Evolutionary plasticity of developmental gene regulatory network architecture

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Sea stars and sea urchins evolved from a last common ancestor that lived at the end of the Cambrian, approximately half a billion years ago. In a previous comparative study of the gene regulatory networks (GRNs) that embody the genomic program for embryogenesis in these animals, we discovered an almost perfectly conserved five-gene network subcircuit required for endoderm specification. We show here that the GRN structure upstream and downstream of the conserved network kernel has, by contrast, diverged extensively. Mesoderm specification is accomplished quite differently; the Delta–Notch signaling system is used in radically distinct ways; and various regulatory genes have been coopted to different functions. The conservation of the conserved kernel is thus the more remarkable. The results indicate types of network linkage subject to evolutionary change. An emergent theme is that subcircuit design may be preserved even while the identity of genes performing given roles changes because of alteration in their *cis*-regulatory control systems.

echinoderm | gene regulation | *Asterina miniata* | *cis* regulation | Delta

Development is driven by the spatial progression of regulatory states, the output of expression of genes encoding transcription factors. Dozens of such genes typically interact with one another in the processes by which each embryonic domain is specified as to its future identity, its signaling behavior, and ultimately its differentiation. The inherited genomic program determining the architecture of the networks of these interactions is structurally embodied in the *cis*-regulatory control sequences of regulatory genes and their primary targets. Thus, as been evident *a priori* for many years, evolutionary change in animal body plans must occur through *cis*-regulatory sequence changes that result in rewiring of developmental gene regulatory networks (GRNs; refs. 1–5). Furthermore, when the hierarchical nature of GRNs is considered, it can be seen that the consequences of such changes will differ depending on what aspects of network architecture they individually affect (5). But despite its importance for understanding evolutionary process, there is very little direct comparison of GRN architecture between animals of known descent from a common ancestor available so far.

In previous work, we compared GRN architectures for early specification of endomesoderm in a sea star, *Asterina miniata*, and a sea urchin, *Strongylocentrotus purpuratus* (6). The last common ancestor of echinoid and asteroid echinoderms existed ≈500 million years ago in the Late Cambrian (7, 8). The comparison revealed an extraordinarily conserved GRN structure, a subcircuit of five regulatory genes that are triply linked by positive feedback loops. This subcircuit, an example of a network "kernel" (6), is required upstream of initial endomesoderm specification, and if expression of any one of the genes is compromised endomesoderm specification is abrogated. This subcircuit, and its recursive wiring, such that each gene has multiple targets within the same kernel, may explain its evolutionary immutability. The fact that sea star and sea urchin embryonic development are manifestly different in several ways ensures that their GRNs cannot in general be identical. But how diverse in structure they really are was revealed only in the further comparison of network linkages outside of the kernel that we now describe.

Results

A primary and fundamental difference between sea star and sea urchin embryos is the presence of a skeletogenic lineage in the sea urchin. This lineage is a precociously specified polyclone descendant from four fifth cleavage micromeres arising at the vegetal pole of the embryo. It is the sole normal source of the embryo/larval skeleton, to which all of the cells of this lineage contribute their unique function. The sea star embryo not only lacks micromeres but also makes no embryonic skeleton. Because none of the other five echinoderm classes possesses a skeletogenic micromere lineage, the sea star represents in this respect the pleisiomorphic state, and the sea urchin represents the derived state. The GRN underlying specification and differentiation of the skeletogenic lineage of *S. purpuratus* is exceptionally well known (4, 9) and is, of course, not expected to exist in the sea star. But in sea urchins the micromere lineage has a function in addition to skeletogenic differentiation: it transcribes the *delta* gene, generating a short-range Delta signal that is essential to specification of the adjacent vegetal plate cells as mesoderm (10, 11). The architecture of the micromere GRN explains why the *delta* gene is expressed specifically and uniquely in the micromeres in early development in sea urchins. This gene is activated specifically in these cells through the action of a double negative transcriptional gate, i.e., repression in these cells of an otherwise global repressor (12). Sea stars also produce vegetal plate mesoderm, that just as in sea urchins, is topologically central to the surrounding ring of cells, which become endoderm, but they must use a different mesodermal specification mechanism.

