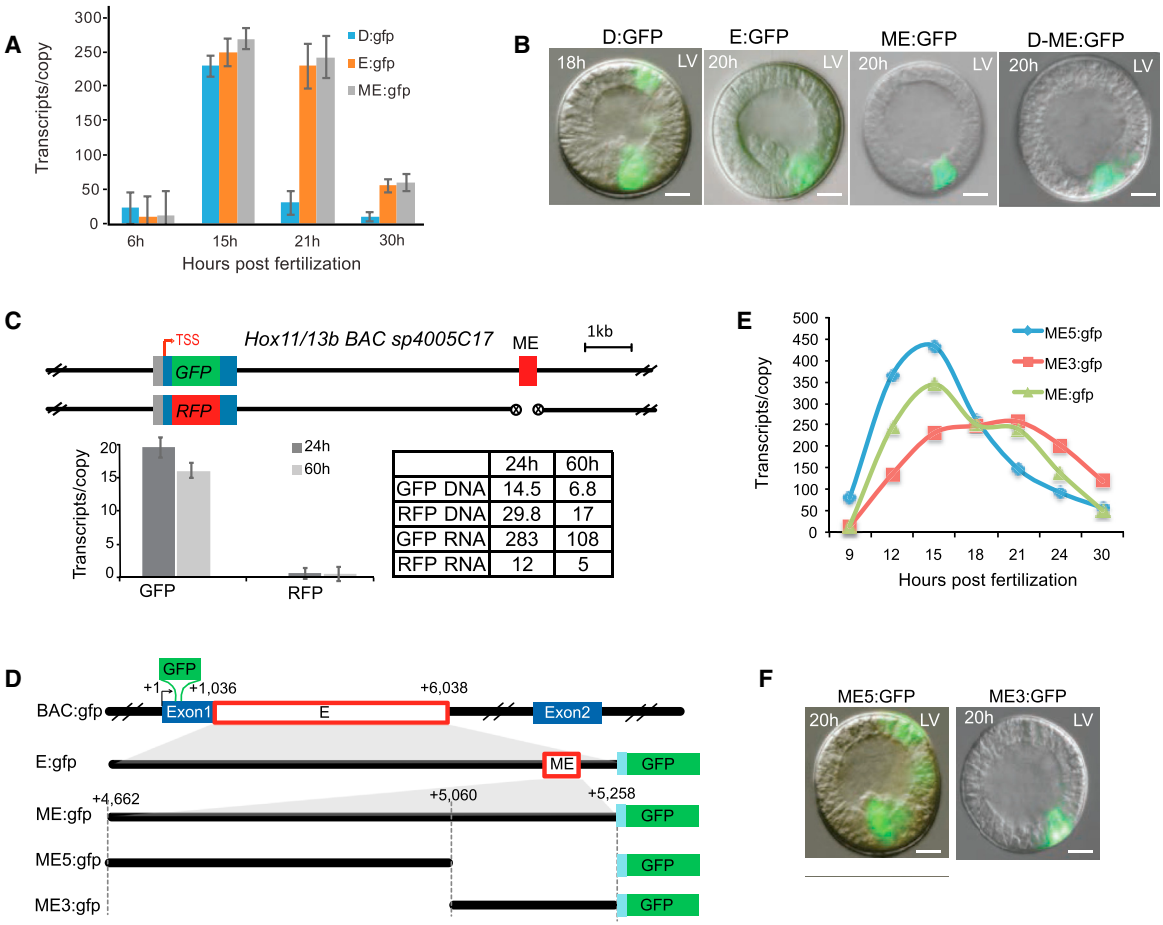


Figure 3. Module ME Recapitulates *hox11/13b* Expression in Pre-gastrular Embryos

(A) GFP expression in embryos injected with constructs of modules D, E, or ME was analyzed by qPCR.

(B) Spatial expression of GFP in embryos injected with D:GFP, E:GFP, ME:GFP, and D-ME:GFP. Scale bar, 20 μm.

(C) Reporter gene expression controlled by wtBAC:GFP or ΔME-BAC:RFP was analyzed by qPCR, shown in the table as transcripts/embryo (RNA) and BAC copy number (DNA) and shown in the diagram as normalized expression levels.

(D) Map showing modules E, ME, ME5, and ME3.

(E) Time course expression of ME:GFP, ME5:GFP, and ME3:GFP.

(F) Spatial expression of ME5:GFP and ME3:GFP at 20 hr. LV, lateral view. Scale bar, 20 μm. n = 3–5, error bars indicate SD.

pre-gastrular development (Figures 3A, 3B, and S2C). To test whether module ME is necessary for *hox11/13b* expression, we deleted module ME from the recombinant *hox11/13b* BAC and replaced GFP with a red fluorescent protein (RFP) reporter gene. When sea urchin embryos were co-injected with ΔME BAC:RFP and wild-type BAC:GFP, expression of GFP was detected by qPCR at both 24 hr and 60 hr, whereas no RFP expression was observed at both time points (Figure 3C). Module ME therefore encodes the *cis*-regulatory functions necessary as well as sufficient to control *hox11/13b* expression during early sea urchin development.

To test whether module D contributes to the activation of *hox11/13b* expression, a D-ME:GFP reporter construct was generated containing modules D and ME upstream of a GFP reporter gene. In embryos injected with D-ME:GFP, reporter gene expression levels increased by 40% at 15 hr compared to embryos injected with ME:GFP (Figure S3A). However, spatial expression of GFP in embryos injected with D-ME:GFP was similar to the expression pattern controlled by module ME (Figure S3B). Thus, although module D may contribute to the rapid increase of *hox11/13b* expression levels during initial activation, it is not required and possibly contributes to the control of other nearby genes. Most importantly, module D does not affect the spatial expression of *hox11/13b*, and module ME alone controls the spatial expression of *hox11/13b* in three developmental phases in response to presumably distinct regulatory contexts (Figure 1B, phases I–III). We next analyzed the binding sites within module ME required for transcriptional activation of *hox11/13b* expression in each developmental phase.

