A conserved gene regulatory network subcircuit drives different developmental fates in the vegetal pole of highly divergent echinoderm embryos ☆

Brenna S. McCauley, Erin P. Weideman, Veronica F. Hinman *

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213, USA

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ABSTRACT

Comparisons of orthologous developmental gene regulatory networks (GRNs) from different organisms explain how transcriptional regulation can, or cannot, change over time to cause morphological evolution and stasis. Here, we examine a subset of the GRN connections in the central vegetal pole mesoderm of the late sea star blastula and compare them to the GRN for the same embryonic territory of sea urchins. In modern sea urchins, this territory gives rise to skeletogenic mesoderm; in sea stars, it develops into other mesodermal derivatives. Orthologs of many transcription factors that function in the sea urchin skeletogenic mesoderm are co-expressed in the sea star vegetal pole, although this territory does not form a larval skeleton. Systematic perturbation of *erg*, *hex*, *tbr*, and *tgif* gene function was used to construct a snapshot of the sea star mesoderm GRN. A comparison of this network to the sea urchin skeletogenic mesoderm GRN revealed a conserved, recursively wired subcircuit operating in both organisms. We propose that, while these territories have evolved different functions in sea urchins and sea stars, this subcircuit is part of an ancestral GRN governing echinoderm vegetal pole mesoderm development. The positive regulatory feedback between these transcription factors may explain the conservation of this subcircuit.

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Introduction

The gene regulatory network (GRN) for early specification of the sea urchin endomesoderm is extremely well resolved and provides one of the most insightful accounts of the mechanisms of development in any animal system. This network explains the development of the vegetal domain of the sea urchin from its specification by maternal factors through the final differentiation of cells as endoderm or one of several types of mesoderm (Davidson, 2006; Davidson et al., 2002b; Oliveri et al., 2008; Smith and Davidson, 2008). A comparison of this GRN to the orthologous endomesoderm network in the sea star, *Asterina miniata*, revealed that some of the regulatory connections are conserved between these divergent echinoderms (Hinman and Davidson, 2007; Hinman et al., 2003a). Presumably, these interactions have been retained from a very ancient ancestor that existed some 500 million years ago (Wada and Satoh, 1994). Other connections, however, have diverged in the time since these organisms last shared a common ancestor. The conserved regulatory interactions found in these two GRNs exhibit a high degree of positive feedback between the transcription factors involved. Such feedback is thought to stabilize expression of target genes and may function as a mechanism

to "lock down" early specification events (Davidson, 2006). These observations led to speculation that positive feedback within GRN subcircuits may be refractory to evolutionary change and thus might provide a molecular mechanism for the constraint that results in a "phylotypic" developmental plan (Davidson and Erwin, 2006). Hints of positive feedback regulatory loops are also found within other conserved developmental programs (Davidson, 2006; Olson, 2006), but there are currently very few direct comparisons of GRN architecture. Therefore, the hypothesis that recursive wiring is causative of evolutionary constraint remains speculative.

In this study, the comparison of endomesoderm GRN architecture in sea urchins and sea stars is expanded with a focus on mesoderm development. Unlike the endoderm, which forms morphologically similar larval digestive tracts in these echinoderms, mesodermal cell types have evolved in the time since sea urchins and sea stars diverged. Sea urchins have at least two mesodermal cell types, pigment cells and micromere-derived skeletogenic mesoderm, that are absent in the young/larval sea star. Indeed, these cell types are not present in the larvae of other groups of echinoderms, and are likely an evolutionary novelty of modern sea urchins (euechinoids). Thus, the comparison of mesoderm regulatory networks in sea urchins and sea stars may prove insightful about how novel cell lineages arise during evolution.

Endomesoderm is derived from the vegetal pole of echinoderm embryos. In sea stars, cell divisions are equal, and mesoderm and endoderm segregate by the late blastula stage (Fig. 1). Fate mapping shows that mesodermal precursors are located at the central vegetal pole, and endoderm is found in a ring surrounding them (Kuraishi and Osanai, 1992). During gastrulation, mesoderm is internalized as part

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* Corresponding author. Department of Biological Sciences, 634 Mellon Institute, Carnegie Mellon University, 4400 5th Ave., Pittsburgh, PA 15213, USA. Fax: +1 412 268 7129.

E-mail address: veronica@cmu.edu (V.F. Hinman).

A Sea urchin
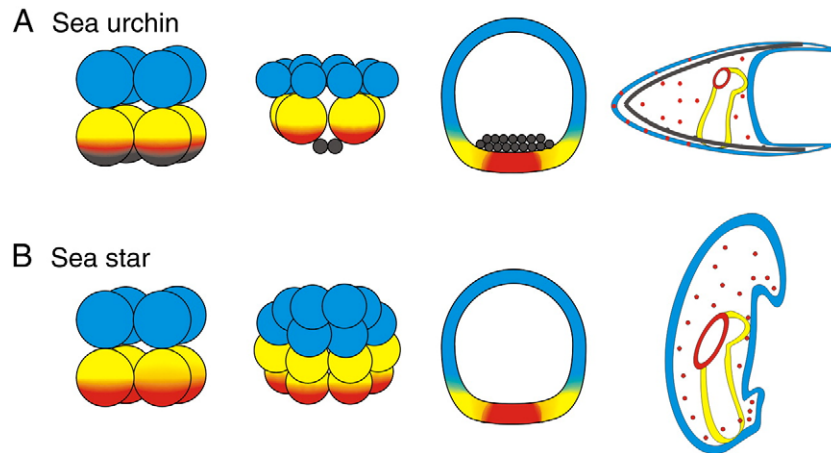
B Sea star

Fig. 1. Schematic of sea star and sea urchin development through the early larva. Sea urchin embryonic development is depicted in A and sea star development in B. During early cleavage, sea urchin and sea star embryos are similarly organized: vegetal blastomeres give rise to endomesoderm (yellow and red), while the animal blastomeres become ectoderm (blue). The 4th embryonic cleavage is unequal in sea urchins, dividing the vegetal half of the embryo in macromeres (endomesoderm) and micromeres (shown in grey), which give rise to the larval skeleton. Cleavage is equal in sea stars and no micromeres form. The blastula of sea urchins and sea stars are organized similarly, with mesodermal progenitors (red) in the central vegetal pole, surrounded by presumptive endoderm (yellow); the remainder of the embryo will become ectoderm. Prior to gastrulation, the skeletogenic mesoderm has ingressed in sea urchins, while sea star blastulae contain no mesenchyme. By the larval stage, sea stars and sea urchins have formed a gut tube (archenteron) and have mesodermally derived coelom, blastocoelar cells, and muscle. Sea urchin larvae also are pigmented and have a skeleton (shown in grey).

of the archenteron, and only after gastrulation is nearly complete does any mesenchyme migrate into the blastocoel as blastocoelar cells (Byrne and Barker, 1991; Kuraishi and Osanai, 1992). A large population of mesoderm remains at the tip of the archenteron and forms the coelomic pouches, which will eventually give rise to the adult rudiment (Hyman, 1955). Larval circumesophageal muscle is also derived from the coelomic mesoderm. This mode of development is thought to be basal among echinoderms.

