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Review

Evolution of gene regulatory network architectures: Examples of subcircuit conservation and plasticity between classes of echinoderms

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ABSTRACT

Developmental gene regulatory networks (GRNs) explain how regulatory states are established in particular cells during development and how these states then determine the final form of the embryo. Evolutionary changes to the sequence of the genome will direct reorganization of GRN architectures, which in turn will lead to the alteration of developmental programs. A comparison of GRN architectures must consequently reveal the molecular basis for the evolution of developmental programs among different organisms. This review highlights some of the important findings that have emerged from the most extensive direct comparison of GRN architectures to date. Comparison of the orthologous GRNs for endomesodermal specification in the sea urchin and sea star, provides examples of several discrete, functional GRN subcircuits and shows that they are subject to diverse selective pressures. This demonstrates that different regulatory linkages may be more or less amenable to evolutionary change. One of the more surprising findings from this comparison is that GRN-level functions may be maintained while the factors performing the functions have changed, suggesting that GRNs have a high capacity for compensatory changes involving transcription factor binding to cis regulatory modules.

1. Introduction: comparative developmental gene regulatory networks and evolution

Developmental gene regulatory networks (GRNs) are epistatic maps of the interactions between regulatory gene products and their cis regulatory elements, as well as between signaling ligands and their receptors. Ideally, GRNs display the flow of regulatory information throughout embryogenesis, starting with anisotropies in the zygote that initiate alternate blastomere identities and ending with the activation of differentiation products within appropriate cell lineages. Although GRNs can incorporate many years' worth of already available data, they also require targeted experimental approaches to determine the direct regulatory targets of transcription factors. For this reason GRNs are not technically trivial to elucidate and are most fruitfully undertaken in embryonic systems in which functional cis regulatory analyses can be performed readily. However, the recent abundance of literature contributing to the growth of such models in various organisms attests to the power of this approach for understanding development [1–12].

The intention of this review is to demonstrate how a comparison of orthologous GRNs from among appropriately chosen taxa is enormously revealing of evolution and also further illuminating of developmental mechanisms. Since the genomic toolkit for development is well conserved, the prominent mode of evolutionary change is through altered uses of orthologous genes [13,14]. A comparison of GRN architectures reveals precisely how regulatory interactions are modified during evolution and how these changes translate into novel developmental programs. Most regulatory interactions (or "nodes") of a GRN represent the binding of a transcription factor protein to a specific sequence within the cis regulatory DNA region of the target gene. Evolutionary changes in sequence that affect the ability of a transcription factor to bind DNA specifically (either through changes to the protein or DNA binding site) will lead to the gain or loss of a particular node, resulting in a change in the trajectory of the GRN and, consequently, the developmental outcome. A GRN approach to understanding evolution improves on the more traditional comparative developmental methods in that it considers the effect of changing the direct, immediate targets of the transcription factor in the context of the whole developmental process explained by the GRN.

While the utility of GRNs for inferring animal development is well established, little is known about how these networks evolve. The vast majority of studies examining the evolution of regulatory networks are performed in bacteria and yeast. Engineered regulatory networks, particularly in bacteria, have revealed that artificial GRNs can tolerate fairly extensive rewiring of particular connections without overt deleterious effects on the organism [for example, 15,16]. For example, a recent study in yeast has shown that turnover of Mcm1 binding sites occurs fairly often and, in one species, is correlated with the gain pathogenicity [17]. However, because these predictions have not been

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made in the context of a developing multicellular organism, they may not, in fact, reflect the changes that are possible in such a situation. In developmental systems, most notably Drosophila, computational models have been used to explore the phenotypic effects of particular mutations within the context of a GRN [for example, 18]. Thus, while we can make predictions about how networks may evolve (under artificial selection or modeling), there is little experimental evidence as to the types of changes a GRN can tolerate.