Early Expression in Endomesoderm Controlled by Ubiquitous Activation and Tcf-Mediated Spatial Repression: Phase I

Despite systematic perturbation of all transcription factors known to be specifically expressed in endoderm precursors

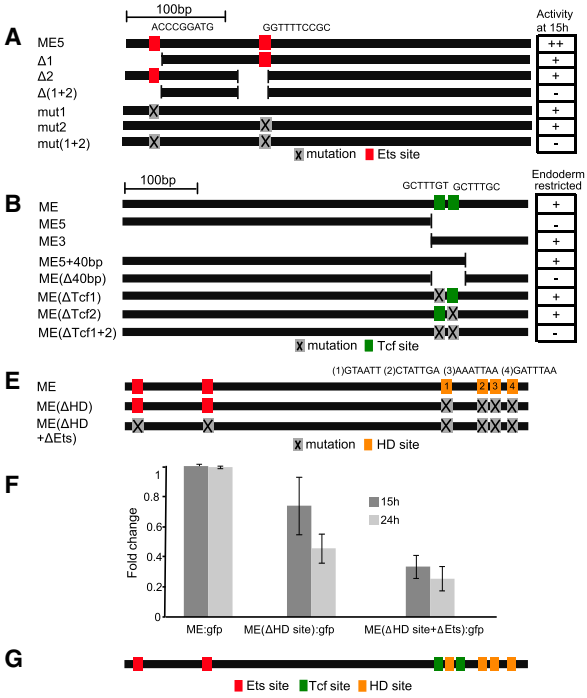


Figure 4. Binding Sites for Ets, Tcf, and HD Transcription Factors Control Activity of Module ME

(A) Map of ME5 constructs with Ets site (red box) deletions and mutations, showing GFP expression levels compared to wt ME5 on the right. -, 0%–33%; +, 33%–66%; ++, 67%–100%. Complete results are shown in Figure S4B.

(B) Map of ME constructs with Tcf site mutations and deletions, showing to the right endodermal expression of GFP in embryos injected with mutated constructs. See Figure S5B for data.

(C) GFP expression in embryos injected with ME:GFP and MEΔTcf:GFP constructs, showing that mutation of Tcf sites results in ubiquitous (15 hr) and pan-veg1 (24 hr) expression. Dashed line shows the endoderm/ectoderm boundary. *Foxa* expression marks the anterior endoderm. LV, lateral view. Scale bar, 20 μm.

(D) *Hox11/13b* and GFP expression levels in embryos injected with ME:GFP and co-injected with Eve morpholino (MASO) versus control (Ctl) morpholino.

(E) Map showing ME constructs with mutations in HD and Ets sites.

(F) GFP expression levels in embryos injected with ME:GFP constructs containing mutations in HD sites or HD and Ets sites.

(G) Map of all functional binding sites discovered in module ME.

(H) Embryo scheme showing spatial expression of ME:GFP with HD site mutation; see Figures S5D–S5F for data and Figure S7A for the sequence of module ME. n = 3–5, error bars indicate SD.

during pre-gastrular development, no regulatory input in addition to Tcf was identified to be controlling *hox11/13b* expression before 21 hr (Peter and Davidson, 2010). To identify transcription factor binding sites necessary for early expression of *hox11/13b*, we generated serial deletion constructs of module ME and injected them into sea urchin embryos (Figure S3C). When analyzed at 15 hr, a construct containing 200 bp of the 3′ end of ME (fragment ME3) showed reduced expression levels compared to module ME, but a similar spatial distribution (Figures 3D–3F and S3E). On the other hand, deletion of ME3 from module ME (ME5) resulted in ubiquitous GFP expression, without affecting expression levels (Figures 3D–3F and S3D). These results indicate that the 5′ fragment of module ME controls ubiquitous activation, whereas spatial regulation of gene expression is encoded in the 3′ fragment.

To analyze the regulation of ubiquitous *hox11/13b* expression, we generated sequential 40-bp deletions throughout ME5 (Figure S4A). Two ME5 deletion constructs showed decreased expression levels compared to the ME5 construct at 15 hr, and simultaneous deletion of both sequences resulted in >3-fold reduction in reporter gene expression levels (Figures 4A and S4B). Within each of the two 40-bp regions, sequences resembling the Ets factor-binding motif were identified (Weirauch et al., 2014). To test the function of the predicted Ets sites, ME5 constructs with mutation in either one or both motifs were injected into embryos, resulting in a reduction of expression levels comparable to the corresponding deletion constructs (Figures 4A and S4B). These results suggest that a ubiquitously expressed transcription factor, possibly an Ets factor, serves as a direct activator of *hox11/13b* expression during early sea urchin development. Five regulatory genes encoding Ets family transcription factors are expressed ubiquitously during early embryogenesis: *ets1/2*, *tel*, *erf*, *ets4*, and *elk* (Rizzo et al., 2006). Most of these genes are expressed maternally, which precludes effective perturbation experiments in this system. The identity of the responsible ubiquitous transcription factor(s) therefore remains unresolved.

To identify the mechanism controlling spatial expression of *hox11/13b* in the endomesoderm, we generated ME reporter constructs with serial 3′ end deletions and tested them in sea urchin embryos (Figure S5A). The results show that extending the ubiquitously active ME5 by only 40 bp restricted GFP expression to the endomesoderm (Figures 4B and S5B). In turn, deletion of these 40 bp from module ME resulted in ubiquitous expression of GFP. Within these 40 bp, we found two sites matching the Tcf consensus motif. Although mutation of either Tcf motif alone did not affect GFP localization, simultaneous mutation of both presumptive Tcf sites led to ubiquitous GFP expression at 15 hr (Figures 4B, 4C, and S5B). Thus, similar to other regulatory genes in the endoderm GRN, Tcf is responsible for the spatial control of gene expression (Ben-Tabou de-Leon and Davidson, 2010; Peter and Davidson, 2010, 2011; Smith et al., 2008). In sea urchin embryos, nuclear β-catenin is maternally localized to the skeletogenic mesoderm and the veg2 endomesoderm, enabling expression of Tcf target genes in these cells (Logan et al., 1999). In the absence of nuclear β-catenin, binding of

Tcf to the co-repressor Groucho (Range et al., 2005) leads to repression of *hox11/13b* in all other cells of the embryo.

