© 2018. Published by The Company of Biologists Ltd | Development (2018) 145, dev167288. doi:10.1242/dev.167288

RESEARCH ARTICLE

Conserved regulatory state expression controlled by divergent developmental gene regulatory networks in echinoids

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ABSTRACT

Evolution of the animal body plan is driven by changes in developmental gene regulatory networks (GRNs), but how networks change to control novel developmental phenotypes remains, in most cases, unresolved. Here, we address GRN evolution by comparing the endomesoderm GRN in two echinoid sea urchins, *Strongylocentrotus purpuratus* and *Eucidaris tribuloides*, with at least 268 million years of independent evolution. We first analyzed the expression of twelve transcription factors and signaling molecules of the *S. purpuratus* GRN in *E. tribuloides* embryos, showing that orthologous regulatory genes are expressed in corresponding endomesodermal cell fates in the two species. However, perturbation of regulatory genes revealed that important regulatory circuits of the *S. purpuratus* GRN are significantly different in *E. tribuloides*. For example, mesodermal Delta/Notch signaling controls exclusion of alternative cell fates in *E. tribuloides* but controls mesoderm induction and activation of a positive feedback circuit in *S. purpuratus*. These results indicate that the architecture of the sea urchin endomesoderm GRN evolved by extensive gain and loss of regulatory interactions between a conserved set of regulatory factors that control endomesodermal cell fate specification.

KEY WORDS: Regulatory states, GRN evolution, Subcircuits, Echinoderms, Embryogenesis

INTRODUCTION

Gene regulatory networks (GRNs) encode the mechanisms that are needed for developmental organization of the animal body plan, and alterations in developmental GRNs must therefore be responsible for evolutionary differences in animal development and morphology (Erwin and Davidson, 2009; Peter and Davidson, 2011a, 2015). Several mechanisms may account for the evolution of GRNs, such as the co-option of transcription factors to novel developmental functions or the acquisition of novel roles for transcription factors, for example further upstream within a given developmental GRN, by intercalary evolution. However, obtaining experimental evidence for the precise changes that occur in developmental GRNs during evolution remains challenging, mostly because of the lack of well characterized GRNs and because of the difficulty in finding two or more species with a degree of similarity and experimental accessibility to support the identification of network change.

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Received 1 May 2018; Accepted 15 November 2018

The GRN that underlies early endomesoderm development in the purple sea urchin *Strongylocentrotus purpuratus* has been experimentally characterized in great detail (Materna et al., 2013; Oliveri et al., 2008; Peter and Davidson, 2010, 2011b, 2015). This GRN consists of more than 40 transcription factors and their regulatory interactions that control the expression of cell fate-specific combinations of transcription factors, or regulatory states (Peter, 2017), and the early specification of several mesodermal and endodermal cell fates. Before gastrulation, the endomesoderm of *S. purpuratus* embryos consists of: the skeletogenic mesoderm (SM), which is responsible for the formation of the larval skeleton later in development; the oral and aboral non-skeletogenic mesoderm (NSM), which gives rise to muscle cells, pigment cells, coelomic pouches and other cell fates; the anterior endoderm (AE), which gives rise to the foregut and midgut; and the posterior endoderm (PE), which provides the progenitors of the hindgut (Cameron et al., 1991; Ransick and Davidson, 1998; Ruffins and Ettensohn, 1996). The accuracy and completeness of the endomesoderm GRN model in capturing the temporal and spatial expression of regulatory genes in the sea urchin endomesoderm has been confirmed by a Boolean computational model (Peter et al., 2012). Given the extensive experimental and computational information available for the endomesoderm GRN, this network provides an excellent opportunity to evaluate the change in network architecture that occurred over long periods of evolutionary time.

Several recent studies that have compared GRNs in sea urchins and in the sea star *Patiria miniata* indicate that the endomesoderm GRN was shaped by conservation as well as by change of regulatory circuits, as these two species last shared a common ancestor more than 500 million years ago (Hinman and Davidson, 2007; Hinman et al., 2003; McCauley et al., 2010). However, because of the extensive evolutionary time separating sea urchins and sea stars, and because of the fact that sea star larvae lack the skeletogenic cells and mesodermal pigment cells that are present in sea urchins, major evolutionary change occurred, in particular in the mesoderm GRN, after the divergence of the two species. Therefore, the early specification of distinct mesodermal cell fates in sea urchins represents a more recent evolutionary innovation (Gao and Davidson, 2008; Hinman and Davidson, 2007; McCauley et al., 2012). Analysis of the evolutionary changes that occurred throughout the endomesoderm GRN, including the more recently evolved mesodermal GRNs, therefore requires a species with a closer evolutionary relationship to purple sea urchins. The cidaroid sea urchin *Eucidaris tribuloides* belongs to the same class of echinoids as purple sea urchins, and embryos of both species form similar skeletogenic mesoderm as well as pigment cells (Schroeder, 1981; Wray and McClay, 1988). However, *E. tribuloides* and *S. purpuratus* belong to distinct subclasses of echinoids, the cidaroids and the euechinoids, respectively, that diverged at least 268 million years ago (mya) (Thompson et al., 2015).

At the phenotypic level, the endomesoderm GRN of *E. tribuloides* and *S. purpuratus* controls the specification of

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similar endomesodermal cell fates. However, several differences have been observed, in particular in the development of SM cells. In purple sea urchins, exactly four SM precursor cells form at fifth cleavage at the vegetal pole, and they divide twice to give rise to 16 cells that ingress into the blastocoel before gastrulation (Cameron et al., 1987). In cidaroid sea urchins, a variable number of skeletogenic cells forms at the vegetal pole, and SM cells and several NSM cell fates ingress only after the onset of gastrulation (Erkenbrack and Davidson, 2015; Schroeder, 1981; Wray and McClay, 1988; Yamazaki et al., 2014). A recent comparison of the skeletogenic GRN in *S. purpuratus* and *E. tribuloides* showed that the mechanisms for early specification of SM cells have indeed undergone evolutionary changes since the divergence of echinoid taxa, consistent with the observed phenotypic changes in the development of these cells (Erkenbrack and Davidson, 2015). Even though other endomesodermal cell fates might be more similar at the phenotypic level, evolutionary changes could nevertheless have affected the architecture of the endomesoderm GRN, given the significant evolutionary distance separating the two species. We therefore assessed the endomesoderm GRN at two levels, comparing the regulatory nodes of the network and comparing the regulatory interactions that ultimately determine GRN function. We analyzed the expression of twelve transcription factors and signaling molecules that are crucial components of the *S. purpuratus* GRN, and show that these factors are indeed all expressed in the endomesoderm of *E. tribuloides*. We then analyzed the function of selected regulatory interactions in *E. tribuloides* by focusing on regulatory circuits that have particular importance for endomesoderm development in *S. purpuratus* (Peter and Davidson, 2017). The results indicate that the echinoid endomesoderm GRN consists of a conserved set of transcription factors and signaling molecules that control the expression of similar cell fate-specific regulatory states, even though the architecture of this GRN has undergone considerable evolutionary rewiring since the two major echinoid sub-classes diverged from each other at least 268 million years ago.

RESULTS

Conserved expression of regulatory genes in endomesodermal cell fates before gastrulation

To determine whether transcription factors of the endomesoderm GRN in *S. purpuratus* are also expressed in the endomesoderm in *E. tribuloides*, we selected twelve regulatory genes that are expressed in the following endomesodermal cell fates in *S. purpuratus*: *alx1* (SM), *ets1* (SM), *myc* (oral NSM and AE), *delta* (NSM), *gcm* (aboral NSM), *gatae* (aboral NSM and AE), *foxa* (AE), *blimp1* (AE), *eve* (PE), *hox11/13b* (AE and PE), *brachyury* (*bra*; AE and PE) and *wnt8* (PE). As *E. tribuloides* develop at room temperature, they develop at a slightly faster rate than *S. purpuratus*, which grows at 15°C. Therefore gastrulation initiates at 20 h in *E. tribuloides* compared with 30 h in *S. purpuratus* (Fig. S1A). When expression of the orthologs of the twelve regulatory genes was analyzed in *E. tribuloides* during pre-gastrular development from 0-20 h by qPCR, we found that all genes are expressed before gastrulation (Fig. S1B).