On the other hand, there are extensive examples of comparative development that identify molecular changes underlying morphological evolution. While these studies provide valuable insights into the proximal mechanisms by which a trait may arise in evolution, most do not seek to understand the molecular changes in the context of a GRN. There are a few examples, however of comparative development analyses that focus on a GRN-level understanding. For example, is a comparison of dorsal–ventral patterning in Drosophila melanogaster and Anopheles gambiae (mosquito) [19]. Mosquitoes, like many insects, have two distinct extraembryonic membranes that arise from the dorsal ectoderm — the amion and the serosa. Flies, however, possess a single amnioserosa tissue derived from the dorsal ectoderm. Goltsev et al. [19] suggest that this derived characteristic of Drosophila may be attributed a narrowing of Dpp signaling due to an expanded expression of *sog*, a *Dpp* repressor. In addition, a loss of a regulatory linkage between *zen* and *ttk* in Drosophila permits *Dpp* activation of the amnion specific genes *tup* and *Doc1/2*. In Anopheles, *zen* might activate *ttk*, allowing for *ttk* to partition the dorsal ectoderm into two distinct tissues, via repression of *tup* and *Doc1/2*. This example nicely illustrates how the developmental, and hence morphological, consequence of a change in transcription factor function usage can only be properly understood in the context of the surrounding GRN.

There is also a good example of a very broad-scale GRN comparison, that of heart field specification *Drosophila* and vertebrates, which revealed conserved regulatory interactions among *nk2* (*tinman*), *tbx* (*mid-H15*), *gata* (*pannier*), *hand* and *mef2* [reviewed in 20]. This is a very important observation, as it suggests that regions of a GRN may be deeply conserved. Such distant comparisons, however, are not going to be informative of the precise nature of evolutionary regulatory changes underlying morphological variation.

Finally, recent work in understanding the evolution of trichome pattern in Drosophila has implicated cis regulatory changes in a transcription factor, *shavenbaby*, thought to act directly downstream of patterning subcircuitry, as being critical for novel trichome positioning in flies [21]. This is a striking example of understanding regulatory evolution in the context of a GRN; however, the vast majority of the network was unaffected by these changes. In order to study more closely how these networks evolve, we must focus on understanding changes more central to the developmental program.

2. The modularity of GRNs

Before considering in more detail how GRNs have evolved in echinoderms, it is important to realize that, in general, developmental regulatory networks, for all their complexity, are modular in organization. GRNs may be conceptualized as a temporal series of interconnected subcircuits, where each subcircuit is defined as a set of regulatory interactions among several transcription factors that can execute a particular function [22,23]. If subcircuits indeed operate as functional units then evolution may act on them as a whole. GRN comparisons may reveal, therefore, the redeployment and maintenance of entire subcircuits. Davidson [24] provides many examples of GRN subcircuits that execute specific developmental functions. These include the use of positive feedback to stabilize transcription initiated by a transient signal, exclusion of alternative regulatory states, subdivision of territories following input from a signaling pathway, and defining tissues and setting their boundaries. Oliveri and Davidson [22] stress that the function of the subcircuit is determined by the architecture of the regulatory connections and consequently the individual transcription factors executing the function are interchangeable. For example, it is the positive regulatory feedback between two, or more, transcription factors that provides the stabilizing function of the subcircuit rather than something specific to the structure of the factors themselves. This is because the function of a transcription factor is somewhat trivial; each transcription factor in the GRN performs a very straight-forward operation: to either activate or repress expression by binding to its cis regulatory DNA target. This is in stark contrast to genes acting at the termini of the GRN, which are involved in differentiation and have highly particular functions related to the very specific properties of the protein.

Davidson and Erwin [25] suggest that GRNs are composed of different types of subcircuits that fulfill specific roles in a given developmental program. Because of their diverse functions, these subcircuits differ in their capacity for evolutionary change. They outline the categories of subcircuit as including: 1) kernels, 2) plug-ins, 3) input/output switches and 4) differentiation gene batteries. Kernels are defined as subcircuits that initiate the development of animal body parts and are highly resistant to evolutionary change. Such subcircuits provide a molecular explanation as to why phyletic body plans have remained essentially unchanged since their first appearance in the Cambrian or Precambrian [26]. Kernels likely operate near the top of a gene network hierarchy, while differentiation gene batteries operate at the periphery of the gene network, serving as its functional output. The protein products of battery genes confer structural and enzymatic functions to differentiated cell types. For example, cell-type specific tasks such as building muscles, skeleton, and nervous systems are all derived from battery gene output. Importantly, differentiation battery gene products do not affect the function of other subcircuits in the GRN. Thus, changes to differentiation gene batteries can only alter the functional capacity of a body part and therefore are most likely to produce changes in development and evolution at the species level. Davidson and Erwin [25] predict that because they do not affect other GRN subcircuit functions, changes in differentiation gene batteries will occur frequently.