In contrast, in modern sea urchins, an unequal 4th cleavage divides the vegetal half of the embryo into polar micromeres and overlying macromeres (Fig. 1). The large micromere daughters give rise to the skeletogenic mesoderm, ingress into the blastocoel prior to gastrulation, and later form the larval skeleton. Starting at late cleavage stages, the micromeres produce the Delta ligand, inducing the overlying macromere descendents to become other mesodermal cell types (Sherwood and McClay, 1999; Sweet et al., 2002; Sweet et al., 1999) including coelom, circumesophageal muscle, blastocoelar cells, and pigment cells. The remaining macromere descendents become the larval endoderm (reviewed in Davidson et al., 1998). From a phenomenological viewpoint, it therefore appears as if the entire micromere/skeletogenic mesoderm lineage is a novelty of euechinoids.

Thus, in both sea stars and sea urchins, endomesoderm precursors form at the vegetal pole of the embryo; subsequent segregation places the mesoderm at the central vegetal pole, surrounded by endoderm. Although much is known about mesoderm development and differentiation in sea urchins, the molecular details of sea star mesoderm formation have only begun to be resolved. A handful of transcription factors are known to be expressed in the mesoderm progenitors at the central vegetal pole of *A. miniata* blastulae, namely *ets1/2, gatac, otx,* and *tbr* (Hinman and Davidson, 2007; Hinman et al., 2003a; Hinman et al., 2003b). Orthologs of these genes participate in the formation of both skeletogenic and non-skeletogenic mesoderm in sea urchins (Chuang et al., 1996; Davidson et al., 2002a; Davidson et al., 2002b; Fuchikami et al., 2002; Kurokawa et al., 1999; Oliveri et al., 2002). Additionally, in sea urchins, *hesc* has an early function in repressing micromere cell fate by blocking the expression of key transcription factors in this territory (Oliveri et al., 2008; Revilla-i-Domingo et al., 2007).

To understand the basal mode of mesoderm development in echinoderms, we analyzed the regulatory interactions between five sea star transcription factors orthologous to genes with known function in the development of the sea urchin skeletogenic mesoderm. Surprisingly, although the vegetal pole domains in these

organisms have very different developmental fates, both utilize a conserved, recursively wired subcircuit downstream of initial specification events. This suggests that a conserved subcircuit can drive distinct developmental outcomes in divergent organisms.

Methods

Isolation of sea star genes from a mid-gastrula cDNA library

Heterospecific probes were prepared by PCR amplifying approximately 0.7-1 kb regions of *hex, erg, tgif,* and *foxn2/3* from *Strongylocentrotus purpuratus* cDNA. Primer sequences are available upon request. PCR products were radiolabeled and hybridized to an *A. miniata* late gastrula cDNA library as described (Hinman and Davidson, 2007). 5′ RACE was performed on a gastrula stage RACE library to extend gene sequences to include the start codon using the GeneRacer system (Invitrogen; Carlsbad, California). Primer sequences are available upon request. Final cDNA sequences were deposited in Genbank (Accession Numbers: *AmHex* GU251972, *AmErgS* GU251974, *AmErgL* GU251975, *AmTgif* GU251973, *AmFoxn2/3a* GU251977, *AmFoxn2/3b* GU251978).

Analysis of gene expression patterns by whole mount in situ hybridization (WMISH)

Spatial gene expression patterns were determined by WMISH as described (Hinman et al., 2003b), except the color reaction contained 10% dimethylformamide to reduce background. Embryos were photographed with DIC optics on a Leica DMI4000B at 200× magnification using the Leica Application Suite software (Leica; Wetzlar, Germany).

Gene perturbation experiments

Gene expression was blocked by injecting zygotes with translation-blocking morpholino antisense oligonucleotides (MASOs; Gene Tools LLC; Philomath, OR). Injections were performed as described (Hinman et al., 2003a), with the addition of 0.5 mg/mL rhodamine green dye in the injection solution. The AmTbr MASO was described previously (Hinman and Davidson, 2007); sequences for other MASOs available upon request. Sibling embryos were injected with the Gene Tools standard control MASO to ensure gene-specific results. In some experiments, MASOs were injected into a single blastomere of the two cell embryo to generate an internal control. The plane of first cleavage

reflects the plane of bilateral symmetry in the blastula; thus half of the embryo develops normally and the other half with MASO-mediated knockdown. Spatial gene expression patterns were determined in blastulae by WMISH. Additionally, quantitative real time PCR (qPCR) was used to assess changes in gene expression in perturbed embryos as described (Hinman et al., 2003a); transcript levels were normalized to *ubiquitin*. Primer sequences available upon request.

Results

Isolation of erg, foxn2/3, hesc, hex, and tgif orthologs from the sea star

Orthologs of the transcription factors *hex*, *erg*, *tgif*, *foxn2/3*, and *hesc* were isolated from the sea star *A. miniata* and were named *Am-Hex*, *AmErg*, *AmTgif*, *AmFoxn2/3*, and *AmHesc*. The orthology of the newly identified sea star proteins was confirmed by phylogenetic analysis (Supplemental Figure 1). *hex* and *tgif* both encode homeodomain transcription factors. *AmHex* is predicted to encode a 280 amino acid protein of the NK1 superfamily, while a partial open reading frame for AmTgif, a TALE family member, has 316 predicted amino acids. *AmErg* encodes at least two proteins using distinct start codons; both isoforms have Pointed and ETS domains. *AmErgS* encodes a 478aa long protein, while *AmErgL* encodes a 560aa protein. PCR revealed that *AmErgS* is present maternally and *AmErgL* is only

expressed zygotically (not shown). Two different isoforms of *Am-Foxn2/3* were also recovered, neither of which contained the full coding sequence. AmFoxN2/3a and b have identical forkhead box (FOX) domains; comparison of the AmFoxN2/3 proteins to SpFoxN2/3 revealed that isoform a is more structurally similar to the sea urchin protein, and was thus used for subsequent analyses. *AmHesc* encodes a 286 amino acid member of the hairy/enhancer of split (HES) basic helix loop helix (bHLH) family; like SpHesC, AmHesC has a histidine residue at position 6 of the basic domain, rather than the proline found in this position of all other HES family proteins (Dawson et al., 1995).