To compare spatial expression of the twelve regulatory genes between the two sea urchin species, we first focused on a developmental stage immediately before the onset of gastrulation. Spatial gene expression was analyzed in *E. tribuloides* at 18 h by whole-mount *in situ* hybridization (WMISH; Fig. 1A and Fig. S2). The results show that *Et-alx1* and *Et-delta* are expressed in a few cells at the vegetal pole, corresponding to SM cells, as previously reported in Erkenbrack and Davidson (2015). Surrounding these cells are several rings of cells: an inner ring that expresses *Et-myc*, *Et-gcm*, *Et-gatae* and *Et-ets1/2*, a middle ring that expresses *Et-gatae*, *Et-myc*, *Et-blimp1*, *Et-foxa* and *Et-hox11/13b*, and an outer ring that expresses *Et-bra*, *Et-hox11/13b*, *Et-eve* and *Et-wnt8*.

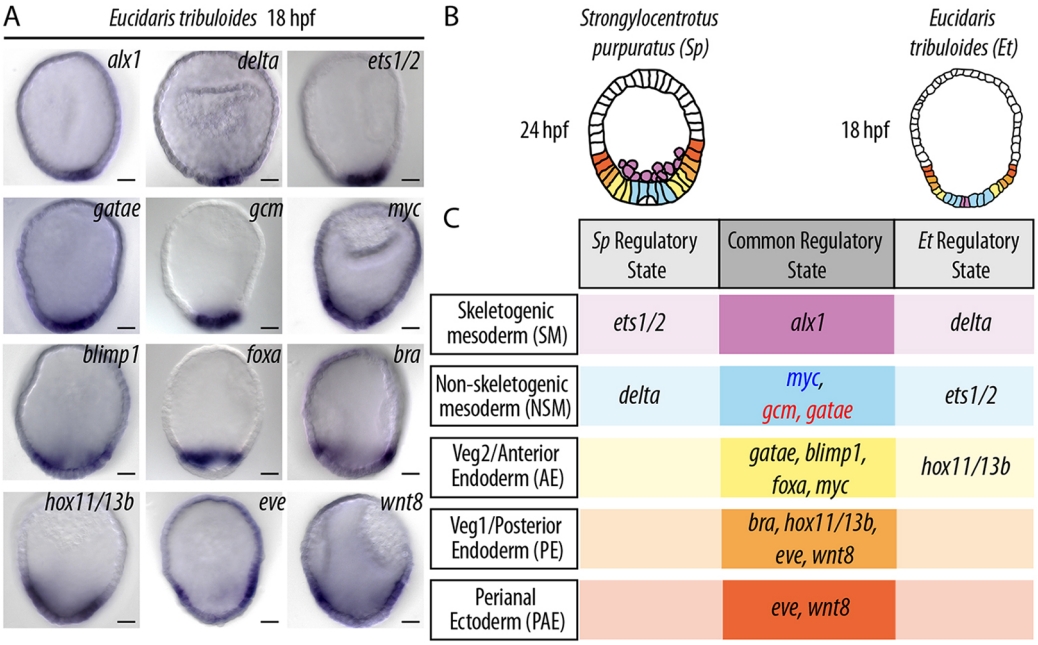

Fig. 1. Spatial expression of endomesodermal regulatory states at onset of gastrulation. (A) WMISH staining of *E. tribuloides* showing spatial expression of the twelve regulatory genes at 18 h. (B) Schematics of *S. purpuratus* and *E. tribuloides* showing the spatial arrangement of endomesodermal cell fate domains. In *S. purpuratus*, skeletogenic cells have ingressed into the blastocoel whereas in *E. tribuloides* these cells are at the vegetal pole. (C) Comparative analysis of *S. purpuratus* and *E. tribuloides* pregastrular regulatory states. Cell fate-specific expression of regulatory genes in *S. purpuratus* (left column; *Sp*), *E. tribuloides* (right column; *Et*), or both (center column) showing high similarity in endomesodermal regulatory states. Purple, SM; light blue, NSM; yellow, AE; orange, PE; red, PAE. Scale bars: 20 µm.

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We compared the gene expression patterns that are observed in 18 h *E. tribuloides* embryos with previous data from 24 h *S. purpuratus* embryos. The results indicated that the combination of transcription factors expressed in each of the rings in *E. tribuloides* is reminiscent of the regulatory states that are expressed in the endomesoderm of *S. purpuratus* (Fig. 1B,C). In both embryos, skeletogenic cells at the vegetal pole express *alx1* (Ettensohn et al., 2003; Oliveri et al., 2008). In *S. purpuratus*, the skeletogenic cells are surrounded by NSM cells that are subdivided into oral and aboral portions: oral NSM expresses *Sp-myc* and aboral NSM expresses *Sp-gcm* and *Sp-gatae* (Materna et al., 2013). In *E. tribuloides*, only one NSM domain forms before gastrulation, and this co-expresses *Et-myc*, *Et-gcm*, *Et-gatae* and *Et-ets1/2*. The subdivision of NSM cell fates was shown to occur shortly after the onset of gastrulation (Erkenbrack, 2016). In the endoderm, both species express *gatae*, *myc*, *blimp1* and *foxa* in AE, and *bra*, *hox11/13b*, *eve* and *wnt8* in PE (Peter and Davidson, 2011b). Furthermore, the endoderm is flanked in both species by cells that express *eve* and *wnt8*, but not *hox11/13b*: these cells give rise to the perianal ectoderm (PAE) (Fig. 1C).

In *S. purpuratus*, most of the twelve regulatory genes continue to be expressed in the respective endomesodermal cell fates throughout embryogenesis. To analyze whether cell fate-specific transcription factors also continue to be expressed during late gastrula stages in *E. tribuloides*, we performed WMISH at 28 h and 40 h (Fig. S3). We identified SM, NSM, AE and PE cell fates in late gastrulae based on embryonic morphology. Indeed, *Et-alx1* continues to be expressed in SM cells, which enter the blastocoel only after the invagination of the archenteron (Erkenbrack and Davidson, 2015; Wray and McClay, 1988). *Et-gcm*, *Et-gatae* and *Et-ets1* are expressed in NSM cells at the tip of the archenteron, and *Et-ets1* is expressed broadly in the mesoderm, possibly including SM and NSM cells. *Et-foxa*, *Et-blimp1* and *Et-gatae* show broad expression in the endoderm including fore- and midgut precursor cells, whereas *Et-hox11/13b* and *Et-bra* are expressed exclusively in future hindgut endoderm. Thus also at gastrula stage, the combinations of transcription factors expressed in corresponding morphological structures are similar in the two sea urchin species.

These results show that all twelve regulatory genes analyzed here are not only important components of the endomesoderm GRN in *S. purpuratus*, but are also, based on their expression, components of the endomesoderm GRN in *E. tribuloides*. Thus, the nodes of this network, as far as are analyzed here, have been conserved for at least 268 million years. Furthermore, the spatial expression of these regulatory genes indicates that this GRN specifies similar endomesodermal cell fate domains in *E. tribuloides* and *S. purpuratus* during pre-gastrular development.

Variations in developmental gene expression patterns

We then focused on comparing spatial gene expression during earlier stages of pre-gastrular development. In *S. purpuratus*, the earliest cell divisions proceed in a synchronized manner with orchestrated cleavage planes, which results in the same geometrical organization of cells in every embryo (Cameron et al., 1991; Cameron et al., 1987). Cell fate specification relies, at least in part, on this spatial organization and occurs according to cell lineage in purple sea urchins. Thus endomesodermal cell fates are specified in concentric rings of cells around the skeletogenic cells at the vegetal pole, first separating the SM from all other cell fates (Oliveri et al., 2002), then separating anterior and posterior endoderm (Peter and Davidson, 2010), NSM and AE (Peter and Davidson, 2010), and finally PE and PAE (Li et al., 2014).