The remaining types of subcircuits are plug-ins (i.e. flexible subcircuits that are repeatedly co-opted for use in diverse developmental events) and input/output switches (i.e. subcircuits that serve as devices to activate or repress other subcircuits). These subcircuits are thought to be evolutionary labile, due to the small number of cis-regulatory changes necessary for their incorporation into a GRN. Changes in these types of subcircuits are thought to account for novel characteristics that arise at the class, order, and family taxonomic levels [25].

Currently, there is little direct evidence that such discrete subcircuits are subject to differential selective forces. Of these subcircuits, kernels have been the most well-characterized, though the evidence is largely drawn from two sources: a comparison of heart field specification in Drosophila and vertebrates [20,24 and see above] and the echinoderm studies presented herein. Work with echinoderm larvae is beginning to elucidate other types of conserved GRN functions, as well as reveal specific divergences in subcircuit function within the context of orthologous regulatory networks.

3. Sea stars and sea urchins as model organisms for comparative GRN analyses

The purple sea urchin (Strongylocentrotus purpuratus) is an important model organism for elucidating GRN architecture partly due to the ease with which cis regulatory analyses may be performed, allowing for verification of predicted GRN architectures. As a case in point, the highly developed GRN for endomesodermal specification in the sea urchin is rapidly approaching a state in which all nodes are verified at the cis regulatory level [27–36]. Sea urchins (Cl. Echinoidea) are one of the five classes of echindoderms (Fig. 1). The phylogenetic history of echinoderms is well known, in part due to the excellent fossil record left by their calcareous spines, tests, and teeth [37]. Crinoids are

the most basal group, but embryos are difficult to obtain and almost all known extant species have a derived form of development (a notable exception being the newly described larva of Metacrinus rotundus [38]). The other classes of echinoderms are grouped together as the Eleutherozoa, and sea urchins and sea stars are the most divergent of these classes, having last shared a common ancestor around 500 million years ago [39,40]. The sea star, Asterina miniata (Cl. Asteroidea), has proven to be an excellent comparative model organism for GRN studies for several technical and phylogenetic reasons [41–45]. Gene function perturbation and cis regulatory analyses are relatively straightforward, and sea stars are at a perfect evolutionary distance from sea urchins for meaningful comparisons of network architecture. As will be shown below, there are regions of the GRN involving orthologous regulatory genes that are highly conserved during development of these two echinoderms and other regions that are divergent [41,44]. This allows us to delve into an analysis of the evolutionary capacity for change of different subcircuit types.

While the adult forms look entirely different, the early development of sea urchin and sea star larvae is quite similar; although some important differences exist [42]. The endomesodermal territories form at the vegetal pole in both taxa in what is termed the vegetal plate (Fig. 2A). The invagination movements of gastrulation proceed from the center to the periphery of this plate. Mesoderm progenitors are central-most in the vegetal plate and therefore invaginate first and sit at the top of the archenteron; the outer tiers of cells will then progressively invaginate to form fore-, mid- and hind-gut [46,47]. In these ways the sea urchin and sea star are very similar. However, modern sea urchins (such as S. purpuratus) undergo early unequal cleavage to produce a vegetal-most micromere cell lineage. This lineage ingresses into the blastocoel prior to invagination and forms the larval skeleton. Sea stars undergo equal cleavage, do not have a population of early ingressing cells, and do not from a larval skeleton. As only modern sea urchins posses this micromere cell lineage, it is clearly an evolutionarily derived state within this group of echinoderms [48]. These developmental programs provide an exceptional opportunity to compare GRN architectures that underlie both conserved aspects of development and the evolution of derived programs.