hex, erg, tgif, and foxn2/3 are expressed in the vegetal pole of sea star blastulae and later in the endoderm and mesoderm

Expression of *AmErg*, *AmFoxn2/3*, *AmHex*, and *AmTgif* was examined using WMISH at blastula and mid gastrula stages (Fig. 2). At the blastula stage (Figs. 2A–H), transcripts from all genes are expressed within the vegetal pole domain, which will form the endoderm and mesoderm of the later larva. *AmHex*, *AmTgif* and *Am-Foxn2/3* are expressed throughout the presumptive endomesoderm at this stage (Fig. 2). This expression pattern is reminiscent of that of *AmTbrain* (*tbr*), which is also expressed throughout the vegetal pole of blastulae (Hinman et al., 2003a). In contrast, *AmErg* expression is restricted to the central vegetal pole, and is thus expressed more
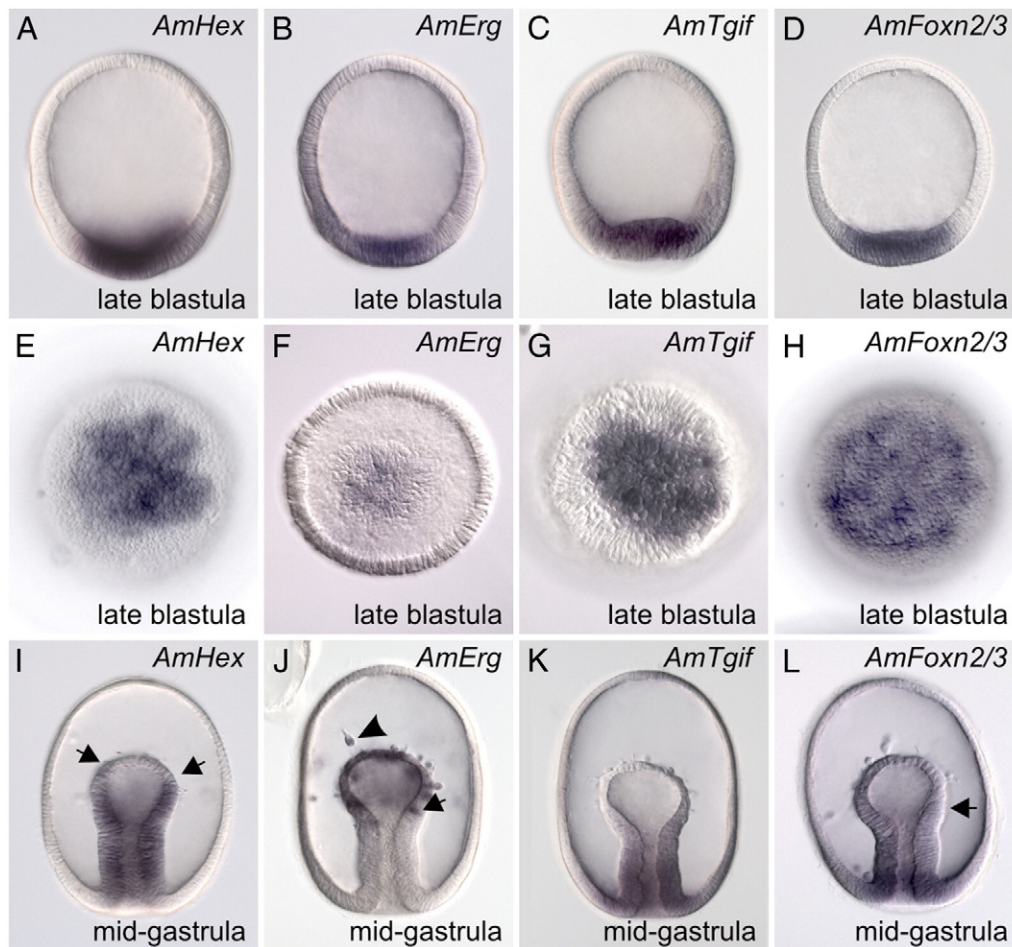
Fig. 2. Sea star orthologs of genes expressed in sea urchin micromeres are co-expressed in the vegetal pole. WMISH reveals that *AmHex* (A, E), *AmErg* (B, F), *AmTgif* (C, G), and *AmFoxn2/3* (D, H) are specifically expressed in the vegetal pole of blastula stage embryos. A-D show lateral views of the embryos, oriented with the animal pole up; E–H depict vegetal views. *erg* expression is restricted to the central vegetal pole, in the presumptive mesoderm, while the remaining transcription factors are expressed throughout the cells fated to become endomesoderm. By the mid gastrula stage, these genes are expressed in distinct domains. *AmHex* is expressed throughout the archenteron, with some clearing evident at the tip of the mesodermal bulb (between the arrows in I). *AmErg* is expressed in individual cells in the mesodermal bulb (arrow) and in mesenchyme (arrowhead) (J). Both *AmTgif* and *AmFoxn2/3* are expressed in the endoderm: *tgif* expression extends into the archenteron (K), while *foxn2/3* is restricted to the blastopore region and weakly but reproducibly in the presumptive foregut (arrow) (L). Embryos are oriented with the animal pole up.

specifically in mesodermal precursors (Figs. 2B, F). By the mid gastrula stage, these genes have distinct expression domains in the endoderm and mesoderm (Figs. 2I–L). *AmHex* and *AmTgif* are expressed in the archenteron. *AmHex* shows a slight clearing from a sub-population of mesoderm cells at the tip of the archenteron (Fig. 2I), while *AmTgif* is no longer expressed in the mesoderm or presumptive foregut (Fig. 2K). *AmFoxn2/3* expression persists in the presumptive foregut, albeit weakly, and in a ring around the blastopore (Fig. 2L). Conversely, *AmErg* is expressed in the mesodermal progenitors at the top of the archenteron and in mesenchyme cells that migrate from here (Fig. 2J). *AmEts1/2* was previously shown to be expressed within the central vegetal plate of the blastula and later within the top of the archenteron and in migrating mesenchyme (Hinman and Davidson, 2007); thus *AmEts1/2* and *AmErg* appear to co-localize during at least blastula and gastrula stages. Although *AmErg*, *AmFoxn2/3*, *AmHex*, and *AmTgif* have distinct expression domains at the gastrula stage, they are co-expressed in the central vegetal pole of the sea star blastula.