To analyze developmental gene expression in *E. tribuloides*, we performed WMISH of all twelve regulatory genes at 2 h time intervals between 10 and 20 h (Fig. 2A, see Fig. S2 for complete dataset). We then compared these expression patterns with the expression patterns in *S. purpuratus* by approximately aligning developmental stages in these two species (Fig. 2B,C and Fig. S4). However, because the alignment of developmental stages might not be accurate, we only focused on differences between the two species that cannot be explained by minor temporal shifts. Even on first glance, the expression patterns in *E. tribuloides* look different from those in *S. purpuratus*: instead of concentric rings, gene expression occurs in irregular nested patches of cells. Throughout pre-gastrular development, a small number of SM cells at the vegetal pole express *Et-alx1* and *Et-delta*, and both genes are initially also expressed in the corresponding cell fate in *S. purpuratus* until *Sp-delta* expression turns off at 21 h. All three NSM regulatory genes, *Et-gcm*, *Et-ets1/2* and *Et-gatae*, are expressed in a broad area in *E. tribuloides* embryos at 12 h, including both SM and NSM domains, before expression clears from the SM and becomes restricted to NSM (Fig. 2A,C). In contrast, the early regulators of NSM specification, *Sp-gcm* and *Sp-gatae*, are not expressed in SM cells in *S. purpuratus*, even though *Sp-ets1/2* is expressed exclusively in SM up to 24 h. Similarly, the endodermal regulatory genes *Et-blimp1*, *Et-hox11/13b*, *Et-wnt8* and *Et-eve* are first expressed in a broad domain in *E. tribuloides* embryos, including SM, NSM and AE (Fig. 2A,C and Fig. S2). After 14 h, expression patterns gradually resolve into exclusive rings, similar to those observed in *S. purpuratus*, as shown by double fluorescent WMISH (Fig. S5). *Et-eve* clears from most vegetal cells and is expressed in cells that surround the *Et-blimp1* and *Et-hox11/13b* expression domain. By 16 h, expression of all endodermal regulatory genes has cleared from the SM, and by 18 h also from the NSM. Unlike *Sp-foxa*, *Et-foxa* is never expressed in mesodermal cell fates and expression is restricted to the AE throughout pre-gastrular development. Furthermore, similar to *S. purpuratus*, expression of *Et-hox11/13b* and *Et-bra* turns off in AE and is restricted to PE immediately before gastrulation.

Despite these changes in developmental expression patterns, endomesodermal cell fate specification appears to occur in a similar order in these distantly related species. In *E. tribuloides*, SM is first separated from other endomesoderm fates (10 h), then NSM from AE (12 h), then AE from PE (14 h) and finally PE from PAE progenitor fates (16 h), similar to in *S. purpuratus* (Fig. 2B). However, these differences in developmental gene expression also suggest that evolutionary alterations occurred in the regulation of these genes. We therefore selected specific regulatory circuits of the endomesoderm GRN in *S. purpuratus* that are important for the control of these genes and/or the specification of endomesodermal cell fates, and tested whether the selected regulatory features are also functional during pre-gastrular development in *E. tribuloides*.

Specification of NSM cells independent of Delta/Notch signaling

Early specification of NSM cell fates in *S. purpuratus* involve several well elaborated circuits, summarized in Fig. 3A. Most importantly, the specification of all NSM cell fates depends on Delta/Notch signaling, induced by expression of *Sp*-Delta in adjacent SM cells (Materna and Davidson, 2012; Ransick and Davidson, 2006). Current experimental evidence suggests that Delta/Notch signaling is required for pigment fate specification throughout euechinoids (Ohguro et al., 2011; Ransick and Davidson, 2006; Sherwood and McClay, 1999; Yamazaki and

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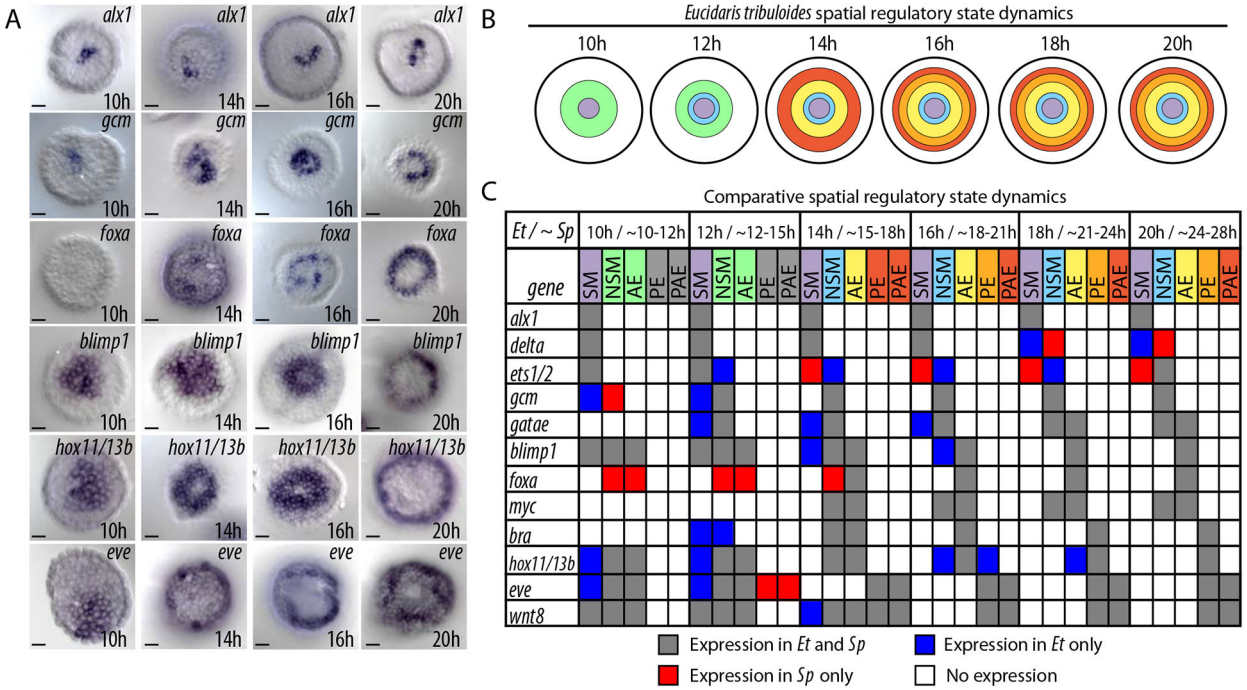

Fig. 2. Developmental expression of regulatory genes in the endomesoderm. (A) *E. tribuloides* embryos at selected developmental stages were analyzed by WMISH to show the spatial expression of endomesodermal regulatory genes. For complete dataset see Fig. S2. (B) Schematic representations of *E. tribuloides* (vegetal view) showing endomesodermal cell fate domains as formed during pre-gastrular development. Green, endomesoderm; purple, SM; blue, NSM; yellow, AE; orange, PE; red, PAE. (C) Gene expression matrix showing expression of regulatory genes in each cell fate domain at selected stages of pre-gastrular development, indicating expression in *S. purpuratus* only (red), in *E. tribuloides* only (blue), in both species (gray) or no expression (white). Separate gene expression matrices for *E. tribuloides* and *S. purpuratus* are shown in Fig. S4. Scale bars: 20 µm.

Minokawa, 2016). We therefore tested whether Delta/Notch signaling is also required for pigment cell specification in *E. tribuloides*. We found that blocking Delta/Notch signaling either by injection of *Et-delta* morpholinos or by treatment with the γ-secretase inhibitor DAPT (Hughes et al., 2009) did not interfere with the development of pigment cells, indicating that pigment cell specification is independent of Delta/Notch signaling (Fig. 3B).

In *S. purpuratus*, the specification of NSM and pigment cells depends on the expression of *Sp-gcm* in NSM cells. *Sp*-Gcm is required not only for the early specification of NSM cells but also for the differentiation of pigment cells later in development, and Gcm was shown to directly control the expression of pigment cell differentiation genes (Calestani and Rogers, 2010; Ransick and Davidson, 2006, 2012). In the absence of Gcm, *S. purpuratus* larvae lack pigment cells (Ransick and Davidson, 2006). In *E. tribuloides*, *Et-gcm* is expressed in NSM cells in early embryos as well as in pigment cells, which are embedded within the ectoderm at 40 h (Fig. S3). To test whether *Et*-Gcm is required for the formation of pigment cells, we injected *E. tribuloides* embryos with *gcm* morpholinos. The results show that, similar to *S. purpuratus* embryos, *E. tribuloides* embryos do not form pigment cells in the absence of Gcm (Fig. 3B), indicating that Gcm is required for pigment cell development in cidaroids, as in euechinoids.