Taken together, expression of *hox11/13b* in the early endomesoderm (Figure 1, phase I) is activated by a ubiquitously expressed presumptive Ets family factor and by maternally localized Tcf/β-catenin. Because mutation of either Ets- or Tcf-binding sites alone does not entirely abolish the activity of module ME, we conclude that both transcriptional activation functions operate in OR logic, in which the presence of either input is sufficient to drive gene expression, although perhaps at lower levels (Figure S5C). Because the regulatory input provided by Ets is sufficient to activate ubiquitous *hox11/13b* expression throughout the embryo, spatial control of *hox11/13b* expression relies exclusively on the transcriptional repression function of Tcf/Groucho, which operates throughout the ectoderm during early development.

Control of *hox11/13b* Expression in Anterior Endoderm: Phase II

After initial expression in all endomesoderm cells, *hox11/13b* expression becomes restricted to the anterior endoderm and clears from the mesoderm, similar to other endodermal regulatory genes (Figure 1, phase II; Peter and Davidson, 2010, 2011; Sethi et al., 2012). Our results show that mutation of Tcf-binding sites within module ME leads to ectopic expression of GFP in mesodermal as well as other cells of the sea urchin embryo, indicating that Tcf-mediated repression is responsible for clearance of *hox11/13b* expression in mesodermal cells (Figure 4C). In this embryo, nuclear β-catenin is detected in the entire endomesoderm until about 15 hr, when the localization of nuclear β-catenin starts being controlled by Wnt signaling (Cui et al., 2014; Logan et al., 1999). Despite abundant expression of Wnt ligands in the mesoderm, nuclear β-catenin clears from the mesoderm, presumably due to the absence of *frizzled* expression (Cui et al., 2014). Thus, our results suggest that repression of *hox11/13b* in veg2 mesoderm during phase II is again controlled by Tcf/Groucho and the clearance of nuclear β-catenin in the veg2 mesoderm, leading to specific expression of *hox11/13b* in the anterior endoderm. The same mechanism has been shown to control expression of other endodermal regulatory genes (Peter and Davidson, 2011; Sethi et al., 2012; Ben-Tabou de-Leon and Davidson, 2010).

Control of Posterior Endoderm Expression and Specification of the Endoderm/Ectoderm Boundary by Lineage-Specific Activator and Tcf: Phase III

Hox11/13b is only transiently expressed in the anterior endoderm, and by 24 hr, is cleared from this domain and instead exclusively expressed in the posterior endoderm (Figure 1, phase III). The activation of *hox11/13b* expression in these cells initiates posterior endoderm specification and defines the endoderm/ectoderm boundary in the sea urchin embryo (Li et al., 2014; Peter and Davidson, 2011). Previous perturbation analyses identified Eve and Wnt signaling as activators of *hox11/13b* expression at 24 hr (Peter and Davidson, 2011; Cui et al., 2014).

Similar to endogenous *hox11/13b*, module ME is expressed in the posterior endoderm by 24 hr (Figure S2C). *Eve* is at first expressed broadly across the vegetal half of the embryo, but becomes restricted to the veg1 lineage, composed of posterior endoderm and veg1 ectoderm, after 15 hr (Peter and Davidson, 2010). To test whether the activation of module ME is also dependent on Eve, we analyzed whether expression of the ME construct is affected by Eve perturbation. Indeed, co-injection of the ME construct with Eve morpholino resulted in reduced reporter gene expression at 24 hr when compared to control morpholinos (Figure 4D), showing that module ME is activated downstream of Eve. The decreased expression of ME5 constructs after 15 hr suggests that activation of *hox11/13b* at 24 hr is controlled by sequences in ME3 (Figure 3E). Within the 200 bp of ME3, four predicted homeodomain (HD) factor-binding motifs were identified. Mutation of all four sites in the ME construct decreased reporter gene expression levels by about 50% at 24 hr (Figures 4E and 4F). These results suggest that Eve activation of *hox11/13b* expression in the posterior endoderm occurs directly through the HD-binding sites within module ME. Furthermore, we tested whether the Ets sites also contribute to the regulatory activity of module ME. Indeed, simultaneous mutation of Ets- and HD-binding sites resulted in further reduction of module ME expression levels to approximately 30% at 15 hr and 24 hr (Figure 4F).

Although Eve activates the expression of *hox11/13b*, Eve alone is not sufficient to control *hox11/13b* expression because Eve is expressed in all veg1 cells, including veg1 ectoderm, as early as 15 hr, without activating *hox11/13b*. Indeed, the two Tcf-binding sites in module ME are located in close proximity to the HD-binding sites (Figure 4G), and mutation of these Tcf sites results in decreased expression at 15 hr and even more prominently at 21 hr (Figure S5C). To test the function of these Tcf sites, embryos were injected with a ME-ΔTcf construct. When GFP expression was analyzed at 24 hr by whole mount in situ hybridization (WMISH), ectopic expression was observed in veg1 ectodermal cells (Figure 4C). This indicates that in the absence of Tcf binding, activation by Eve is sufficient to support *hox11/13b* expression in the veg1 ectoderm, and that Tcf/Groucho is necessary for restricting expression to the posterior endoderm.

Simultaneous to the activation of *hox11/13b* expression in posterior endoderm, expression ceases in anterior endoderm cells (Peter and Davidson, 2011). Previous results indicate that blocking expression of Hox11/13b proteins by morpholino injection interferes with the clearance of *hox11/13b* transcripts in anterior endoderm (Peter and Davidson, 2011). If module ME encodes the mechanism for repressing *hox11/13b* in the anterior endoderm, it should also be affected by perturbation of Hox11/13b. Indeed, co-injection of the ME construct with Hox11/13b morpholino resulted in 60% of embryos showing GFP expression in the anterior endoderm at 30 hr versus 20% of embryos co-injected with a control morpholino (Figure S5D). Because Eve and Hox11/13b recognize similar binding motifs, we examined the spatial expression driven by ME constructs with mutated HD sites (Figures S5E and S5F). As expected, embryos injected with the mutated construct failed to activate reporter gene expression in the posterior endoderm, whereas 60% of embryos injected with the wild-type ME construct showed GFP expression in this domain at 24 hr. On the other hand, about