AmHesc is expressed in the vegetal pole of sea stars and does not repress transcription factors involved in vegetal development

In sea urchins, *SpHesc* functions to repress micromere state specification in the endomesoderm and ectoderm of the early embryo by directly repressing expression of transcription factors, including *SpEts1* and *SpTbr*, in these territories. *SpHesc* is expressed in all cells of the embryo except the large micromeres during early sea urchin development because it is itself repressed by the micromere-localized factor Pmar1 (Revilla-i-Domingo et al., 2007). AmHesC function was assessed to determine if the role of SpHesC in vegetal development is an ancestral feature of echinoderms. By WMISH, *AmHesc* was found to be expressed in the vegetal pole of the sea star blastula, as well as throughout most of the ectoderm, though a small clearing is observed in the vegetal ectoderm (Figs. 3A, E). This is in strict contrast to what

occurs in the sea urchin, in which *SpHesc* expression clears from the vegetal pole in an expanding torus during the specification of this territory (Smith and Davidson, 2008). Knockdown of *AmHesC* did not cause any visible defects in endomesoderm formation (not shown), nor did it affect gene expression in the vegetal pole. As shown in Fig. 3, loss of HesC function did not alter expression of *AmEts1/2* (B, F) or *AmTbr* (C, G). In contrast, *AmGcm*, a gene expressed in the ectoderm (Hinman and Davidson, 2007), was upregulated upon *AmHesc* knockdown (Fig. 3D, compared to H). This demonstrates that AmHesC functions as a repressor but does not repress mesoderm formation. Therefore, the functional role of HesC is dramatically different in the early development of sea urchins and sea stars.

A GRN governing mesoderm specification in the sea star

We determined the epistatic relationships between the four transcription factors isolated here (i.e. AmErg, AmTgif, AmHex, and AmFoxN2/3) and also AmTbr and AmEts1/2. Systematic analysis of gene expression in Erg, Hex, Tbr, and Tgif morphant embryos was used to construct a preliminary sea star mesoderm GRN. Zygotes were injected with translation-blocking MASOs against *AmErg*, *AmHex*, *AmTbr*, and *AmTgif* transcripts and changes in expression of other genes with endomesodermally restricted expression were assessed by WMISH and, in most instances, also by qPCR (Fig. 4 and Supplemental Figure 2). In some cases, the MASO was injected into one cell of a 2 cell embryo, generating half-morphant embryos with internal controls, which were analyzed by WMISH.