Absence from Sea Star Mesoderm GRN of a Delta–Notch (N) Signaling Subcircuit Found in Sea Urchins. In sea urchins the Delta signal emitted by the skeletogenic cells activates maternal N receptor on the surface of the future mesodermal cells (13). The target of the N signal transduction system is the *cis*-regulatory control system of the *gcm* gene (11), which in the sea urchin GRN is an upstream mesodermal specifier. Interference with Delta–N signaling by any means blocks mesodermal *gcm* transcription. Expression of *gcm* is directly required for subsequent appearance of pigment cells, one of the major mesodermal cell types of the echinoid embryo (4, 10, 11, 14). But, as we show here, in sea stars this particular mesodermal specification circuitry does not exist at all.

The sea star *delta* gene is expressed within the mesodermal progenitors of the central vegetal plate and scattered in the cells of the ectoderm at the blastula stage (Fig. 1*A*). But the Delta-expressing cells are what become the definitive vegetal plate

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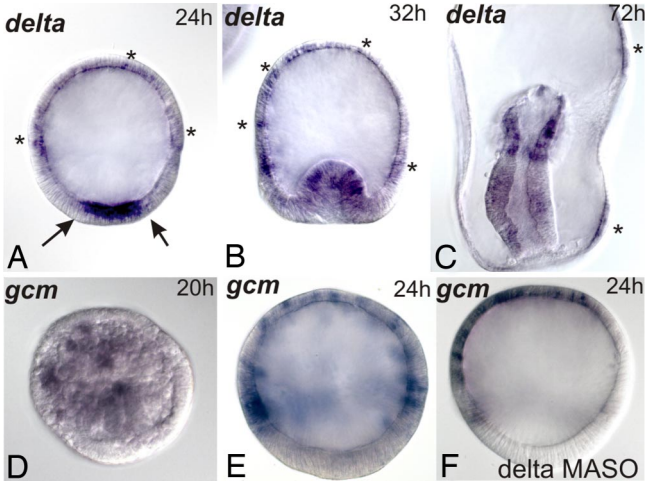

Fig. 1. Expression of *delta* and *gcm* in *A. miniata* visualized by WMISH. (*A–C*) *delta*. (*A*) Blastula stage. (*B*) Early gastrula stage. (*C*) Larval stage. *Delta* is expressed in the central vegetal plate in blastulae (arrows) and in cells scattered within the ectoderm (*). (*D* and *E*) *gcm*. (*D*) Early blastula. (*E*) Blastula. *Gcm* is not expressed within the vegetal plate, but rather in cells scattered within the ectoderm. (*F*) *Gcm* expression continuing normally in blastula in which Delta translation has been blocked with a targeted MASO. (Magnification: ×200.)

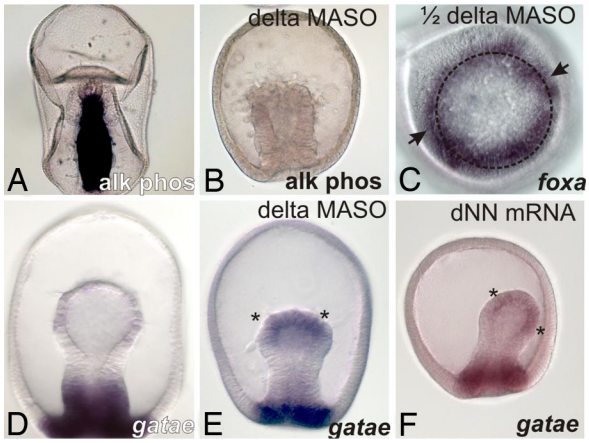

Fig. 2. Delta requirement in specification of endoderm. (*A*) Alkaline phosphatase (alk phos) expression in gut of normal 3-day larva. (*B*) Same-stage larva bearing Delta MASO, identically processed for alkaline phosphatase but showing no expression. (*C*) Vegetal pole view of *foxa* expression in a blastula-stage embryo in which one of two blastomeres of the two-cell embryo was injected with Delta MASO; top half (above arrows) of the blastula is Delta deficient, whereas the bottom half is normal. (*D–F*) *Gatae* expression in gastrulae. (*D*) Normal. (*E*) Delta deficient. (*F*) N signaling blocked by introduction of dominant negative N mRNA. *Gatae* expression is lost from the midgut and hindgut but up-regulated at the top of the archenteron (* in *E* and *F*). (Magnification: ×200.)