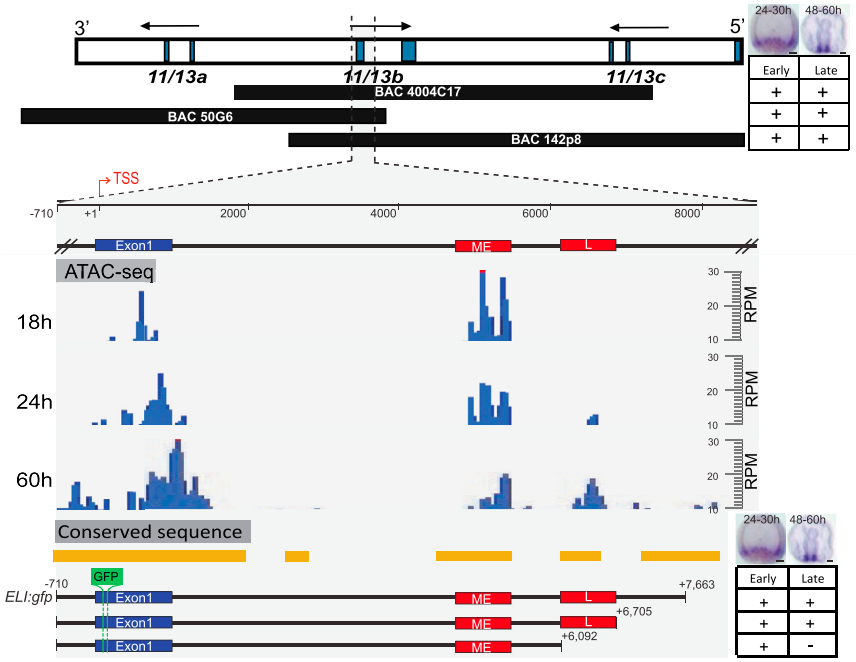


Figure 5. Identification of Second Intronic Module Required for Hindgut Expression

Map of three recombinant BACs controlling GFP expression at 24 hr and 48 hr, marked with "+." ATAC-seq data at 18 hr, 24 hr, and 60 hr and sequences conserved in comparison with *L. variegatus* are shown in the genome browser. Bottom: map of ELI:GFP constructs, with endodermal expression on the right showing that deletion of module L abolishes late expression. RPM, reads per million. Scale bar, 20 μm.

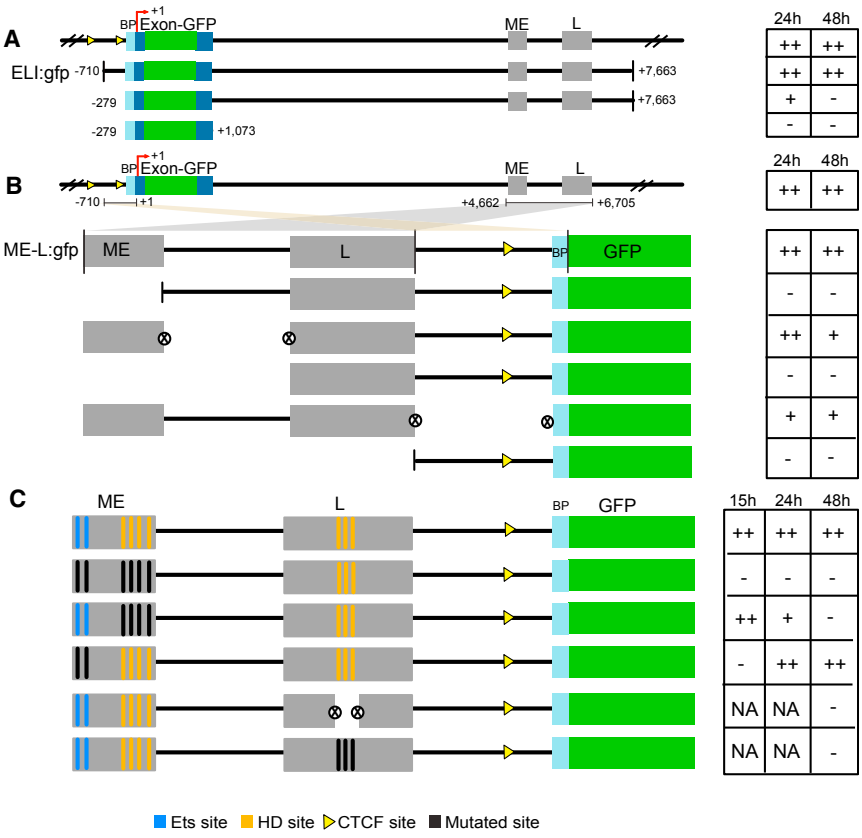
40% of embryos injected with the wild-type ME construct show GFP expression in the anterior endoderm at 30 hr versus 70% of embryos injected with the mutant construct (summarized in Figure 4H). These results indicate that the HD sites in module ME control both the activation of *hox11/13b* expression in posterior endoderm in response to Eve and the repression in anterior endoderm in response to Hox11/13b.

Taken together, these results indicate that the specific expression of *hox11/13b* in the posterior endoderm during phase III is activated by Eve, which is expressed broadly in the veg1 lineage, whereas spatial expression in posterior endoderm after 21–24 hr is controlled by Tcf. Consistent with the idea that the presence of β-catenin prevents Tcf-dependent repression of *hox11/13b*, levels of nuclear β-catenin increase in the endodermal portion of veg1 descendants at this developmental stage (Logan et al., 1999). Expression of Wnt1 and Wnt16 in the anterior endoderm was previously shown to activate *hox11/13b* expression in adjacent posterior endoderm cells (Cui et al., 2014). Nuclearization of β-catenin in response to Wnt signaling from adjacent anterior endoderm cells and subsequent expression of *hox11/13b* controlled by module ME thus defines the endoderm/ectoderm boundary in the sea urchin embryo. Subsequently, Hox11/13b activates the expression of *wnt1* and *wnt16* in posterior endoderm cells, and continuous expression of *hox11/13b* is controlled by a community effect circuit among Wnt1, Wnt16, and Hox11/13b (Cui et al., 2014). Because mutation of Tcf-binding sites leads to ectopic expression of the ME reporter gene in veg1 ectodermal cells, Eve alone is sufficient for activating *hox11/13b* expression, and operates in OR logic with Tcf/β-catenin. Thus, the spatial and temporal expression pattern of *hox11/13b* in the posterior endoderm again is controlled by Tcf-mediated repression, restricting the function of a transcriptional activator, here Eve, which is present more broadly and earlier in development. In addition, the HD sites in module ME not only mediate the activation of *hox11/13b* expression in the posterior endoderm, but also the repression of *hox11/13b* expression in the anterior endoderm, which is controlled by a negative auto-regulatory feedback.