Previous studies in *S. purpuratus* have shown that the mechanism for pigment cell specification involves activation of *Sp-gcm* expression downstream of Delta/Notch signaling (Materna and Davidson, 2012; Ransick and Davidson, 2006). We therefore tested whether Delta/Notch signaling is also required in *E. tribuloides* to activate expression of *Et-gcm* in pigment cell precursors. To interfere with Delta/Notch signaling, embryos were injected with *Et-delta* morpholinos or treated with DAPT. The effect of this perturbation was determined by analyzing expression of *Et-gcm* as well as *Et-alx1*, which has previously been shown to be upregulated in the absence of Delta/Notch signaling (Erkenbrack and Davidson, 2015). In embryos that were injected with *Et-delta* morpholinos, we found that *Et-alx1* was ectopically expressed in NSM cells, which was consistent with earlier results (Erkenbrack and Davidson, 2015), whereas *Et-gcm* expression in NSM cells was not affected (Fig. 3C). Similarly, embryos that were treated with DAPT showed qPCR expression levels of *Et-gcm* comparable with control embryos that were treated with DMSO, whereas the expression of *Et-alx1* was significantly upregulated (Fig. 3D). These results indicate that although Delta/Notch signaling is active in NSM cells, it is not required for the activation of *Et-gcm* expression and the specification of pigment cells in *E. tribuloides*.

NSM specification occurs downstream of maternal factors

As expression of *Et-gcm* occurs independent of Delta/Notch signaling, we investigated how *Et-gcm* is regulated in *E. tribuloides* embryos. Curiously, the expression pattern of *Et-gcm* is reminiscent of the expression pattern of endodermal genes in *S. purpuratus*. Expression of *Et-gcm* is first detectable in SM cells, before it expands to the NSM and clears from SM cells (Fig. 2C and Fig. S2). In *S. purpuratus*, several endodermal genes are first expressed in SM cells at the vegetal pole before being activated in endodermal progenitor cells (Smith et al., 2008). Expression of these endodermal genes in *S. purpuratus* is regulated by two maternal transcription factors, Otx and Tcf/β-catenin. To test the function of Otx, we injected *E. tribuloides* embryos with a fusion construct between *Et*-Otx and Engrailed repressor domain (*Et*-Otx-En; Erkenbrack and Davidson, 2015). In embryos injected with *Et*-Otx-En, potential target genes of Otx are expected to be repressed. Indeed, endodermal genes such as *Et-foxa*, *Et-blimp1*, *Et-bra* and *Et-*

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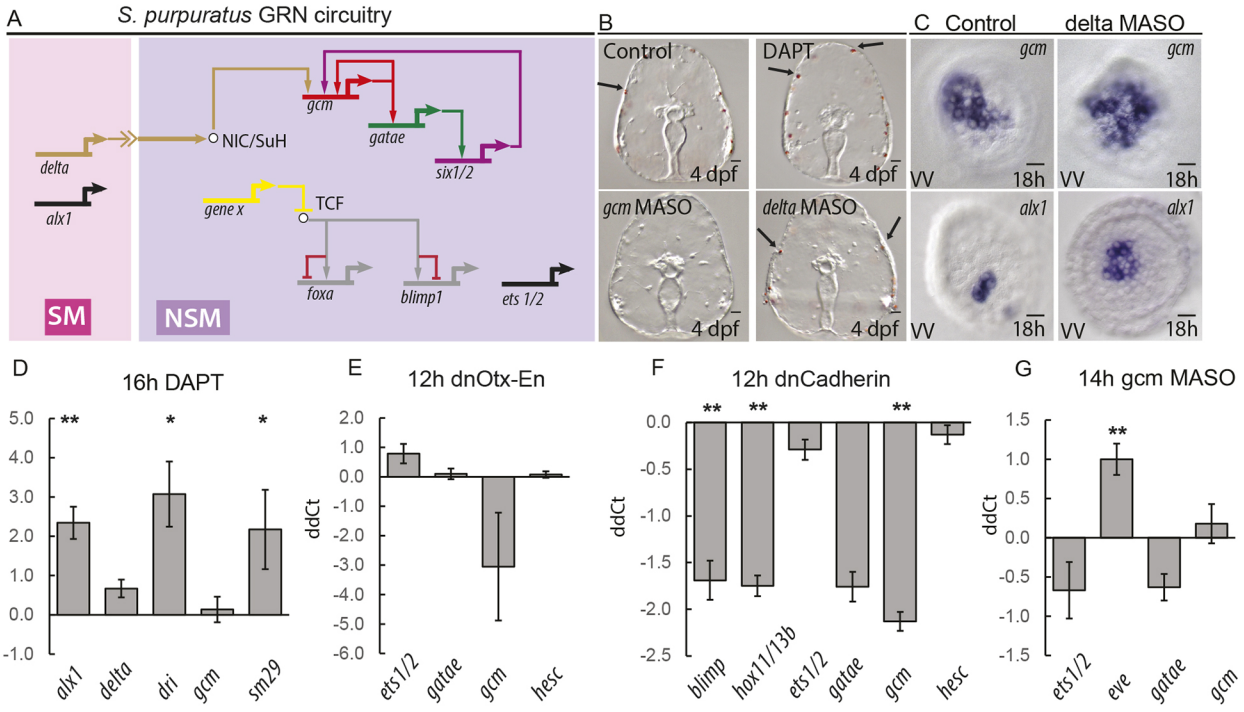

Fig. 3. Perturbation of mesodermal GRN circuitry in *E. tribuloides*. (A) BioTapestry diagram of selected regulatory circuits of the *S. purpuratus* NSM GRN. (B-G) Perturbation of selected regulatory linkages in *E. tribuloides*. (B) Development of red pigment cells (arrows) is not affected by loss of Delta/Notch signaling through injection of *Et-delta* MASOs or treatment with DAPT injection, but pigment cells are absent in embryos injected with *Et-gcm* MASOs. (C) WMISH detecting expression of *Et-gcm* and *Et-alx1* in embryos injected with *Et-delta* MASOs or control MASOs. Perturbation of Delta/Notch signaling does not interfere with expression of *Et-gcm* but expression of *Et-alx1* expands to NSM, which demonstrates that Delta/Notch signaling is functional in NSM cells. (D) qPCR analysis at 16 hpf showing that *Et-gcm* expression is not affected by perturbation of Delta/Notch signaling through DAPT treatment, whereas genes in SM GRN (*Et-alx1*, *Et-dri*, *Et-sm29*) are upregulated. *n*=3. (E) qPCR analysis of embryos injected with mRNA that encoded dnOtx-En showing a decrease of *Et-gcm* expression, but no effect on other NSM regulatory genes. *n*=2. (F) qPCR analysis of embryos injected with mRNA that encoded Δ-Cadherin (dnCadherin) showing downregulation of endodermal and NSM genes, including *Et-gcm*. *n*=2. (G) qPCR analysis in embryos that were injected with *Et-gcm* MASOs showing an increase in *Et-eve* expression but no significant effect on *Et-gatae*. ddCt, normalized difference in qPCR cycles compared with control embryos. *n*=4. Error bars represent standard deviation. Individual measurements are shown in Table S1. **P*<0.05, ***P*<0.01 (two-tailed *t*-test). Scale bars: 20 µm.

hox11/13b were significantly downregulated, whereas expression of *Et-gcm* was slightly downregulated but not significantly affected (Fig. 3E, Table S1). Expression of the NSM genes *Et-ets1*, *Et-gatae* and *Et-hesc* (Erkenbrack and Davidson, 2015) remained unaffected. To perturb the activity of Tcf, we injected *E. tribuloides* with mRNA that encoded a dominant negative form of Cadherin, Δ-Cadherin, which interferes with β-catenin nuclearization (Erkenbrack and Davidson, 2015). In *S. purpuratus*, injection of *Δ-Cadherin* mRNA leads to downregulation of endodermal genes including *Sp-blimp1* and *Sp-hox11/13b* (Davidson et al., 2002). Similarly, injection of *E. tribuloides* embryos with *Δ-Cadherin* mRNA resulted in significantly reduced expression of *Et-blimp1* and *Et-hox11/13b*, and significantly downregulated *Et-gcm* and *Et-ets1/2* (Fig. 3F, Table S1). These results suggest that expression of *Et-gcm* is regulated, directly or indirectly, downstream of maternal Tcf/β-catenin.

Absence of a signal-dependent positive feedback circuit

In *S. purpuratus* embryos, Delta/Notch signaling is active transiently for only a few hours. Once the signal turns off, continuous expression of *Sp-gcm* is subsequently controlled by a three gene positive feedback circuit (Ransick and Davidson, 2012). In this feedback circuit, *Sp*-Gcm activates the expression of *Sp-gatae*, and Sp-Gatae activates the expression of *Sp-six1/2*, the product of which in turn activates *Sp-gcm* expression (Fig. 3A) (Materna et al., 2013; Ransick and Davidson, 2012). In addition, *Sp*-Gcm activates its own expression by an autoregulatory feedback (Ransick and Davidson, 2012). To test whether a positive feedback circuit also regulates expression of *Et-gcm*, we injected embryos with *Et-gcm* morpholinos and analyzed expression of *Et-gcm* and *Et-gatae* (Fig. 3G and Table S1). The results show that blocking *Et*-Gcm did not affect the expression of these genes, indicating that neither Delta/Notch signaling nor the positive feedback is deployed in *E. tribuloides* during pre-gastrular development. Even after the onset of gastrulation, when *Et-gcm* expression is restricted to a subset of NSM cells, perturbation of *Et*-Gcm expression leads to ectopic expression of *Et-gcm*, indicating that a negative feedback circuit operates instead of a positive feedback during later development (Fig. S6 and Table S1).