Knockdown of Erg

Loss of Erg function had a broad effect on gene expression in the vegetal pole. As seen in Fig. 4, a decrease in *AmFoxn2/3* (compare C and H), *AmHex*, (D vs. I) and *AmTgif* (E and J) expression was observed in hatched blastulae by WMISH. This effect was seen throughout the vegetal pole, not just in the more restricted domain of *AmErg* expression, which could mean that Erg-dependent signaling is
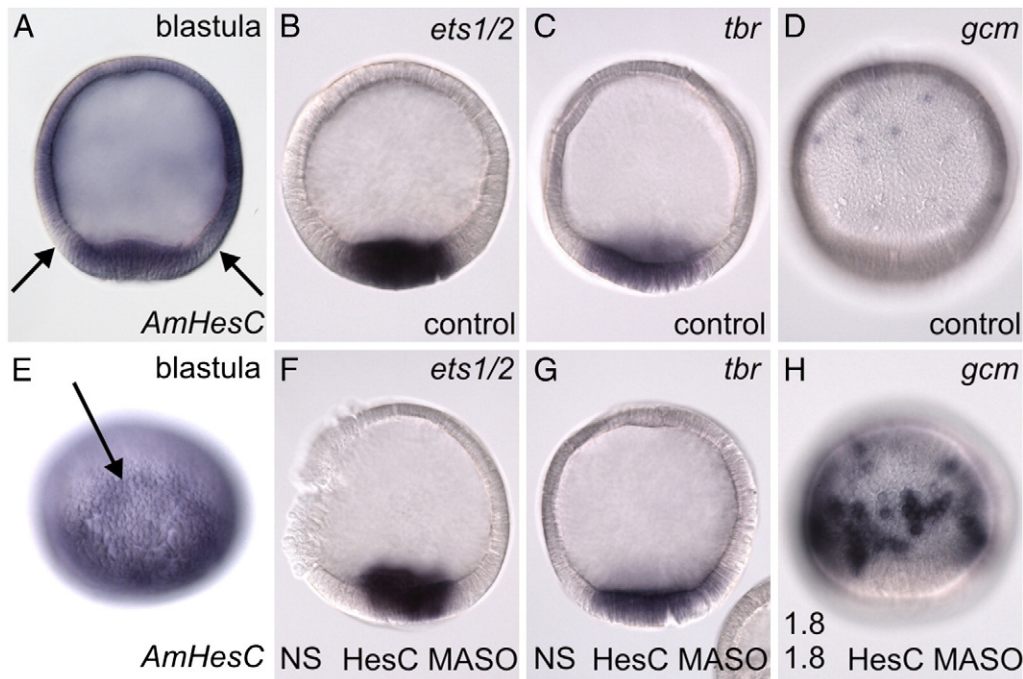
Fig. 3. The sea star ortholog of *hesc* is expressed in the vegetal pole and does not repress expression of other genes in this territory. WMISH shows that *AmHesc* is expressed in the vegetal pole and broadly throughout the ectoderm, except for a clearing from the vegetal-most ectoderm (panel A). *AmHesc* is not cleared from the vegetal pole at the hatched blastula stage (panel E). Knockdown of *hesc* in the sea star has no effect on the expression of *ets1/2* (compare B to F) or *tbr* (compare C and G), which are targets of *hesc* in the sea urchin (Revilla-i-Domingo et al., 2007). In the sea star, *hesc* represses expression of *gcm* (D and H), which is expressed in the ectoderm; the numbers in the bottom left corner are qPCR results. Expression of *AmGcm* was originally reported by Hinman and Davidson (2007).
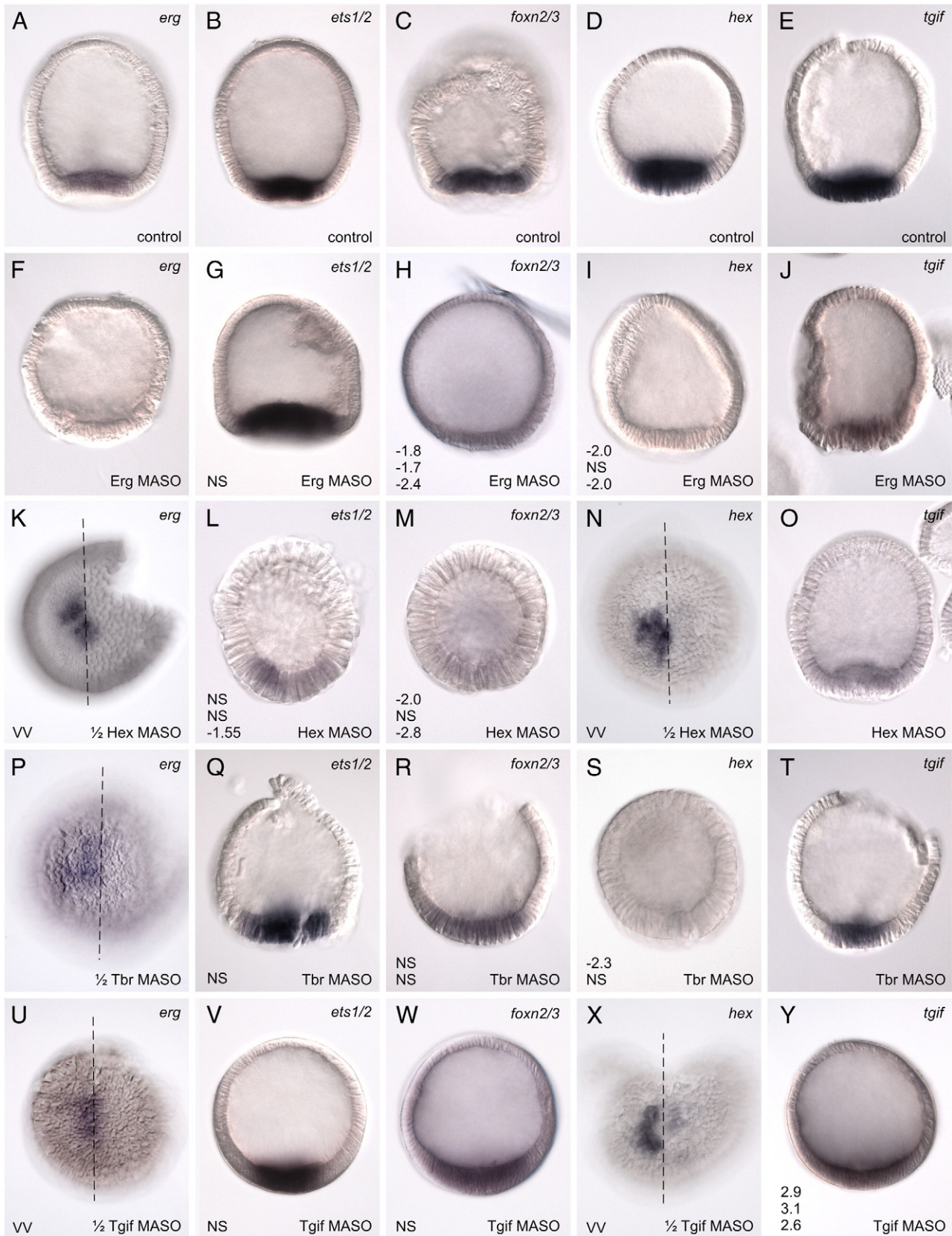
Fig. 4. Changes in expression of *erg*, *ets1/2*, *foxn2/3*, *hex*, and *tgif* upon knockdown of Erg, Hex, Tbr, and Tgif. WMISH and qPCR in morphant embryos show the regulation of *erg*, *ets1/2*, *foxn2/3*, *hex*, and *tgif* in sea star blastulae. qPCR data are presented in the bottom left cornel of the panels; NS indicates not significant. *AmErg* decreases when Erg, Hex, Tbr, and Tgif are knocked down (A and F, K, P, and U). Likewise, *hex* is downregulated in Erg, Hex, Tbr, and Tgif morphants (D and I, N, S, X). Hex knockdown causes a decrease in *AmTgif* expression (E, P); knockdown of Tgif upregulates *tgif* expression (Y). *ets1/2* expression expands upon knockdown of Erg and slightly when Hex function is blocked (B and G, B and M). *foxn2/3* expression is decreased in Erg and Hex morphants (C and H, C and N). Embryos are shown in the lateral view, except where indicated by "VV," signifying vegetal view. In K, N, P, Q, and X, Hex, Tbr, and Tgif were knocked down in half the embryo; the injected half is shown on the right.

required for normal gene expression in the presumptive endoderm, or that an early loss of Erg function downregulates gene expression throughout the endomesoderm. A decrease in *AmErg* expression was also observed, indicating that this gene autoregulates (Fig. 4, compare A to F). Interestingly, there was a slight but reproducible expansion in the expression domain of *AmEts1/2* (Fig. 4, compare B to G), suggesting that an Erg-dependent signaling event normally functions to restrict *AmEts1/2* expression within the vegetal pole.

Knockdown of Hex

Molecular analysis of Hex morphants indicates that this transcription factor has a central role in activating or maintaining gene expression in the vegetal pole. Shown in Fig. 4, a dramatic decrease in the expression of *AmFoxn2/3* (Fig. 4C vs. M) was observed upon Hex knockdown and confirmed by qPCR. *AmErg*, *AmHex*, and *AmTgif* expression were also downregulated, although not as dramatically (Fig. 4A vs. K for *erg*; D vs. N for *hex*; and E vs. O for *tgif*; additional data in Supplemental Figure 2). *AmEts1/2* appeared somewhat decreased by WMISH and its expression domain seemed slightly expanded, though not as dramatically as seen in Erg morphangs (Fig. 4B vs. L). Thus, Hex seems to function early in the determination of endomesoderm.