Identification of CRMs Driving Expression in the Hindgut

Surprisingly, none of the 51 fragments covering the *hox11/13b* BAC induced GFP expression after the onset of gastrulation at 30 hr, although the *hox11/13b* BAC contains regulatory sequences driving gene expression in the hindgut (Figures 1, Phase IV and 2C). In addition, two other BACs that are largely non-overlapping with the original *hox11/13b* BAC drive reporter gene expression during later development. However, all three BACs contain the coding regions and part of the intron of *hox11/13b* (Figure 5), and we thus examined the regulatory activity of individual fragments of the 30-kb region included in all three BACs. A fragment of about 11 kb starting at 710 bp upstream of the transcription start site and encompassing approximately 8 kb of intronic sequence, including module ME, produced accurate spatial reporter expression at 60 hpf (early and late intronic fragment [ELI]; Figure S6A). The identified ELI fragment thus contains regulatory sequences supporting early as well as late expression of *hox11/13b*.

To identify active regulatory sequences, we analyzed chromatin accessibility at 18 hr, 24 hr, and 60 hr by ATAC-seq (Buenrostro et al., 2013). Although module ME is accessible at all three time points, sequences downstream of module ME are accessible at 60 hr but remain inaccessible at 18 hr and 24 hr (Figure 5). Deletion of these sequences in the ELI construct showed that a 700-bp DNA fragment ("module L") located 700 bp downstream of module ME is required for late but not early reporter gene expression (Figures 5 and S6B). Module L also shows sequence conservation when compared to orthologous sequences in a related sea urchin species, *Lytechinus variegatus* (Figure 5), whereas the 700-bp sequence between modules ME and L is not conserved and shows no increase in accessibility in the ATAC-seq experiment. An additional sequence fragment positioned just upstream of the *hox11/13b* basal promoter and spanning approximately 400 bp shows ATAC-seq peaks specifically at 60 hr. Indeed, injection of embryos with an ELI construct



24h	48h
++	++
++	++
+	-
-	-

24h	48h
++	++

++	++
-	-
++	+
-	-
+	+
-	-

15h	24h	48h
++	++	++
-	-	-
++	+	-
-	++	++
NA	NA	-
NA	NA	-

Figure 6. Binding Sites in Modules ME and L Function Combinatorially to Control Late Expression

(A) Deletion constructs of ELI:GFP, showing that deletion of the promoter proximal region leads to a decrease in GFP expression at 24 hr and 48 hr. Symbols are as in Figure 4A, and data are shown in Figure S6B.

(B) Deletion constructs of ME-L:GFP, showing that modules ME and L as well as the inter-modular sequence and promoter proximal region are required for early and late expression. Symbols are as in Figure 4A, and data are in Figure S6C.

(C) Mutation of binding sites within ME-L:GFP showing that HD sites in modules ME and L are required for late expression. NA, not analyzed. See Figure S6E for data and Figure S7B for sequence of module L.

lacking this promoter proximal region resulted in reporter gene expression levels comparable to that of the negative control construct (Figures 6A and S6B).

Because one of the initial fragments, fragment 17, despite containing module L did not drive late gene expression, we concluded that module L is not sufficient to drive expression of *hox11/13b* in the hindgut endoderm. Furthermore, deletion of module ME from the *hox11/13b* BAC not only abolished GFP expression at 24 hr but also at 60 hr, suggesting that module ME is also required during phase IV of *hox11/13b* expression (Figures 2A and 3C). On the other hand, module ME by itself is not sufficient either to drive late expression at 48 hr and 60 hr, indicating that modules ME and L are both required for *hox11/13b* expression after the onset of gastrulation.

To find the minimal regulatory sequence supporting expression of *hox11/13b* in the hindgut, we generated construct ME-L by placing a 1.5-kb fragment containing modules ME and L upstream of the promoter proximal region and the basal promoter (Figures 6B and S6C). Indeed, when injected into embryos, this construct activates reporter gene expression at both 24 hr and 48 hr. As expected, deletion of module ME abolishes this activity at both developmental time points. More surprisingly, deleting the sequences between modules ME and L also reduces expression levels at 48 hr but not at 24 hr (Figures 6B and S6C). Although we cannot exclude the possibility that the fragment between modules ME and L is itself bound by specific transcription factors, the absence of ATACseq peaks at several stages between 24 hr and 60 hr (not shown) suggests that this sequence might play a different role in the regulation of *hox11/13b*. Furthermore, deleting the promoter proximal region from this construct reduces the transcriptional activity both early and late in development. These results indicate that the late expression of *hox11/13b* is controlled by two CRMs that not only require the sequence between them, but also rely on a promoter proximal region, even when placed directly upstream of the basal promoter, suggesting that the two CRMs may operate simultaneously by looping interactions. Indeed, the promoter proximal region does contain a predicted CTCF-binding site (Figure S6B), and looping of both CRMs to the basal promoter region was confirmed by 4C sequencing analysis (Figure S6D).

Modules ME and L Function Combinatorially in the Hindgut: Phase IV

To identify the specific binding sites in module ME contributing to late gene expression, we first mutated binding sites of the early activators Ets and Eve, while leaving all other sequences within the ME-L construct intact. As expected, GFP expression driven by the ME construct with mutated Ets/HD sites was significantly decreased at 15 hr and 24 hr, but, surprisingly, also at 48 hr (Figures 6C and S6E). Mutating either the two Ets-binding sites or the four HD-binding sites within the ME-L construct showed that the HD sites, but not the Ets sites, are necessary for late expression of *hox11/13b* (Figures 6C and S6E).