Alternative mechanisms for exclusion of endoderm fate in NSM

In addition to activation of mesoderm specification, Delta/Notch signaling also leads to the repression of endodermal regulatory genes in euechinoids (Croce and McClay, 2010; Peter and Davidson, 2010, 2011b). In *S. purpuratus*, clearance of endodermal transcripts occurs at 15-18 h downstream of Delta/Notch signaling and independent of Gcm (Croce and McClay, 2010; Peter and Davidson, 2010). In *E. tribuloides*, clearance of *Et-blimp1* and *Et-hox11/13b* transcripts from NSM cells occurs at 16-18 h (Fig. 2C). However, treatment with DAPT did not interfere with clearance of *Et-blimp1* or *Et-bra* from NSM cells (Fig. 4A,B), which indicates that clearance of endodermal genes is independent

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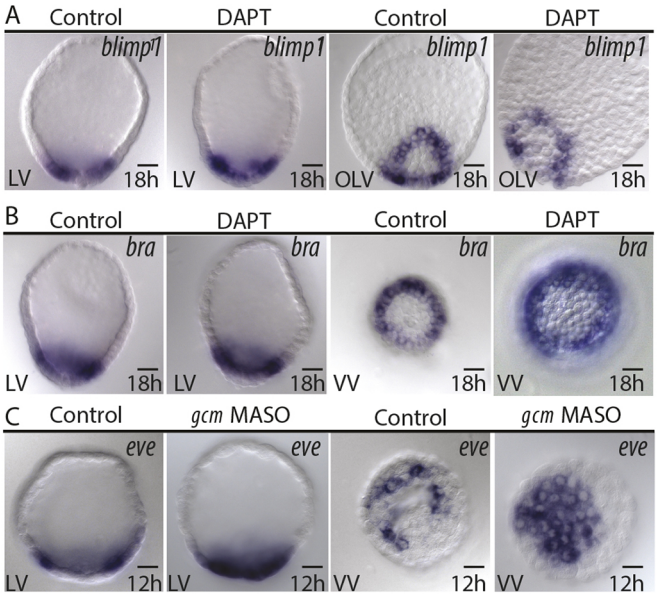

Fig. 4. Altered mechanisms for cell fate exclusion. (A,B) WMISH of *E. tribuloides* showing that perturbation of Delta/Notch signaling by DAPT does not interfere with clearance of *Et-blimp1* (A) and *Et-bra* (B) transcripts from mesoderm. (C) WMISH detecting *Et-eve* expression in *E. tribuloides* embryos showing ectopic expression in NSM in embryos injected with *Et-gcm* MASOs but not in embryos injected with control MASOs. LV, lateral view; OLV, oral-lateral view; VV, vegetal view. Scale bars: 20 µm.

of Delta/Notch signaling. Indeed, in *E. tribuloides* embryos that were injected with *Et-gcm* morpholinos, *Et-eve* expression is increased throughout the vegetal plate, including in NSM cells (Figs 3G and 4C). The exclusion of alternative cell fates is therefore regulated by different mechanisms in the two species. In *S. purpuratus*, Delta/Notch signaling is crucial for the exclusion of endoderm fates, whereas in *E. tribuloides* Delta/Notch signaling contributes to the exclusion of skeletogenic fates (Erkenbrack and Davidson, 2015). Furthermore, *Et*-Gcm mediates the repression of *Et-eve* in the NSM, a regulatory linkage that has not been observed in *S. purpuratus*.

Conserved activation of endoderm regulatory genes downstream of maternal factors

The endoderm GRN in *S. purpuratus* specifies distinct anterior and posterior endoderm cell fates during early stages of development, as shown in Fig. 5A (Peter and Davidson, 2010, 2011b). Both endodermal GRNs in *S. purpuratus* are initially activated by maternal Tcf/β-catenin. Previous results showed that expression of *Sp-blimp1*, *Sp-hox11/13b*, *Sp-foxa* and *Sp-bra* is controlled by maternal Tcf/β-catenin, with additional input from maternal Otx and from Hox11/13b into *Sp-blimp1*, *Sp-foxa* and *Sp-bra* (Ben-Tabou de-Leon and Davidson, 2010; Cui et al., 2014; Peter and Davidson, 2010, 2011b; Smith et al., 2008). To test the function of Tcf/β-catenin in endodermal gene regulation in *E. tribuloides*, nuclearization of β-catenin was perturbed by injection of *Δ-Cadherin* mRNA and gene expression was analyzed at 8 h, 12 h and 16 h. Expression of *Et-blimp1* and *Et-hox11/13b* was significantly reduced at 8 h, indicating that activation of the endodermal GRN depends on maternal Tcf/β-catenin (Fig. 5B and Table S1). Similarly, injection of mRNA that encoded dominant negative (dn)Otx-En significantly downregulated *Et-blimp1*, *Et-bra* and *Et-foxa*, but not *Et-eve* (Fig. 5C and Table S1). Therefore, initial activation of regulatory genes in the anterior endoderm occurs in

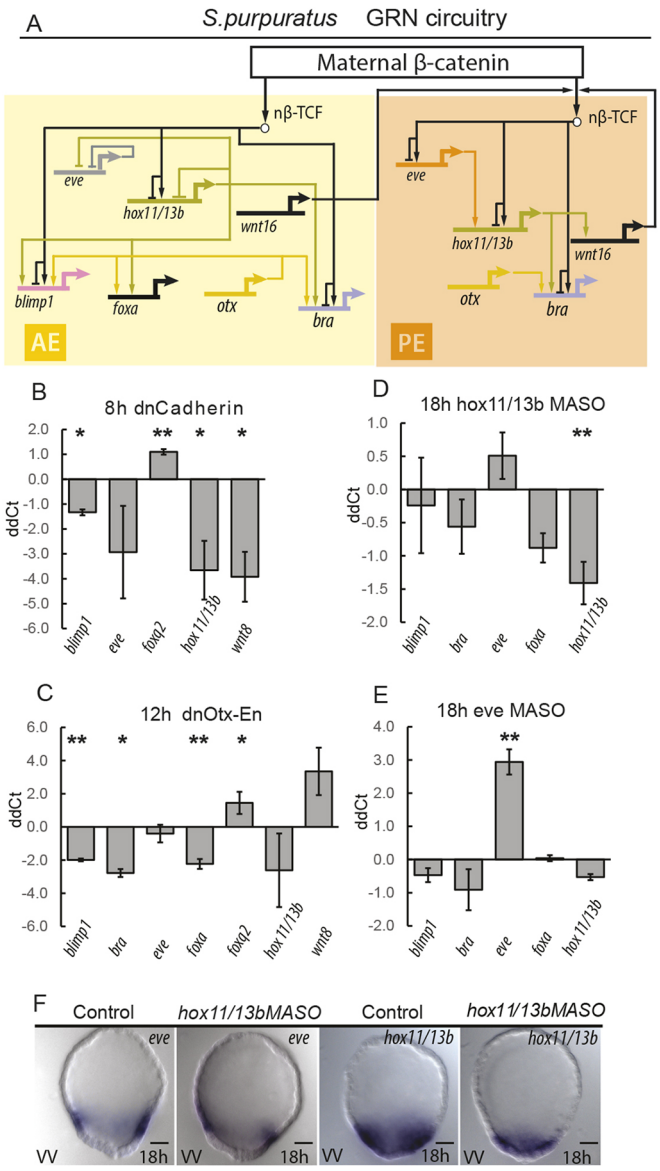

Fig. 5. Perturbation of potential endodermal GRN circuitry in *E. tribuloides*. (A) BioTapestry diagram displaying endodermal GRN circuits operating in *S. purpuratus* that are tested in *E. tribuloides*. (B) qPCR analysis in embryos injected with mRNA that encoded Δ-Cadherin, showing the downregulated expression of endodermal regulatory genes (*Et-blimp1*, *Et-hox11/13b*) but not of apical regulatory genes (*Et-foxq2*). *n*=2. (C) qPCR analysis in embryos injected with mRNA that encoded dnOtx-En, showing the effect on expression of several endomesodermal regulatory genes. *n*=2. (D) qPCR analysis in embryos injected with *Et-hox11/13b* MASOs, showing no significant effect on *Et-foxa*, *Et-bra* and *Et-blimp*, but a decrease of *Et-hox11/13b* expression. *n*=3. (E) qPCR analysis in embryos injected with *Et-eve* MASOs indicating increase in *Et-eve* expression, but no effect on *Et-hox11/13b* expression. *n*=3. (F) WMISH on embryos that were injected with *Et-hox11/13b* morpholinos or control MASOs showing no change in the spatial expression of *Et-eve*, but that expression of *Et-hox11/13b* is restricted to AE and absent from PE. ddCt, normalized difference in qPCR cycles compared with control embryos. Error bars represent standard deviation. Individual measurements are shown in Table S1. **P*<0.05, ***P*<0.01 (two-tailed *t*-test). Scale bars: 20 µm.