Knockdown of Tbr

Tbr was previously shown to drive endomesoderm specification in the sea star by activating expression of *AmOtx* and *AmDelta* (Hinman and Davidson, 2007). Additionally, we have found that loss of Tbr function results in a downregulation of *AmErg* and *AmHex* (Fig. 4A vs. P for *erg* and D vs. S for *hex*); thus, Tbr functions to activate these genes as well. In contrast, expression of *AmEts1/2*, *AmFoxn2/3*, and *AmTgif* is unaffected in Tbr morphants (Fig. 4B vs. Q, C vs. R, and E vs. T, respectively).

Knockdown of Tgif

Tgif knockdown had only a minimal effect on gene expression in blastula stage embryos: as seen in Fig. 4, *AmErg* (A compared to U) and *AmHex* (D vs. X) were downregulated; additional embryos are shown in Supplemental Figure 2. An upregulation of *AmTgif* was observed by qPCR, though WMISH in morphant embryos was inconclusive (Fig. 4E vs. Y), but no additional changes in gene expression were found (*AmEts1/2* compare B and V; *AmFoxn2/3* C vs. W). Based on the later expression of *AmTgif*, we additionally tested transcription factors known to be restricted to the ring of endodermally fated territory (i.e., *AmBra*, *AmFoxa*, and *AmGatae*) (Hinman and Davidson, 2003a, 2003b; Hinman et al., 2003a); these also showed no change in expression (not shown).

The sea star mesoderm GRN subcircuit

The above data were combined into a regulatory network depicting the molecular events of mesoderm development in *A. miniata* (Fig. 5). A striking feature of this network is the recursively wired subcircuit formed among *AmHex*, *AmErg*, and *AmTgif*. Given the critical roles of *AmErg* and *AmHex* in mesoderm formation (described in Supplemental Figure 3), this subcircuit may function in the
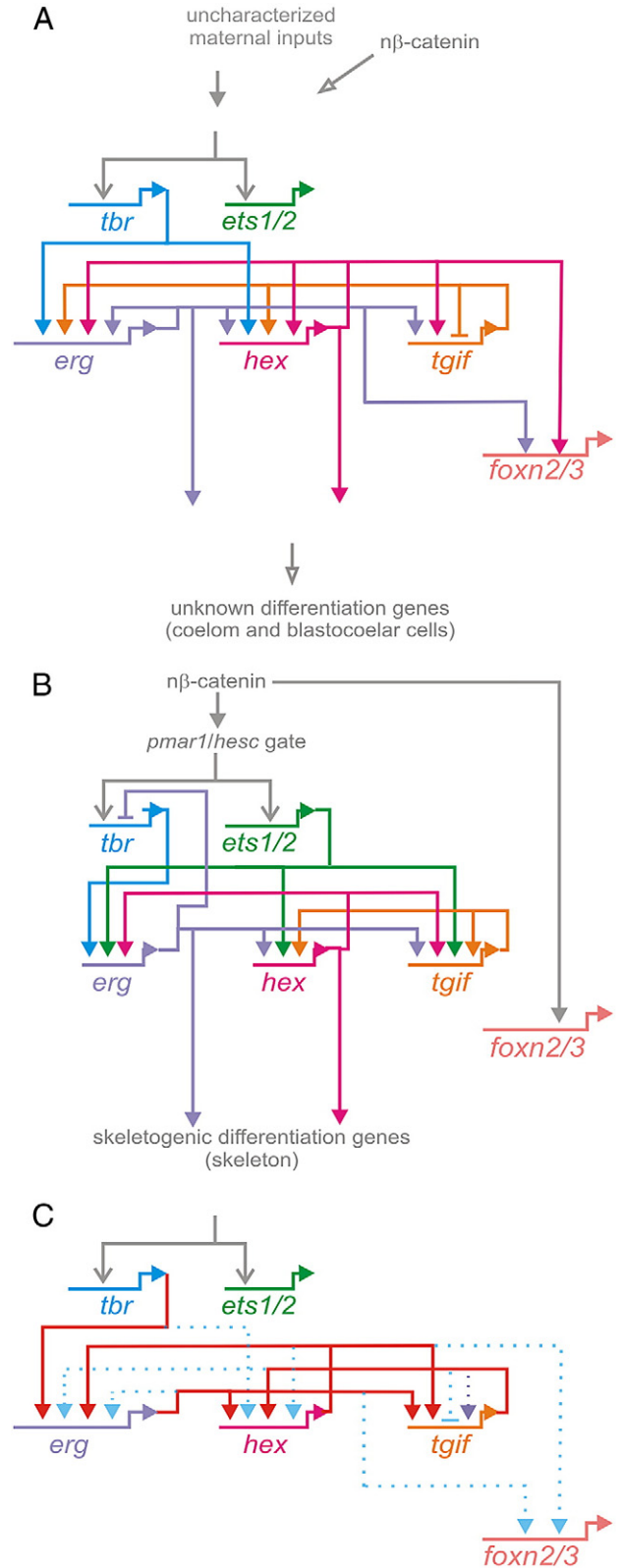
Fig. 5. A conserved subcircuit operates in sea star mesoderm and sea urchin micromeres. (A) A wiring diagram depicting the epigenetic interactions between *tbr*, *erg*, *hex*, *tgif*, *ets1/2*, and *foxn2/3* in the late star sea star blastula. Arrows represent positive regulation; bars represent repression, and the color of the arrow matches the gene providing the input. A positive feedback subcircuit is formed between *erg*, *hex*, and *tgif*, which receives input from *tbr*. The correct expression of *ets1/2* and *foxn2/3* is further influenced by *erg* and *hex*. The "interactions" shown in grey place the subcircuit within the context of sea star vegetal development (while making no assumptions about the nature of the interactions). (B) Orthologous genes are depicted in the sea urchin micromere GRN. As in sea stars, a positive feedback subcircuit exists between *erg*, *hex*, and *tgif*, though all of the interactions are not precisely conserved. Grey interactions place this subcircuit within the micromere GRN: *tbr* and *ets1/2* are activated directly downstream of the *pmar1/hesc* gate, while the only known input into *foxn2/3* is provided by nuclearized β-catenin (nβ-catenin). *hex* and *erg*, in turn, are thought to directly activate skeletogenic differentiation genes. (C) A comparison of the sea urchin micromere and sea star central vegetal pole subcircuits. Conserved interactions are shown in red; species-specific interactions are shown as dashed lines, blue for the sea star and purple for the sea urchin. Interactions only examined in one species (i.e., *ets1* in the sea urchin) are not shown. This comparison clearly shows conserved epigenetic interactions between *erg*, *hex*, and *tgif* in sea urchins and sea stars, highlighting another example of positive feedback conservation. This conservation further suggests that the sea urchin micromere GRN may be derived from an ancestral echinoderm central vegetal pole mesoderm GRN.