To identify regulatory sequences within module L driving *hox11/13b* expression during hindgut formation, we focused on a 142 bp with highest accessibility in the ATAC-seq analysis. Deletion of this 142-bp fragment in the ME-L construct showed that this sequence is required for regulating expression of *hox11/13b* at 48 hr (Figures 6C and S6E). Within these 142 bp, we found three putative HD-binding sites. Simultaneous mutation of all three putative HD sites strongly reduced GFP

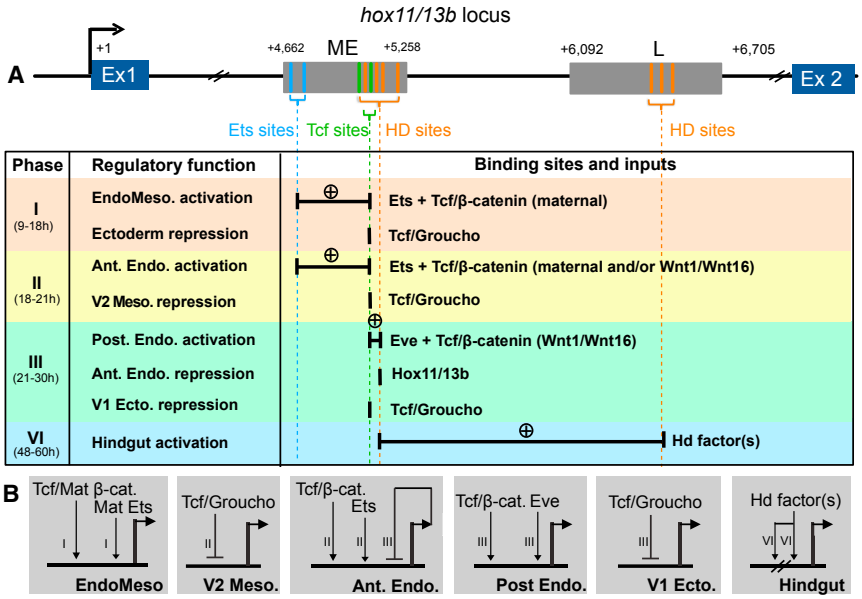


Figure 7. Summary Diagram Showing Developmental Control of *hox11/13b* Expression

(A) Map showing the organization of transcription factor binding sites within intronic modules ME and L and their contribution to the control of *hox11/13b* expression in each phase of endoderm development. Sites being used in each phase are marked as black vertical lines. Black horizontal lines connect the different sites needed for the same regulatory function. Phases I–IV are as defined in Figure 1.

(B) Regulatory inputs controlling *hox11/13b* expression in each spatial domain. The developmental phase (I–IV) indicates temporal activity of each regulatory input. EndoMeso, endomesoderm; V2 Meso, veg2 mesoderm; Ant. Endo., anterior endoderm; Post. Endo., posterior endoderm; V1 Ecto., veg1 ectoderm; Mat, maternal.

expression levels at 48 hr to a similar extent as the deletion of the entire 142-bp sequence (Figure 6C). Several HD transcription factors are expressed in the developing sea urchin hindgut at 48 hr, which could function as activators of *hox11/13b* expression, including Eve, Cdx, and Hox11/13b (Annunziata and Arnone, 2014; J. Valencia and I.S.P., unpublished data). Although the specific transcription factor(s) regulating module L remain to be resolved, these results show that expression of *hox11/13b* in the hindgut endoderm is controlled by transcription factors binding to the HD sites in module ME and in module L, operating by combinatorial AND logic over a distance of more than 1 kb.

DISCUSSION

Despite the complex developmental expression of *hox11/13b* and the multiple sequentially acting network circuits determining this expression pattern, we identified a single CRM that is necessary, and, during pre-gastrular development, also sufficient to control *hox11/13b* expression during more than 50 hr of development. In addition, after the onset of gastrulation, this CRM operates together with a second CRM located in close proximity to drive *hox11/13b* expression in the hindgut.

The organization of transcription factor binding sites controlling *hox11/13b* expression during endoderm development is shown in Figure 7A. The function of these sites on *hox11/13b* expression is controlled by the specific regulatory states expressed in each developmental context. For instance, Tcf-binding sites function in the activation of *hox11/13b* expression in endomesoderm progenitors at 10 hr, their anterior endoderm descendants at 18 hr, and in the posterior endoderm at 24 hr. In addition, Tcf sites mediate the switch from activation to repression of *hox11/13b* in mesodermal cell fates at 18 hr, the switch from repression to activation in posterior endoderm cells at 24 hr, and continued repression in all ectodermal cells throughout early development (Figure 7B). On the other hand, HD sites within module ME function in the activation of *hox11/13b* expression in posterior endoderm and, together with HD-binding sites in module L, in the activation of *hox11/13b* in the prospective hindgut endoderm. Furthermore, HD sites also mediate the repression of *hox11/13b* expression in the anterior endoderm in response to the negative feedback of Hox11/13b on itself.

It might seem peculiar that two transcription factors with similar sequence-recognition motifs, Hox11/13b and Eve, serve to control antagonistic regulatory functions in the anterior and posterior endoderm through the same HD-binding sites. However, this design provides an elegant solution to a conflicting regulatory logic. That is, auto-repression of *hox11/13b* should operate only in anterior endoderm, but not in posterior endoderm, where this gene continues to be expressed throughout development of the gut. Our results suggest that direct competition between the activating Eve input and the auto-repressive Hox11/13b input for interaction with the same HD-binding sites might provide a mechanism to prevent auto-repression and ensure continued expression of *hox11/13b* in posterior endoderm cells. In addition, the HD-binding sites within module ME operate together with HD-binding sites within module L in an AND logic that functions despite a distance of approximately 1 kb.