4. The GRN for endomesodermal specification in sea urchins

The remainder of this review will focus on a comparison of the GRN architecture in these two echinoderms. The GRN model in sea urchin [7–8] explains how cells at the vegetal pole of the embryo are fated to become endoderm and mesoderm, distinct from the remainder of the embryo, which forms various ectodermal cell types. The overall process described by the GRN (the pertinent regions of which will be explained more fully below) starts with an explanation of how initial anisotropies within the zygote are sufficient to specifically activate certain transcription factors within the vegetal region of the embryo. These transcription factors then activate or repress additional sets of transcription factors to further lock down the specification of the vegetal blastomeres and distinguish them from the animal half of the embryo. Vegetal cell types are further subdivided into endoderm or mesoderm through signals from adjacent cells that direct the regulation of additional transcription factors within only subsets of cells that receive the signal. Eventually each cell territory has a unique regulatory state and therefore will activate distinct batteries of differentiation genes appropriate to their lineage.

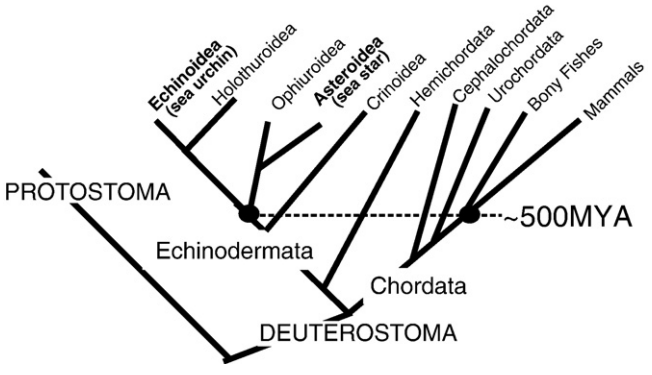

Fig. 1. Phylogenetic relationships among the deuterostomes. Bilateral animals are broadly classified as either protostomes or deuterostomes. Echinodermata, along with Hemichordata, Chordata and the less well known Xenoturbella (not shown) comprise the deuterostomes. The crinoids (Crinoidea) are the most basal class within the echinoderms. Sea urchins (Echinoidea) and sea stars (Asteroidea) are distantly related within the Eleutherozoa and last shared a common ancestor around 500 million years ago. This corresponds to approximately the same period in which the bony fishes first emerged.

5. A comparison of the early GRN for endomesoderm specification in sea urchin and sea star reveals an example of a kernel

The earliest process by which vegetal cells are distinguished in the sea urchin is initiated by the localization of both β-catenin and Otx to the nuclei of vegetal blastomeres [49–57] (Fig. 2). Extensive gene perturbation and direct functional cis regulatory analyses revealed that these factors activate expression of the transcription factor *blimp1* and the ligand *wnt8*, the latter of which provides a signal that drives the further nuclearization of β-catenin [29,36,52,53]. Blimp1 activates expression of *wnt8* and *otx* [29,32]. Thus positive regulatory interactions are formed between the *otx*, *blimp1*, and *wnt8* genes (via, in part, the Wnt/nuclear β-catenin signaling pathway). Embryological manipulations also demonstrate that sea star embryos have a vegetally localized maternal determinant required to specify endomesoderm and that β-catenin is preferentially nuclearized in cells at the vegetal pole [54–56]. It has not been experimentally verified that this nuclearization also functions in early segregation of these lineages but it seems likely as β-catenin has a role in early axis specification in many animals [57,58]. It is also unknown whether β-catenin nuclearization activates the sea star ortholog of *blimp1*, although *blimp1* is transcribed early and in the vegetal pole [59]. Perturbation experiments, however, do demonstrate that *blimp1* and *otx* form a cross-regulatory loop in sea star embryos (Fig. 2), although, as will be discussed later, *otx* in the sea star is initially activated by Tbrain and not the early form of Blimp1 [43]. In both sea urchins and sea stars, *gataE* transcription is activated by Otx, and GataE drives expression of *foxA* and *bra*, transcription factors required for the correct differentiation of endoderm [7,33,41]. In both organisms GataE also subsequently feeds back to positively regulate *otx* expression, which further maintains the endoderm as a distinct embryonic territory [32,41,43]. These two distantly related echinoderms have therefore maintained the recursive regulatory interactions between *blimp1*, *otx* and *gataE* for almost 500 million years. The highly conserved uses of these factors in this recursively-regulated subcircuit define a developmental regulatory kernel [25]. This positive feedback loop operates to stabilize the expression of *gataE* (and subsequently *foxA* and *bra*) downstream of the transient signaling pathways that initially set up the state of the vegetal hemisphere. Gata factors have highly conserved roles in specifying endoderm in many taxa [60] and hence this regulatory kernel may have been maintained in these echinoderms through such extensive periods of evolution to ensure *gata*-gene expression within the endoderm.