establishment of the mesoderm territory. *AmTbr* is an essential activator of both *AmErg* and *AmHex*, and the previously observed defects in mesoderm derivates in Tbr morphant gastrulae (Hinman and Davidson, 2007) may be due, in part, to the loss of *AmErg* and *AmHex* expression. In addition to internal positive feedback, the mesoderm subcircuit regulates the expression of other transcription factors expressed in the vegetal pole. *AmHex* and *AmErg* play key roles in the regulation of *AmEts1/2* and *AmFoxn2/3*. While *AmHex* functions as an activator of *AmFoxn2/3*, *AmErg* activates *AmFoxn2/3* expression and spatially represses *AmEts1/2* expression, probably via inter-territory signaling. Interestingly, these interactions are not conserved in the sea urchin (Fig. 5C). Conversely, *AmTgif*, the other member of the subcircuit, does not regulate the expression of other transcription factors in the presumptive mesoderm. Some of the epigenetic interactions the data suggest may well be indirect. However, it is impossible to determine which of these predicted interactions are direct without a comprehensive *cis*-regulatory analysis. Although *AmHex*, *AmErg*, and *AmTgif* form a recursively wired subcircuit that operates early in mesoderm development, the varied morphologies of morphant gastrulae suggests that these genes have distinct downstream functions in mesoderm development (see Supplemental Figure 3).

Discussion

erg, ets1/2, foxn2/3, hex, tbr, and tgif orthologs are co-expressed in the vegetal pole domain of sea urchins and sea stars

In both sea urchins and sea stars, the transcription factors *erg, ets1/2, foxn2/3, hex, tbr,* and *tgif* are co-expressed in the central vegetal pole of blastulae (Croce et al., 2001; Hinman and Davidson, 2007; Hinman et al., 2003a; Howard-Ashby et al., 2006; Kurokawa et al., 1999; Rizzo et al., 2006; Tu et al., 2006; Zhu et al., 2001; Fig. 2 herein). In sea urchins, the central vegetal pole territory is derived from the micromeres. Although the above transcription factors are expressed in the vegetal pole of both sea urchins and sea stars, and in endomesoderm-derived tissues in gastrulae, aspects of their expression at both these stages have diverged (summarized in Table 1). While the expression domains, and presumably roles, of *erg, ets1/2, foxn2/3, hex, tbr,* and *tgif* are largely conserved in sea urchin and sea star embryos, small changes in expression suggest that these transcription factors have likely evolved other, novel roles in the

development of vegetal tissues. In both organisms, however, the central vegetal pole territory is the only region in which *erg, ets1/2, foxn2/3, hex, tbr,* and *tgif* are co-expressed during embryonic development.

A comparison of the sea urchin micromere GRN and sea star mesoderm network shows striking conservation of regulatory interactions: identification of a conserved echinoderm mesoderm GRN subcircuit

In sea urchins, directly following micromere specification, *ets1* and *tbr* activate a recursively wired *hex/erg/tgif* subcircuit (Oliveri et al., 2008) (Fig. 5B). Interestingly, many of the regulatory interactions downstream of initial specification also occur in the sea star mesoderm (Fig. 5A): *tbr* activates a recursively wired *hex/erg/tgif* subcircuit in *A. miniata* as well. A direct comparison of these regulatory interactions in sea star mesoderm and sea urchin micromeres are shown in Fig. 5C; conserved interactions are depicted in solid red lines, while species-specific interactions are dashed, with those specific to the sea star shown in blue and the sea urchin in purple. This comparison reveals the conservation of the recursively wired *hex/erg/tgif* subcircuit. Positive feedback loops (i.e., *hex* activates *erg* expression and *erg* activates *hex* expression; *hex* activates *tgif* expression and *tgif* expression activates *hex* expression; *erg* activates *tgif* expression and *tgif* activates *erg* expression) between these transcription factors is thought to ensure robust expression of all three genes (Davidson, 2006). Sea urchin micromeres and sea star mesoderm give rise to distinct cell types, yet they are both derived from the central vegetal pole of the embryo. Thus, the conserved GRN subcircuit, in this case, is not directing cells to take on a specific fate, but functions more generally to specify cells as central vegetal pole mesoderm. While the pattern of cross-regulation among these transcription factors is not identical in sea urchins and sea stars, many of the interactions are conserved, and the overall function of cross-regulation is tolerant to additional positive feedback loops between genes in the subcircuit. As of yet, the *cis*-regulatory analyses needed to determine whether these interactions are direct or not have not been performed. However, both the logic of positive feedback and the subcircuit function are conserved regardless of whether the predicted regulatory connections are direct or not.

General features of GRNs: similarities of the echinoderm mesoderm GRN subcircuit and other conserved GRN subcircuits

The mesoderm subcircuit presented here shows striking similarities to the previously described conserved GRN subcircuit that operates early during specification of echinoderm endomesoderm. This latter subcircuit is essential for the formation of endomesoderm in both sea urchins and sea stars and evinces positive feedback between *blimp1, otx, foxa,* and *gatae* (Hinman and Davidson, 2007). In both cases, a recursively wired subcircuit is activated following a transient specification event, which has diverged since the split between sea urchins and sea stars. Additionally, in both cases, transcription factors with broadly conserved roles across metazoa form part of the subcircuit. *foxa* and *gatae* (*gata4/5/6*) function in endoderm formation in many animals (reviewed in Friedman and Kaestner, 2006; Murakami et al., 2005), and are part of critical feedback loops that mediate endoderm specification in echinoderms. Similarly, in the echinoderm mesoderm subcircuit presented here, *erg* participates in positive feedback; orthologs of this gene have roles in the formation of mesenchyme and other mesodermal derivatives in various organisms (reviewed in Maroulakou and Bowe, 2000). While it is interesting to note that transcription factors with broadly conserved roles in endoderm and mesoderm formation are part of these highly conserved, recursively wired subcircuits, this is not to suggest that the specific regulatory interactions in which they participate are also broadly conserved.