The transcriptional control of *hox11/13b* displays several regulatory principles that have been observed in other contexts and apply more generally. For example, the spatial expression of *hox11/13b* is controlled by broadly distributed activators and spatially localized repressors, a combination frequently determining spatial gene expression in early embryos. In the skeletogenic GRN in sea urchin embryos, ubiquitous activators drive the expression of essential early regulatory genes, whereas their specific spatial expression is ensured by HesC-mediated repression in all non-skeletogenic cells (Oliveri et al., 2008). Similarly, in early *Drosophila* embryos, Bicoid provides an activating input into many regulatory genes, whereas their spatial expression is restricted by a system of localized repressors (Chen et al., 2012). Furthermore, spatial repression is mediated by transcription

factors responding to signaling pathways in a binary manner (Barolo and Posakony, 2002; discussed in Peter and Davidson, 2015). Perhaps more often than previously expected, the role of these transcription factors is to repress gene expression in the absence of the signaling input, while additional broadly expressed transcription factors activate gene expression where presence of the signal abolishes repression. Examples include the role of Su(H) in the regulation of genes along the dorsoventral axis in *Drosophila* (Ozdemir et al., 2014) and the role of Gli in the control of gene expression in the vertebrate neural tube (Cohen et al., 2014).

Our results show that the dynamically changing expression patterns of *hox11/13b* in the endodermal lineages is controlled by a limited set of transcription factor binding sites responding to several network circuits. Although expression of a given gene in different developmental contexts is often controlled by separate CRMs, we show that diverse regulatory functions can also be encoded within a single CRM. Thus, module ME controls the response to several developmentally changing regulatory states, operated by only a few transcription factor binding sites, some of which alternatively bind to different transcription factors in different regulatory contexts. The control of *hox11/13b* expression in each developmental phase is therefore executed by distinct subsets of binding sites within this CRM. Together, the binding sites within module ME integrate the information provided by the various regulatory states expressed during gut development, from definition of early progenitor fields to the specification of the hindgut.

These results furthermore indicate that combinatorial regulation of CRM function does not necessarily depend only on the simultaneous binding of transcription factors, but can also be the result of sequential deployment of transcription factors during the developmental process. *cis*-regulatory studies of similar developmental depth are so far limited, but one might expect that CRMs capable of computing developmentally changing regulatory states are a common mechanism to generate continuous gene expression outputs.

EXPERIMENTAL PROCEDURES

Generation of BAC Reporter Constructs

BAC clones Sp_4005C17, 50G8, and 142p8 were sequenced previously (Cameron et al., 2006). The protocol for insertion of GFP or RFP marker genes and for deletion of *cis*-regulatory sequences were adapted directly from published procedures using re-engineered λ phage as a source of recombinase (Holmes et al., 2015). GFP and RFP were inserted into the first exon.

Generation of *cis*-Regulatory Reporter Constructs and Binding Site Mutations

Putative regulatory sequences were inserted upstream of the *hox11/13b* basal promoter into "barcoded" GFP vectors using the Gibson assembly kit (New England Biolabs, E2611L), as reported previously (Nam and Davidson, 2012; Nam et al., 2010). For primer sequences, see Supplemental Information. Site-specific mutation of reporter constructs was generated through Gibson assembly of synthesized double-strand DNA (IDT) containing the desired mutation or deletion. Binding sites were mutated by exchanging "A" with "C" and "G" with "T," except when new sites would have been generated by such change (Figure S7C).

Reporter Assays and Injection of Morpholinos

For injection of small constructs and BAC reporters, the injection solution contained 120 mM KCl, 20 ng/μL of carrier DNA, and linearized DNA constructs at 100 molecules/pl (picoliter). In barcoded GFP reporter experiments, DNA constructs were mixed at an equal molar ratio. The morpholino injection solution contained 120 mM KCL and 300 μM morpholino. Randomized morpholinos (N_{25}) were used as control. The spatial expression of reporter genes was determined by double WMISH using probes against *gfp* and *foxa* as a marker for the endomesoderm (12–18 hr) and the anterior endoderm domain (18–30 hr), according to previous protocols (Peter and Davidson, 2010). GFP fluorescence was detected by an Axioskop 2 plus (Zeiss) compound microscope. See Supplemental Experimental Procedures for additional information.

Quantitative Analysis of Reporter Gene Expression

Barcoded *cis*-regulatory reporter constructs were pooled and injected into fertilized eggs. Approximately 300 injected embryos were collected per time point and used to extract RNA and genomic DNA. Preparation of cDNA and quantification of GFP "barcoded" sequences using specific qPCR primers and Power SYBR Green Master Mix (Life Technology) was performed as reported (Nam et al., 2010), and the reporter gene expression levels were normalized by copy number of integrated constructs (Revilla-i-Domingo et al., 2004). Experiments were performed in 3–5 independent embryonic batches.

ATAC-seq

Embryos at stage 15 hr, 24 hr, and 60 hr were harvested by centrifugation at 1,400 rpm for 5 min in 4°C. Single-cell suspension was prepared by re-suspending embryos in ice-cold dissociation buffer containing 1 M glycine, pH 8, 4 mM EGTA, and protease inhibitors (Roche mini-complete EDTA free) at room temperature for 10 min. The transposition reaction and amplification procedures were performed following a protocol described in Buenrostro et al. (2013). Data are accessible at http://www.echinobase.org/Echinobase/. See Supplemental Experimental Procedures for additional information.

ACCESSION NUMBERS

The accession numbers for the ATAC-seq and 4C-seq data reported in this paper are GEO: GSE95651 and NCBI SRA: SRP097791.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.039.

AUTHOR CONTRIBUTIONS

Conceptualization, M.C. and I.S.P.; Investigation, M.C. and E.V.; Writing – Original Draft, M.C. and I.S.P.; Writing – Review & Editing, M.C. and I.S.P., Funding Acquisition, E.H.D. and I.S.P.

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REFERENCES

Annunziata, R., and Arnone, M.I. (2014). A dynamic regulatory network explains ParaHox gene control of gut patterning in the sea urchin. Development *141*, 2462–2472.

Arenas-Mena, C., Cameron, R.A., and Davidson, E.H. (2006). Hindgut specification and cell-adhesion functions of Sp*hox11/13b* in the endoderm of the sea urchin embryo. Dev. Growth Differ. *48*, 463–472.