6. Subcircuits operating downstream of the kernel: evidence of evolutionary plasticity

The significance of the conserved regulatory kernel is highlighted by the number of evolutionary linkage changes that occur preceding and after the developmental placement of this subcircuit (Fig. 2). This additional comparison provides further evidence that the GRN is

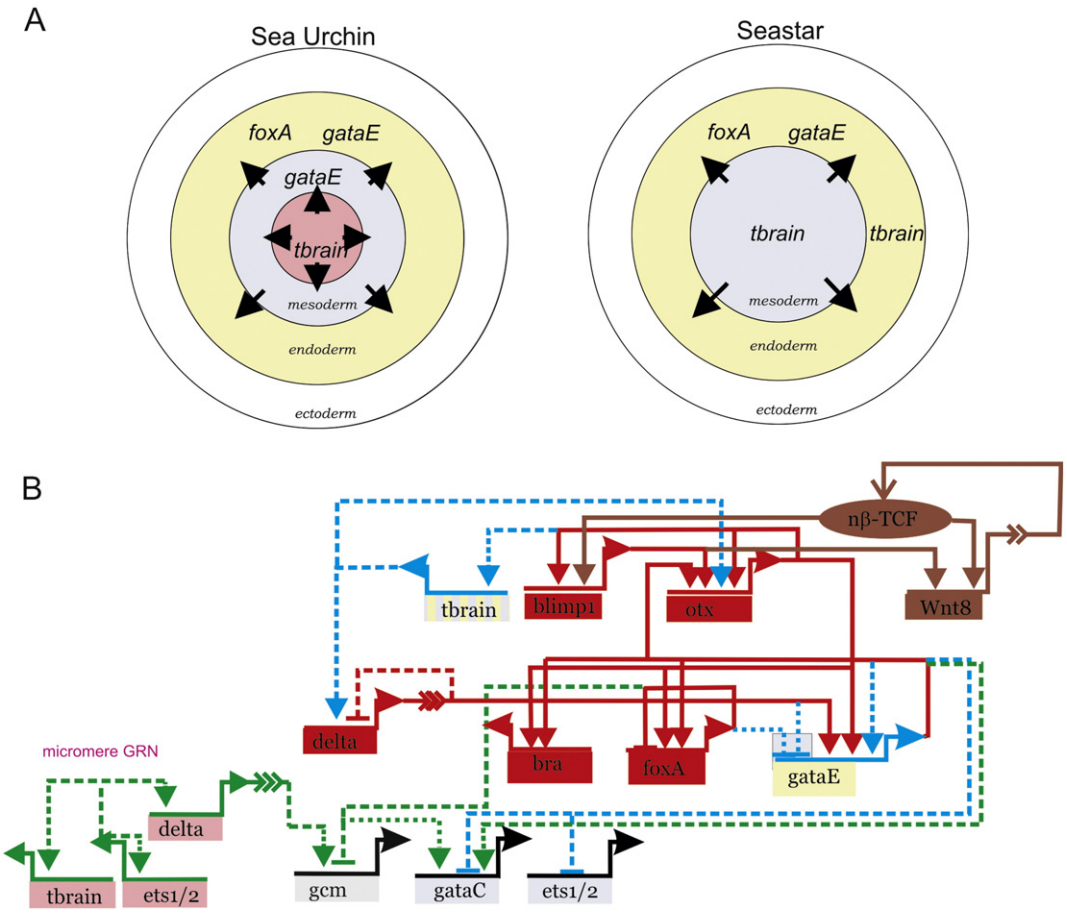

Fig. 2. Conservation and divergence in endomesoderm specification in sea urchins and sea stars. (A) Schematic representation of sea urchin and sea star blastulae viewed from the vegetal pole. In both organisms, the mesoderm (blue disc) is derived from the central vegetal plate region and the surrounding cells will give rise to endoderm (yellow ring). In modern sea urchins, an additional mesodermal lineage (the micromere lineage indicated by the pink disc) is formed at the central-most vegetal plate and will ingress into the blastocoel as the first movement of gastrulation. Some gene names are overlaid in the domain of their expression, e.g. *tbrain* is expressed in the micromere cell lineage in sea urchin and in the mesodermal and endodermal territories in sea star, and *gataE* is expressed within the endoderm and mesoderm in sea urchin but only the endoderm in sea star. The arrows represent Delta-N signaling from one cell territory to another. (B) The GRN depicting endomesoderm specification in sea urchins and sea stars at blastula stage. Many gene names are shown against a background of color that represents their domain of their expression, i.e. micromeres-pink, mesoderm-blue, endoderm-yellow, endoderm and mesoderm-hashed blue/yellow. Each horizontal line above the gene name represents a 'node', i.e. it represents the cis regulatory module on which the protein (represented by the line emanating from another gene) binds. An arrow indicates positive regulation, while a bar represents repression. The regulatory interactions found in common in both taxa are shown in red, while those occurring in only sea urchin are shown dashed in green, and those only occurring in sea star are shown dashed in blue. In sea urchins, the nuclearization of β-catenin is critical for the establishment of endomesoderm and forms a positive feedback loop with *blimp1* (shown in brown). The role of nuclear β-catenin has not been examined in sea stars, but is likely to be conserved. Many of the initial molecular events acting downstream of the nuclearization of β-catenin are conserved in these two echinoderms (i.e. all of the interactions shown in red), and are defined as a kernel. However, there are important differences in the deployment of the endomesoderm kernel and in the specification of mesoderm in sea urchins (green linkages) and sea stars (blue linkages). After [7,44].