Table 1
Comparison of pre- and post-gastrular domains of *erg, ets1/2, foxn2/3, hex, tbr,* and *tgif* expression in sea stars and sea urchins. A comparison of expression domains of *erg, ets1/2, foxn2/3, hex, tbr* and *tgif* in sea urchins and sea stars indicates that their expression is largely conserved, though there are changes in the expression of some genes. Abbreviations: SM, skeletogenic mesoderm; AA-NSM, archenteron-associated non-skeletogenic mesoderm; CM, circumesophageal muscle. Expression patterns were previously characterized as follows: *AmEts1/2* Hinman and Davidson (2007); *AmTbr* Hinman et al. (2003a); *SpErg* Zhu et al. (2001) and Rizzo et al. (2006); *SpEts1/2* Rizzo et al. (2006), *SpFoxn2/3* Tu et al. (2006), *SpHex* Howard-Ashby et al. (2006), *PlSke-T,* the *Paracentrotus lividus tbr* ortholog, Croce *et al.* (2001), and *SpTgif* Howard-Ashby et al. (2006).

	Sea urchin blastula	Sea star blastula	Sea urchin gastrula	Sea star gastrula
erg	SM, NSM	Mesoderm	mesenchyme	mesenchyme, CM
ets1/2	SM, NSM	Mesoderm	SM, mesenchyme, AA-NSM	mesenchyme
foxn2/3	SM, later NSM	Endomesoderm	foregut (weak)	foregut (weak)
hex	SM	Endomesoderm	archenteron (weak)	gut, later mouth
tbr	SM	Endomesoderm	SM, later not expressed	not expressed
tgif	SM, endoderm	Endomesoderm	midgut, AA-NSM	midgut

Recursive wiring is thought to be a feature of subcircuits governing the establishment of an embryonic territory, which have been defined as "kernels" (Davidson and Erwin, 2006). Putative kernels have been identified on the basis of conserved regulatory interactions in the echinoderm endomesoderm (discussed above) and also in bilaterian heartfield specification, which is conserved between *Drosphila* and vertebrates (Hinman and Davidson, 2007; Olson 2006). While the echinoderm endomesoderm kernel drives the development of morphologically similar digestive tracts in sea urchins and sea stars, the outputs of the *Drosophila* and vertebrate heartfield specification GRNs and the echinoderm mesoderm subcircuits are drastically different. Regardless of the developmental output, in all of the currently documented cases, the conserved subcircuit is positioned near the top of the GRN governing territory development. The identification of another conserved, recursively wired subcircuit needed, in this case, for the development of the echinoderm central vegetal pole mesoderm, greatly strengthens the argument that early specification lockdown events may feature positive regulatory feedback and be refractory to evolutionary change.

Changes in the activation of the mesoderm subcircuit in sea urchins and sea stars

In sea urchins, maternal factors activate expression of the transcription factor *pmar1* in the micromeres shortly after their birth (Chuang et al., 1996; Logan et al., 1999; Oliveri et al., 2002). *pmar1* in turn represses expression of the *hesc* repressor in these cells, thus reliving repression of many transcription factors in this territory, including *ets1* and *tbr* (Oliveri et al., 2008; Revilla-i-Domingo et al., 2007). This type of subcircuit, in which one repressor prevents expression of another repressor, has been termed a double negative gate; in sea urchins, it is used to allow specification of the micromeres. Downstream of this double negative gate, the mesoderm subcircuit is activated. In sea stars, the molecular details of initial mesoderm specification are unknown; however, there is no indication in *A. miniata* that HesC functions to repress expression of *ets1* or *tbr* (Fig. 3), nor has a *pmar1* ortholog been isolated from the sea star, despite numerous attempts. Thus, the circuitry governing activation of the recursively wired mesoderm subcircuit has diverged since sea urchins and sea stars last shared a common ancestor, although orthologs of all transcription factors in this subcircuit are similarly expressed in these two taxa (see Table 1).

The evolution of novelty

The comparison of regulatory connections between orthologous transcription factors in the sea star and sea urchin central vegetal pole mesoderm has provided many insights into the manner in which GRNs might evolve and, of equal importance, might be refractory to evolutionary change. The very earliest specification processes, involving the double negative gate, are likely to be a novelty of modern sea urchins and not a deeply conserved feature of echinoderm vegetal pole development. However, we identified a recursively wired subcircuit acting immediately downstream of early specification that has been conserved for almost 500 million years. In both this comparison and previous work (Hinman and Davidson, 2007), such conserved subcircuits have been found to be surrounded by many diverged regulatory connections. The changes that have occurred outside of the subcircuit are found despite the implicit focus on detecting conservation by considering only orthologs of genes known to function within the sea urchin micromere GRN at this time. A general screen of sea star transcription factors will identify additional genes expressed in the mesoderm and bring to light even more differences between the sea urchin micromere and sea star mesoderm developmental programs. Such differences serve to highlight the importance of the connections that remain unchanged and demonstrate that evolutionary co-option of regulatory interactions can occur while downstream gene expression patterns are conserved.

The developmental fates downstream of the conserved, recursively wired subcircuit described here are vastly different in sea stars and sea urchins, although they retain some common functions such as driving an epithelial to mesenchymal transition in both organisms. In the sea urchin only, transcription factors in this subcircuit also direct cells to take on a skeletogenic fate and directly activate genes necessary for the cells to fuse and produce a skeleton (Oliveri et al., 2008). This function of the echinoderm mesoderm subcircuit is considered an evolutionary novelty because, among echinoderms, only modern sea urchins form a larval skeleton from micromeres. Larval skeletogenesis is thought to have evolved by the co-option of the adult skeletogenic program that runs in all echinoderms into an embryonic context in sea urchins (for example, Ettensohn, 2009; Gao and Davidson, 2008; Yajima, 2007). However, like the larval skeleton, adult skeletogenic centers are derived from mesoderm (Hyman, 1955); thus, the skeletogenic network running in these centers is likely modified from an ancestral GRN for mesoderm development.

The types of changes that have allowed the sea urchin micromeres to give rise to a skeleton have yet to be determined. However, it is clear that the evolution of this dramatically novel phenotype did not require a dramatic change in the presence of particular transcription factors. Perhaps only more subtle changes in transcription factor abundance and probably changes in the *cis*-regulatory control of differentiation genes were required for the evolution of a micromere-derived skeletogenic lineage in sea urchins.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.11.020.

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