Barolo, S., and Posakony, J.W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. Genes Dev. *16*, 1167–1181.

Ben-Tabou de-Leon, S.B., and Davidson, E.H. (2010). Information processing at the foxa node of the sea urchin endomesoderm specification network. Proc. Natl. Acad. Sci. USA *107*, 10103–10108.

Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y., and Greenleaf, W.J. (2013). Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat. Methods *10*, 1213–1218.

Cameron, R.A., Rowen, L., Nesbitt, R., Bloom, S., Rast, J.P., Berney, K., Arenas-Mena, C., Martinez, P., Lucas, S., Richardson, P.M., et al. (2006). Unusual gene order and organization of the sea urchin hox cluster. J. Exp. Zoolog. B Mol. Dev. Evol. *306*, 45–58.

Chen, H., Xu, Z., Mei, C., Yu, D., and Small, S. (2012). A system of repressor gradients spatially organizes the boundaries of Bicoid-dependent target genes. Cell *149*, 618–629.

Cohen, M., Page, K.M., Perez-Carrasco, R., Barnes, C.P., and Briscoe, J. (2014). A theoretical framework for the regulation of Shh morphogen-controlled gene expression. Development *141*, 3868–3878.

Cui, M., Siriwon, N., Li, E., Davidson, E.H., and Peter, I.S. (2014). Specific functions of the Wnt signaling system in gene regulatory networks throughout the early sea urchin embryo. Proc. Natl. Acad. Sci. USA *111*, E5029–E5038.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., et al. (2002). A genomic regulatory network for development. Science *295*, 1669–1678.

de Santa Barbara, P., and Roberts, D.J. (2002). Tail gut endoderm and gut/genitourinary/tail development: a new tissue-specific role for Hoxa13. Development *129*, 551–561.

Holmes, S., Lyman, S., Hsu, J.K., and Cheng, J. (2015). Making BAC transgene constructs with lambda-red recombineering system for transgenic animals or cell lines. Methods Mol. Biol. *1227*, 71–98.

Li, E., Cui, M., Peter, I.S., and Davidson, E.H. (2014). Encoding regulatory state boundaries in the pregastrular oral ectoderm of the sea urchin embryo. Proc. Natl. Acad. Sci. USA *111*, E906–E913.

Logan, C.Y., Miller, J.R., Ferkowicz, M.J., and McClay, D.R. (1999). Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. Development *126*, 345–357.

Nam, J., and Davidson, E.H. (2012). Barcoded DNA-tag reporters for multiplex cis-regulatory analysis. PLoS ONE *7*, e35934.

Nam, J., Dong, P., Tarpine, R., Istrail, S., and Davidson, E.H. (2010). Functional cis-regulatory genomics for systems biology. Proc. Natl. Acad. Sci. USA *107*, 3930–3935.

Oliveri, P., Tu, Q., and Davidson, E.H. (2008). Global regulatory logic for specification of an embryonic cell lineage. Proc. Natl. Acad. Sci. USA *105*, 5955–5962.

Ozdemir, A., Ma, L., White, K.P., and Stathopoulos, A. (2014). Su(H)-mediated repression positions gene boundaries along the dorsal-ventral axis of *Drosophila* embryos. Dev. Cell *31*, 100–113.

Peter, I.S., and Davidson, E.H. (2010). The endoderm gene regulatory network in sea urchin embryos up to mid-blastula stage. Dev. Biol. *340*, 188–199.

Peter, I.S., and Davidson, E.H. (2011). A gene regulatory network controlling the embryonic specification of endoderm. Nature *474*, 635–639.

Peter, I.S., and Davidson, E.H. (2015). Genomic Control Process, Development and Evolution (Academic Press/Elsevier).

Peter, I.S., Faure, E., and Davidson, E.H. (2012). Predictive computation of genomic logic processing functions in embryonic development. Proc. Natl. Acad. Sci. USA *109*, 16434–16442.

Range, R.C., Venuti, J.M., and McClay, D.R. (2005). LvGroucho and nuclear beta-catenin functionally compete for Tcf binding to influence activation of the endomesoderm gene regulatory network in the sea urchin embryo. Dev. Biol. *279*, 252–267.

Revilla-i-Domingo, R., Minokawa, T., and Davidson, E.H. (2004). R11: a cis-regulatory node of the sea urchin embryo gene network that controls early expression of SpDelta in micromeres. Dev. Biol. *274*, 438–451.

Rizzo, F., Fernandez-Serra, M., Squarzoni, P., Archimandritis, A., and Arnone, M.I. (2006). Identification and developmental expression of the ets gene family in the sea urchin (Strongylocentrotus purpuratus). Dev. Biol. *300*, 35–48.

Scotti, M., Kherdjemil, Y., Roux, M., and Kmita, M. (2015). A Hoxa13:Cre mouse strain for conditional gene manipulation in developing limb, hindgut, and urogenital system. Genesis *53*, 366–376.

Sethi, A.J., Wikramanayake, R.M., Angerer, R.C., Range, R.C., and Angerer, L.M. (2012). Sequential signaling crosstalk regulates endomesoderm segregation in sea urchin embryos. Science *335*, 590–593.

Smith, J., Kraemer, E., Liu, H., Theodoris, C., and Davidson, E. (2008). A spatially dynamic cohort of regulatory genes in the endomesodermal gene network of the sea urchin embryo. Dev. Biol. *313*, 863–875.

Warot, X., Fromental-Ramain, C., Fraulob, V., Chambon, P., and Dollé, P. (1997). Gene dosage-dependent effects of the Hoxa-13 and Hoxd-13 mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts. Development *124*, 4781–4791.

Weirauch, M.T., Yang, A., Albu, M., Cote, A.G., Montenegro-Montero, A., Drewe, P., Najafabadi, H.S., Lambert, S.A., Mann, I., Cook, K., et al. (2014). Determination and inference of eukaryotic transcription factor sequence specificity. Cell *158*, 1431–1443.

Zacchetti, G., Duboule, D., and Zakany, J. (2007). Hox gene function in vertebrate gut morphogenesis: the case of the caecum. Development *134*, 3967–3973.