composed of functional subciruits that evolve at different rates. As development proceeds, the vegetal plate endomesoderm territory specified by the kernel subcircuit is further subdivided into endodermal versus mesodermal progenitors. In both taxa, the mesoderm forms at the central-most vegetal plate while the endoderm forms in a ring around this [46,47] (Fig. 2A). Both embryos must therefore deploy subcircuitry that functions to set alternative regulatory states within two concentric rings. As discussed above, sea urchin embryos have an evolutionarily derived micromere cell lineage (also known as the primary mesenchyme or skeletogenic cells) at the very center of the vegetal pole (Fig. 2A); this entire lineage is absent in sea stars. While the ultimate developmental outcome of this lineage is to form the larval skeleton, it is also an important source of signals to the overlying secondary mesenchyme cell lineage that forms the remainder of the mesodermal cell types in sea urchin embryos. Thus embryological evidence already indicates that there must be substantial deviation in the mechanisms used to specify mesoderm in these two classes of echinoderms.

In sea urchins, the micromeres produce the membrane-bound Delta ligand that activates the Notch (N) signal transduction pathway in the adjacent secondary mesenchyme cells [61,62]. The transcriptional output of this pathway is the activation of the transcription factor *gcm* that in turn activates the transcription factor *gataC* [27]. These factors are required to specify various mesodermal cells types, including pigment and blastocoelar cells [63,64]. *Delta* expression is turned off in the micromeres as they ingress into the blastocoel, and is activated within the now central-most vegetal tier of cells (the secondary mesenchyme cells) that previously received Delta-N signaling. Delta now signals to N within in the overlying endodermal tier of cells. The transcriptional output of Delta-N signaling within these cells is *gataE* [65]. This second phase of Delta-N signaling thus serves to reinforce specification of endoderm in these cells. Additionally, in sea urchins, FoxA, which is activated downstream of the recursively wired kernel, functions within this endodermal territory to dually activate the expression of other endodermally expressed transcription factors and to repress the expression of transcription factors that function to specify mesoderm [66]. For example, FoxA functions to repress *gcm* in the endoderm, thus restricting the domain of *gcm* expression to the mesoderm. FoxA therefore serves the dual function of activating one state (the endoderm) and excluding an

alternative fate (the mesoderm) in what has been termed an exclusion subcircuit [22,66]. Multiple events therefore operate to ensure the boundary between endoderm and mesoderm in the sea urchin. *GataE* and *foxA* are activated by the kernel subcircuit, an input from Delta-N signaling reinforces *gataE* expression in the endoderm, and finally the dual action of FoxA operating to drive endoderm and further repress mesoderm serves an exclusion function. Oliveri and Davidson [22] argue that this is an example of over-engineering: multiple processes operating to ensure a correct developmental outcome.