both sea urchin species downstream of maternal Tcf/β-catenin and Otx. However, injection of *Et-hox11/13b* morpholino did not significantly affect the expression of *Et-foxa*, *Et-blimp1* and *Et-bra*, which indicates that the regulatory function of Hox11/13b changed

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after the divergence of cidaroids and euechinoids (Fig. 5D and Table S1).

Changes in the activation of the PE GRN

The expression of *Sp*-Hox11/13b initiates the specification of PE and defines the boundary between endoderm and ectoderm in *S. purpuratus* (Cui et al., 2014, 2017; Li et al., 2014; Peter and Davidson, 2011b). The expression of *Sp*-Eve in the precursors of PE, together with Wnt signaling from AE, leads to the activation of *Sp-hox11/13b* expression in PE at 21-24 h (Cui et al., 2014; Peter and Davidson, 2011b). In turn, *Sp*-Hox11/13b activates the expression of *Sp-bra*. Both *Sp-eve* and *Sp-hox11/13b* are initially expressed in AE, and restriction of these genes to PE involves the auto-repression of both genes as well as the repression of *Sp-eve* by *Sp*-Hox11/13b (Fig. 5A) (Peter and Davidson, 2010, 2011b). In *E. tribuloides*, blocking the expression of *Et*-Eve by injection of morpholinos leads to increased levels of *Et-eve* expression at 18 h, as shown by qPCR, which indicates that a negative auto-regulatory feedback also controls the expression of *Et-eve* (Fig. 5E). However, expression of *Et-hox11/13b* was not affected by *Et*-Eve perturbation, indicating that the regulation of *hox11/13b* is different in the two sea urchin species. Injection of *Et-hox11/13b* morpholinos did not significantly affect the expression of *Et-eve* and *Et-bra*, but significantly reduced the expression of *Et-hox11/13b* (Fig. 5D,F), contrary to observations in *S. purpuratus*. Although the negative auto-regulation of *eve* appears to be conserved in echinoids, the repression of *eve* and *hox11/13b* by Hox11/13b, the activation of *hox11/13b* by Eve, and the activation of *bra* by Hox11/13b, all appear to be specific features of the euechinoid lineage that have been either acquired in the euechinoid GRN after divergence from cidaroids or lost in extant cidaroids.

A community effect circuit in PE

After the initial activation of *Sp-hox11/13b* expression in PE by Wnt signaling from the AE, the expression of Wnt ligands turns off in the AE and expression of *Sp-hox11/13b* is subsequently maintained in PE by a community effect circuit, a form of intercellular positive feedback circuit (Cui et al., 2014; Gurdon, 1988). In this circuit, *Sp*-Hox11/13b activates the expression of *Sp-wnt1* and *Sp-wnt16* and, in turn, expression of *Sp-hox11/13b* is activated by signaling from *Sp*-Wnt1 and *Sp*-Wnt16 among PE cells (Cui et al., 2014). To test whether the community effect circuit is functional in *E. tribuloides*, we analyzed the expression of *Et-hox11/13b* in embryos that were injected with *Et-hox11/13b* morpholinos. In perturbed embryos, expression of *Et-hox11/13b* was downregulated when analyzed by qPCR (Fig. 5D), and not detected in PE cells when analyzed by WMISH (Fig. 5F). Therefore, although expression of *Et-hox11/13b* remains unaffected in AE in perturbed embryos, expression in PE depends on the presence of *Et*-Hox11/13b, consistent with the requirement of a positive feedback circuit that is dependent on *Et*-Hox11/13b. Furthermore, expression of *Et-hox11/13b* also depends on Tcf/β-catenin. These observations are consistent with the conclusion that a Wnt- and Hox11/13b-dependent community feedback circuit also controls activation and maintenance of *Et-hox11/13b* expression in PE cells of *E. tribuloides*, similar to *S. purpuratus*.

DISCUSSION

Conservation of regulatory genes in the sea urchin endomesoderm GRN

At the phenotypic level, *S. purpuratus* and *E. tribuloides* display remarkable similarities in endomesoderm development. Both form similar endodermal domains, skeletogenic cells at the vegetal pole, and both form pigment cells that become intercalated within the aboral ectoderm at gastrula stage. Consistent with this observation, regulatory genes that are crucial to the function of the endomesoderm GRN in euechinoids are similarly patterning endomesodermal domains of the distantly related cidaroid *E. tribuloides*, which suggests a remarkable conservation of the regulatory roles of transcription factors that contribute to the specification of endomesodermal cell fates. All twelve regulatory genes that were analyzed from the *S. purpuratus* endomesoderm GRN were found to be expressed also in the *E. tribuloides* endomesoderm, suggesting that the endomesoderm GRN in the last common echinoid ancestor already included this set of regulatory nodes and likely patterned the endomesoderm in a similar way. Furthermore, network function in terms of regulatory state expression and specification of cell fates also shows similarity between the two sea urchin species. Our results indicate that the function of the endomesoderm GRN to generate at least two mesodermal and two endodermal progenitor cell fates was already present in the euechinoid/cidaroid ancestor. It has also been shown, in other developmental contexts, that the expression of regulatory states can be conserved over large evolutionary distances, e.g. in the nervous system (Royo et al., 2011) and in several cell types throughout bilateria (Arendt et al., 2016). Regulatory states might therefore be among the most conserved features of developmental GRNs, indicating their importance to developmental programs (Peter, 2017).

Evolutionary change in the function of Delta/Notch signaling

Our results show that, despite the establishment of similar mesodermal cell fates and expression of similar regulatory states, major changes have occurred in the GRN that controls specification of NSM cells after the divergence of cidaroids and euechinoids, as summarized in Fig. 6. Although Delta/Notch signaling is active in the NSM in both species, the function of this signal in NSM specification has changed during echinoid evolution. In *S. purpuratus*, Delta/Notch signaling is crucially important for the activation of NSM specification and the formation of pigment cells, and for the expression of *Sp-gcm*. Yet in *E. tribuloides*, interfering with Delta/Notch signaling does not affect pigment cell specification or *Et-gcm* expression. A simple explanation would have been that Delta/Notch signaling is not active in NSM cells in *E. tribuloides*, but this is not the case, as absence of Delta/Notch signaling leads to an activation of the skeletogenic cell fate in NSM cells (Erkenbrack and Davidson, 2015).

To reconstruct the evolutionary events that led to this redeployment of Delta/Notch signaling, we first considered regulatory features that were already present in the last common ancestor of cidaroids and euechinoids. Both species possess SM cells that express Delta and that are surrounded by NSM cells in which Delta/Notch signaling is activated. Furthermore, both *S. purpuratus* and *E. tribuloides* produce Gcm in NSM cells, which is required for the specification of pigment cells. Therefore, ancestral echinoids possessed NSM cells with activated Delta/Notch signaling in which Gcm functioned as a transcriptional activator of pigment cell specification. The specific evolutionary change in the function of Delta/Notch signaling that occurred after the divergence of euechinoids and cidaroids therefore specifically affected the regulatory interaction between Delta/Notch signaling and the *gcm* target gene, which was either lost in cidaroids or acquired in the euechinoid lineage.

To determine the function of Delta/Notch signaling that is likely to be ancestral, a comparison with distantly related echinoderms becomes necessary. In embryos of the sea star *P. miniata*, *Pm-delta*

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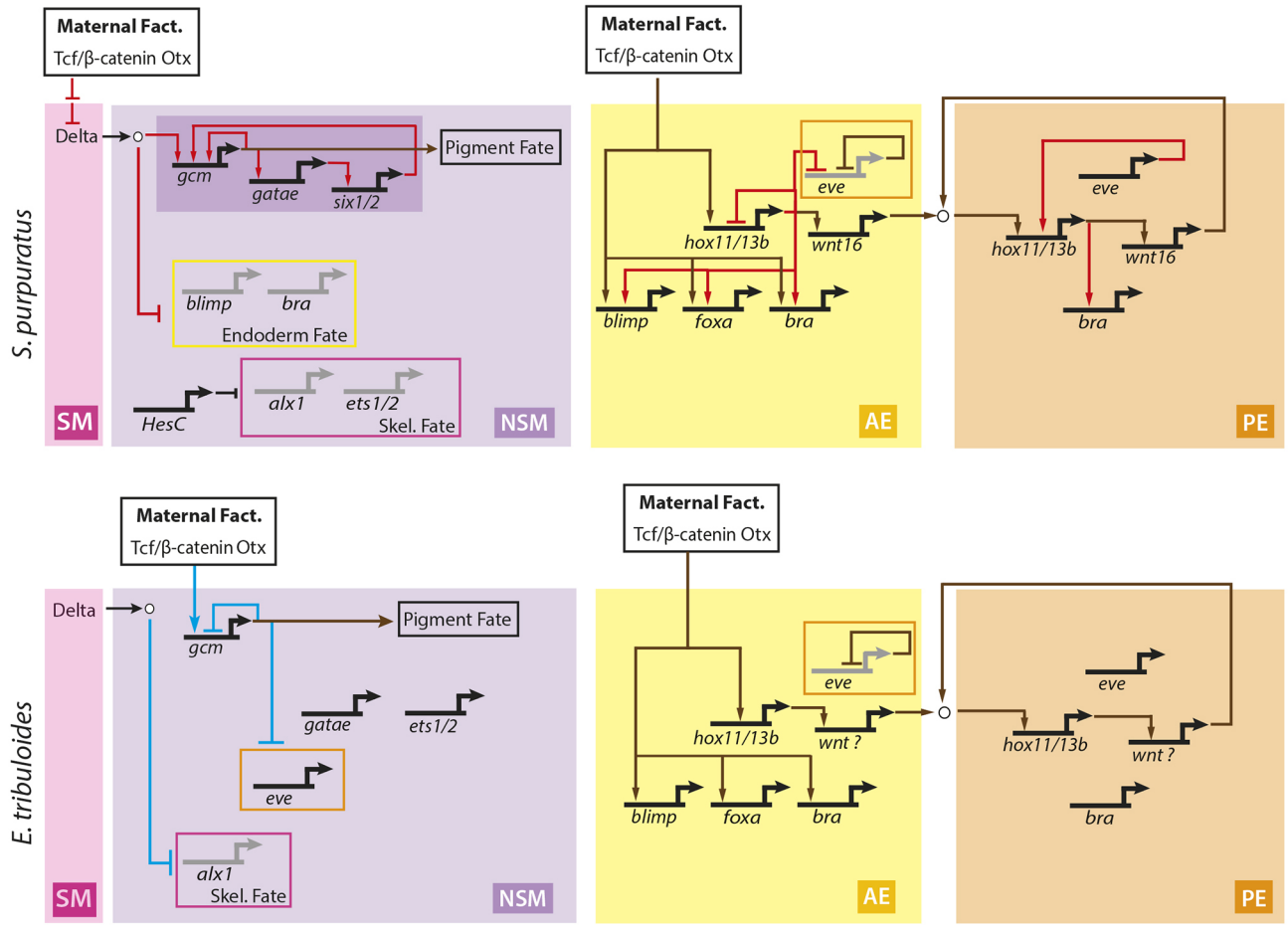

Fig. 6. Evolution of endomesodermal GRN circuits in echinoids. Biotapestry diagram (Longabaugh, 2012; Longabaugh et al., 2005) showing GRN circuits in NSM, AE and PE. Regulatory interactions that function in both species are shown by brown linkages, linkages specific to *S. purpuratus* are shown in red and those specific to *E. tribuloides* are shown in blue. The results show that only a few of the regulatory circuits tested remained conserved in the echinoid endomesoderm GRN since these two species last shared a common ancestor more than 268 million years ago. Circles indicate intercellular signaling interactions.

is expressed in vegetal pole cells, leading to activation of Delta/Notch signaling in adjacent cells despite the absence of skeletogenic and pigment cell fates (Hinman and Davidson, 2007). The function of Delta/Notch signaling in sea star embryos has been shown to lead to the exclusion and not the activation of mesodermal cell fates, similar to the function of Delta/Notch signaling in *E. tribuloides*, (Hinman and Davidson, 2007). These results suggest that the ancestral function of Delta/Notch signaling in the NSM was to suppress alternative cell fates, and that novel functions for Delta/Notch signaling in the specification of mesoderm cell fates were acquired within the euechinoid mesodermal GRN. During euechinoid evolution, the regulatory gene *gcm* became a novel target gene of Delta/Notch signaling during the pre-gastrular stages of embryogenesis. This regulatory interaction most likely formed by the acquisition of binding sites for SuH, the transcription factor responding to Delta/Notch signaling, in the *cis*-regulatory sequences that control *Sp-gcm* expression (Ransick and Davidson, 2006). The evolutionary change therefore led to the co-option of a regulatory interaction between a developmental signal and a target gene that were already both active in the same cells in an ancestral organism.

The important role of Delta/Notch signaling in mesoderm specification that was acquired in euechinoids might be somehow connected to the particular geometry of these embryos (Cameron and Davidson, 1991; Ruffins and Ettensohn, 1996). The first few cell divisions in *S. purpuratus* embryos are synchronized, producing a constant number of skeletogenic cells and a constant number of cells surrounding them in every embryo (Cameron et al., 1991). A ring of 15-16 cells surround the *Sp-delta*-expressing skeletogenic cells and forms the progenitors of the NSM lineage. These NSM precursors are all in contact with *Sp*-Delta-producing cells and express *Sp-gcm* in response to Delta/Notch signaling (Ransick and Davidson, 2006). In *E. tribuloides* however, only few skeletogenic cells are initially specified, and the number of skeletogenic cells varies (Erkenbrack and Davidson, 2015; Schroeder, 1981). As a result, the number of cells that receive contact-dependent Delta/Notch signaling also varies. The lack of control of the number of cells that receive Delta/Notch signaling might limit the function of this signal to the exclusion of alternative cell fates, whereas other regulatory mechanisms control the activation of mesoderm specification in a broader area of cells. Based on this argument, we would predict that the inductive function of Delta/Notch signaling in the NSM has been acquired only after the development of an invariant cleavage pattern during euechinoid evolution.

Acquisition of a positive feedback circuit downstream of Delta/Notch signaling

As with most developmental signaling interactions, Delta/Notch signaling is active only transiently in NSM cells of *S. purpuratus* and,

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subsequently, expression of *Sp-gcm* depends on a positive feedback circuit (Ransick and Davidson, 2012). This network constellation represents no exception. Positive feedback circuits are frequently observed motifs in GRNs, particularly downstream of transient signaling interactions (Narula et al., 2013; Peter and Davidson, 2015; Peter and Davidson, 2017). The proposed function of the signal-induced positive feedback circuit is to ensure that genes activated by a transient signal will continue to be expressed once the signal turns off. In *E. tribuloides*, expression of *Et-gcm* is not controlled by a transient signaling input but instead by maternal transcription factors that are present throughout early embryogenesis. Consistent with the idea that a positive feedback circuit is important, in particular in the context of transient signaling interactions, we found that *Et-gcm* expression does not depend on a positive feedback (Fig. 6). The positive feedback circuit mediated by *Sp-gcm*, *Sp-gatae* and *Sp-six1/2* is therefore a network feature that most likely evolved in the euechinoid endomesoderm GRN after the divergence from cidaroids. The observation that, during euechinoid evolution, *gcm* became a target of Delta/Notch signaling in early embryogenesis as well as a positive feedback circuit is consistent with the assumption that these two regulatory mechanisms are coupled.