How, then, is this same process achieved in sea star embryos in the absence of a micromere lineage? *Delta* is also expressed within the vegetal-most cells of the sea star embryo [44], but these cells are the mesodermal orthologs of the secondary mesenchyme cells, which are the initial recipients of Delta-N signaling in sea urchins (though they are later a source of this signal). When the translation of sea star Delta is blocked, embryos develop an excess of migratory mesodermal cells [44]. Phenotypically, therefore, the exact opposite effect is found upon blocking Delta function: an increase of mesodermal cell types in sea star and a decrease in sea urchin. A comparison of the GRNs explains this dramatic difference in phenotype (see Fig. 2). First Delta-N signaling has no role in activating any of the known sea star mesodermally expressed transcription factors (e.g. it is not needed to activate expression of *A. miniata* orthologs of *gataC*, *tbrain*, or *ets1/2*). Delta, however, functions to activate the sea star *gataE* ortholog within the endoderm. As all evidence indicates that the micromere cell lineage is derived, the evolutionary scenario of this alteration in Delta-N signaling is clear. The ancient ancestor of sea urchins and sea stars used a Delta signal emanating from the central vegetal plate to activate N in the adjacent endoderm progenitors. This signal reinforced the expression of *gataE* and drove endoderm specification. In the echinoid lineage, Delta-N signaling took on a new role in specifying mesodermal cell types and activating *gcm*. Davidson and Erwin [25] suggested that signaling pathways are likely to constitute an evolutionarily promiscuous subcircuitry and are hence likely to be utilized in different contexts. The above example provides one clear instance of this type of evolutionary plasticity with such a signaling "plug-in" subcircuit function.

7. Conservation of network-level functions

In addition to the change associated with Delta-N signaling, there are numerous alterations in regulatory connections associated with specification of the mesoderm in these two echinoderms. In sea stars, *gataE* is repressed from the mesoderm by FoxA [41]. This linkage has been lost in sea urchins and *gataE* is expressed within the mesoderm where it functions to activate mesodermally restricted genes (e.g. *gataC*) [7]. The sea star ortholog of *gataE* can have no such role and indeed acts to repress the formation of markers of mesodermal specification from within the endoderm (e.g. sea star GataE represses *gataC* and *ets1/2* from within endodermal progenitors). Therefore, the sea star embryo also uses an exclusion subcircuit function to segregate endodermal from mesodermal cell fates. FoxA and GataE in both taxa function to specify endoderm and FoxA (only in sea urchin) or GataE (only in sea star) also act to repress mesodermal fate from within endodermal territories.

Strikingly, when the process of endodermal and mesodermal specification in these echinoderms is viewed in terms of GRN-level subcircuitry there is a highly conserved progression of a lockdown feedback subcircuit to specify endomesoderm, signal transduction subcircuit to further drive endoderm specification, and exclusion subcircuitry to reinforce the distinction of mesoderm from endoderm. The regulatory genes used to perform the early lockdown and signal transduction functions are orthologous (i.e. *Delta-N*, *otx*, *blimp1* and *gataE*), while those executing the exclusion function have altered (i.e. FoxA repression of *gcm* in sea urchin, and GataE repression of *ets1/2* and *gataC* in sea star).

8. Compensatory changes

Consideration of the GRN diagram (Fig. 2B) reveals three instances in which the regulatory inputs to orthologous genes are altered between sea urchin and sea star and yet the orthologs are similarly expressed. Thus, *otx*, *delta*, and *gatac* are regulated differently in sea star and sea urchin, yet are expressed in essentially similar patterns; *otx* in the endomesoderm and *gatac* and *delta* in the mesoderm (the earlier micromere lineage phase of sea urchin *delta* expression is of course regulated entirely differently) [41,42,61,67]. Therefore GRN changes upstream of a particular gene are not predictive of a conserved or evolved expression of this gene. There appears to be a high evolutionary capacity for compensatory changes in regulatory input. Ease of transcription factor swapping in this context may be related to the relatively simple function that each factor performs, i.e. to either activate or repress expression by binding to a simple site within a cis regulatory target. The particular example of the evolution in the regulation of *otxβ1/2* demonstrates this idea. *Otxβ1/2* is one of the alternatively spliced transcript forms of the *otx* gene and the orthologs in each taxa are expressed similarly within the vegetal plate and ectoderm of blastulae in both the sea urchin and sea star (although we consider here only the regulation of vegetal plate expression) [42,67]. In sea stars *otxβ1/2* is regulated by Tbrain, while in sea urchins this activation function is taken over by Blimp1 and otxα (another alternatively processed form of the *otx* gene) [43]. *otxα* cannot perform this function in sea star as it is expressed too late during development to activate *otxβ1/2* and Tbrain cannot perform the function in sea urchin as it is expressed within an entirely different cell lineage. Yet expression of *otxβ1/2* in the vegetal plate has been maintained in these echinoderms despite this alteration in regulators. The function executed by otxα or Tbrain is to activate *otxβ1/2* in the right time and place: in sea urchins *otxα* is expressed in the right time and place to perform this function, while in sea stars *tbrain* is expressed appropriately. This evolutionary change in regulation of *otxβ1/2* is due to evolution of binding sites within the *cis* regulatory module of *otxβ1/2* [43].

9. Conclusions

Although the comparison between the GRNs underlying endomesoderm formation in sea urchins and sea stars is only in the earliest stages, it has already provided many insights into how these networks change during evolution. Both echinoderm species use identical GRN functions in the specification and segregation of mesoderm and endoderm: a positive feedback 'lockdown' kernel, inter-territory signaling, and an exclusion subcircuit. While the network logic employed is the same in these organisms, the transcription factors executing certain functions have diverged, and we see compensatory changes in the GRNs, i.e., *foxa* represses mesoderm formation in sea urchins while *gatae* has this role in sea stars. Thus, it seems that in some cases, GRN logic, rather than specific GRN connections, may be acted upon by selection, especially if the function of said connections is to activate a specific transcription factor or subcircuit. In contrast, the transcription factors used in the kernel have been conserved during the 500 million years since sea stars and sea urchins diverged. This demonstrates that GRNs are indeed comprised of functional subcircuits under diverse selective pressures.

These findings are of fundamental importance in understanding how development and evolution interact to generate the great morphological diversity of today's organisms. The principles that govern formation of orthologous structures in echinoderm larvae can be extrapolated to other systems. For example, we have seen that an exclusion subcircuit is used to segregate endoderm from mesoderm, and that exclusion function is conserved. Thus we might predict that gain of a tissue or cell type would require that a novel GRN incorporate such a subcircuit to ensure alternate fate exclusion, and that the network connections might be similar to those seen in the echinoderm endomesoderm. Similarly,

the loss of a tissue would require the dismantling of exclusion circuitry. Additionally, the co-option of Delta-N signaling by the novel micromere cell lineage in sea urchins suggests that there might be constraints or pre-dispositions as to the regulatory genes available for incorporation into a given GRN. In the sea star and, presumably, the ancestral echinoderm, Delta-N signaling was employed in the same embryonic territory that gave rise to the micromeres. Because this pathway was already in place, fewer cis-regulatory changes may have been needed to incorporate it into the new GRN than, for example, Hedgehog-mediated signaling would have required. As such we can hypothesize that during the evolution of a novel cell type, regulatory genes already used in the tissue from which they are derived may be more likely to be incorporated into the new GRN.

The comparison of endomesoderm specification in echinoderms is the first study that examines evolutionary changes in gene regulation during development in the context of an established GRN. As such, it is an instrumental first step in our understanding of how regulatory networks change with time. The evolutionary principles uncovered by studying these simple larvae are more broadly applicable and can be used as a basis for generating hypotheses about the evolution of many other organisms, developmental processes, and cell types.

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