Evolutionary change and conservation in the endoderm GRN

One might expect to see fewer evolutionary changes within the endoderm than the mesoderm GRNs, given the morphological similarities of endoderm development among echinoderms. Indeed, expression of FoxA throughout the endoderm, GataE in the midgut, and Hox11/13b in the hindgut is very similar at gastrula stage in *E. tribuloides* and *S. purpuratus*, and orthologs of all three transcription factors are also involved in the patterning of the vertebrate gut (Zorn and Wells, 2009). However, despite the expression of similar endodermal regulatory genes in the two sea urchin species, we present evidence here that evolutionary rewiring also affected the endoderm GRN. Most regulatory interactions tested here between *eve*, *hox11/13b*, *bra*, *foxa* and *blimp1*, were not functional during pre-gastrular development in *E. tribuloides*. Two important aspects of the endoderm GRN however appear to be shared in the two sea urchin species. First, the initial expression of endodermal regulatory genes depends on Tcf/β-catenin and Otx, which are transcription factors that are initially maternal and later provided by Wnt signaling and *otx* expression. Second, the maintenance of regulatory gene expression in PE cells depends on an intercellular community effect circuit, a circuit that regulates the expression of a common regulatory state within a field of cells in both species (Fig. 6) (Gurdon, 1988). Similarly, in sea star embryos, endodermal expression of *bra* is controlled by Tcf/β-catenin and Wnt16 is expressed in the PE, as in *S. purpuratus* (McCauley et al., 2013; McCauley et al., 2015). The importance of Wnt signaling in the activation of endodermal GRNs is therefore a shared feature among echinoderms and possibly beyond.

Evolutionary history of the endomesoderm GRN

The comparative analysis of the endomesoderm GRNs in *S. purpuratus* and *E. tribuloides* reveals a remarkable conservation of regulatory nodes, as all twelve regulatory genes tested here are expressed in the endomesoderm of both species, as components of similar cell fate-specific regulatory states. However, a considerable rearrangement of network architecture has occurred since these two lineages diverged from each other at least 268 mya. Evolutionary rewiring in the upstream hierarchy of developmental GRNs has also been shown in other animals. For example, the segmentation in early arthropod embryos involves expression of *en* and *wingless* at the anterior and posterior boundary of each parasegment, but whereas the expression of these genes is conserved, the GRN that controls their expression is different in *Drosophila* and *Tribolium* embryos (Choe and Brown, 2009; Damen, 2007; Peel et al., 2005). The novel features of the endomesoderm GRN, which were acquired after the divergence of cidaroids and euechinoids, are the regulatory interactions among signaling molecules and transcription factors that are already co-expressed in given cell fates, thus contributing to the timing and maintenance of regulatory state expression in respect to the developmental geometry of the sea urchin embryo.

In conclusion, this work shows that, despite the remarkable conservation of transcription factors that control development in the sea urchin endomesoderm and in other developmental processes, GRNs may continue to evolve by formation of novel regulatory circuits through gain and loss of regulatory interactions. Developmental GRNs therefore display some degree of flexibility in network architecture, allowing the continuous rewiring of regulatory linkages among conserved sets of regulatory factors without necessarily affecting downstream cell fate-specific gene expression. Through comparison of regulatory functions among homologous developmental GRNs at different evolutionary distances, it therefore becomes possible to reconstruct the evolution of complex developmental control mechanisms that occurred over long periods of evolutionary time.

MATERIALS AND METHODS

Animals and embryo cultures

Eucidaris tribuloides sea urchins were obtained off the coast of Key Largo, FL (SeaLife) and were maintained in room temperature aquaria. Animals were spawned by intracoelomic injection of 0.5 M KCl. Cultures were grown between 22°C and 23°C in Millipore-filtered natural sea water.

qPCR

The qPCR timecourse for the twelve genes of interest in this study was carried out at 23°C over the first 20 h post fertilization (hpf) of *E. tribuloides* development. For each timepoint, 100 embryos were collected and a cDNA template was obtained as previously described (Erkenbrack and Davidson, 2015). To obtain per embryo transcript counts in timecourse samples, each timepoint was spiked with ∼1000 copies of synthetic Xeno RNA (TaqMan Cells-to-Ct Kit, Life Technologies). Microinjected embryos [morpholino antisense oligonucleotides (MASO) and constructs] were prepared as previously described (Erkenbrack and Davidson, 2015), except that the exogenous RNA spike-in was Xeno RNA rather than GFP RNA. Primers to amplify cDNA were designed based on incomplete sequences provided by EchinoBase. cDNAs were amplified, cloned, sequenced and used as templates for probe synthesis. All cDNA sequences have been deposited in GenBank (accession numbers are listed in Table S2). qPCR analysis for each gene was performed using the primer sequences in Table S3. Statistical analyses were carried out on delta Ct values (two-tailed *t*-test) of control and treatment groups of two to four replicates. A P-value <0.05 was considered statistically significant.

WMISH

Chromogenic WMISH was carried out as previously described (Erkenbrack and Davidson, 2015). Embryos were prepared for double fluorescent WMISH (dfWMISH) according to the same protocol and were stained using the Tyramide Signal Amplification Kit (Perkin Elmer). Anti-digoxygenin-AP FAB fragment (Roche, 11093274910) concentration was 1:2000, and both cyanine3 and fluorescein were diluted 1:400 in manufacturer's diluent solution. Staining proceeded for ∼5 min at room temperature. Stained embryos were imaged on an Axioskop II Plus equipped with an Axiocam MRc (Carl Zeiss). WMISH primers used in this study are listed in Table S4.

Microinjection of MASOs, constructs, and RNA

Unfertilized eggs of *E. tribuloides* were prepared essentially as described in Erkenbrack and Davidson (2015). MASOs were synthesized by Gene Tools

and their sequences are provided in Table S5. All MASO injection solutions were 1 mM, and each fertilized egg received ∼10 pl of injection solution. Gene expression in MASO-injected embryos was compared with uninjected embryos of the same batch, as in Erkenbrack and Davidson (2015). Injected embryos were monitored and experiments discarded if embryos showed developmental delays. Embryos for WMISH or qPCR were collected and processed as described above. At least 100 embryos were injected or treated per experiment and, of these, 10-20 embryos were carried forward for imaging and downstream analyses. The *Et*-dnOtx-En mRNA construct consisted of: 5′-T3 RNA polymerase recognition sequence, the 5′ 885 nucleotide (nt) repressor domain of the *Drosophila melanogaster engrailed* coding sequence, the 225 nt homeodomain of *E. tribuloides*, and a 21 nt nuclear localization sequence-3′ (see the supplementary information for details). The complete coding sequence was codon-optimized and synthesized as a single gBlocks fragment (IDT). After addition of dATP nucleotides to the 3′ ends, the construct was directly ligated into pGEM-T vector (Promega) and cloned into *Escherichia coli*. Capped mRNA was synthesized using the T3 mMessage Machine kit (Ambion, Thermo Fisher Scientific) and microinjected into *E. tribuloides* embryos. *Δ-Cadherin* RNA, which blocks β-catenin nuclearization at the vegetal pole, was synthesized with SP6 mMessage Machine kit (Ambion, Thermo Fisher Scientific) and injected at a concentration of 1000 ng/µl. As a control for the effect of the introduction of exogenous RNAs we injected capped GFP mRNA.

Treatment with small molecule inhibitors

Embryos were treated with DAPT (GSI-IX, Selleck Chemicals). Dose response was tested using three concentrations of DAPT (5, 10 and 20 µM) and all experiments were carried out at 10 µM.

Acknowledgements

We thank Deanna Thomas for her contributions to the figures, Jonathan Valencia for contributing an image of *S. purpuratus*, and Rebekah Kitto for helping with WMISH and imaging embryos. We are grateful to Andy Cameron, Parul Kudtakar and the bioinformatics team at the Center for Computational Regulatory Genomics at the Beckman Institute for their computational support.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.M.E., E.H.D., I.S.P.; Formal analysis: E.M.E., I.S.P.; Investigation: E.M.E.; Writing - original draft: E.M.E., I.S.P.; Writing - review & editing: I.S.P.; Supervision: I.S.P.; Funding acquisition: E.H.D., I.S.P.

Funding

This work was supported by the National Institutes of Health (Eunice Kennedy Shriver National Institute of Child Health and Human Development) grant HD037105 and the National Science Foundation (Division of Integrative Organismal Systems) CREATIV grant 1240626. Deposited in PMC for release after 12 months.

Data availability

All sequence data have been deposited in GenBank under accession numbers MF990311, MF990312, MF990313, MF990314, MF990315, MF990316, MF990317, MF990318, MF990319, MF990320 and MF990321 (as listed in Table S2).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.167288.supplemental

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