mesoderm, rather than the cells adjacent to it, as shown in the later stages portrayed in Fig. 1 *B* and *C*. It might still be conceivable that Delta–N signaling would activate the mesodermal specifier *gcm* as in sea urchins within the mesodermal territory, but this possibility is excluded by the expression domain of *gcm* in the sea star. *AmGcm* is not expressed at all within the vegetal plate (Fig. 1 *D* and *E*), instead displaying a spotty pattern of ectodermal expression. Nor does *gcm* expression depend on Delta expression as in sea urchins: in the sea star *gcm* transcription is unaffected in embryos bearing morpholino-substituted antisense oligonucleotides (MASOs) targeting Delta, as illustrated in Fig. 1 *E* and *F* and supported as well by quantitative PCR (qPCR) measurements of transcript levels (data not shown).

Embryonic pigment cells are not formed at all in sea stars as they are in euechinoids. Thus, with respect to sea stars, the whole of the echinoid GRN subcircuit from Delta–N signaling to *gcm* to downstream pigment cell differentiation genes (15) is novel.

Conservation of Mesoderm to Endoderm Delta–N Signaling. Another phase of Delta–N signaling is, in contrast, conserved since divergence of these echinoderms from their Late Cambrian ancestor. In sea urchins, long after initial specification of the mesoderm, the *delta* gene is activated within the mesodermal territory. In *S. purpuratus*, for example, the initial activation of *gcm* in response to the micromere Delta signal occurs at ≈10 h postfertilization, and the activation of the Delta gene within the vegetal plate mesoderm at ≈21 h (10, 16, 17). A *cis*-regulatory target of this second phase of Delta–N signaling is the endomesodermal *gatae* gene (18). *Gatae* is essential for endoderm specification, as it regulates many other endodermally restricted transcription factors (4, 19). At this stage the future endodermal cells surround and are adjacent to the Delta-expressing vegetal plate mesodermal cells, so just as in the initial phase the signal is an inductive one passed from one territory to another. We have already seen, in Fig. 1 *A–C*, that *delta* is transcribed in sea star mesoderm, just as in the second phase of *delta* expression in sea urchin mesoderm.

Alkaline phosphatase activity is a marker of endoderm differentiation in the sea star as in many other embryos, and expression of this marker is extinguished in embryos treated with Delta MASOs (Fig. 2 *A* and *B*). We therefore examined the expression of several endodermally restricted transcription factors in Delta MASO blastulae and gastrulae. Both whole-mount *in situ* hybridization (WMISH; Fig. 2 *C–F*) and quantitative analyses of transcript levels [supporting information (SI) Fig. 6] revealed that Delta is required for expression of *gatae* in the gut and for its own expression. Expression of *gatae* in the blastopore of late embryos is unaffected. *Brachyury (bra)*, which is expressed normally around the blastopore is similarly unaffected (SI Fig. 6). *Foxa*, which is restricted to the posterior hindgut and blastopore until late gastrula is also unaffected when measured quantitatively (SI Fig. 6), although possibly the inner-most ring of expression of *foxa*, fated to the more anterior domain of its hindgut expression, is reduced (Fig. 2*C*). Delta was not required for the expression of several early and broadly endomesodermally expressed transcription factors, i.e., *tbrain* (*tbr*), *otxβ1/2*, or *otxα* (SI Fig. 6).

As in sea urchins, *gatae* expression is essential for the correct specification of endoderm in *A. miniata* embryos (6). Combining that requirement with the data shown here (Fig. 2 and SI Fig. 6), Delta–N signaling is clearly necessary for the correct specification of the gut endoderm through activation of the regulatory gene *gatae*. This regulatory linkage is the same as found in sea urchins. It is likely a pleisomorphic feature of endoderm specification in echinoderms, like the endomesodermal specification kernel (6) into which it feeds, and unlike the Delta–N function in initial mesoderm specification seen in sea urchins.

Delta–N Repression of Mesoderm Specification in Sea Stars. Not only is Delta–N signaling not used for mesoderm specification in sea stars, but instead it functions oppositely, to limit mesoderm specification. In the sea urchin, Delta–N signaling is required not only for pigment cell specification but also for the specification of blastocoelar cells, which are mesenchymal mesoderm cells that delaminate from the top of the invaginating archenteron into the blastocoel (10). There are several pathways by which Delta–N signaling positively affects blastocoelar cell formation. First, in sea urchins *gatae* regulates the expression of the

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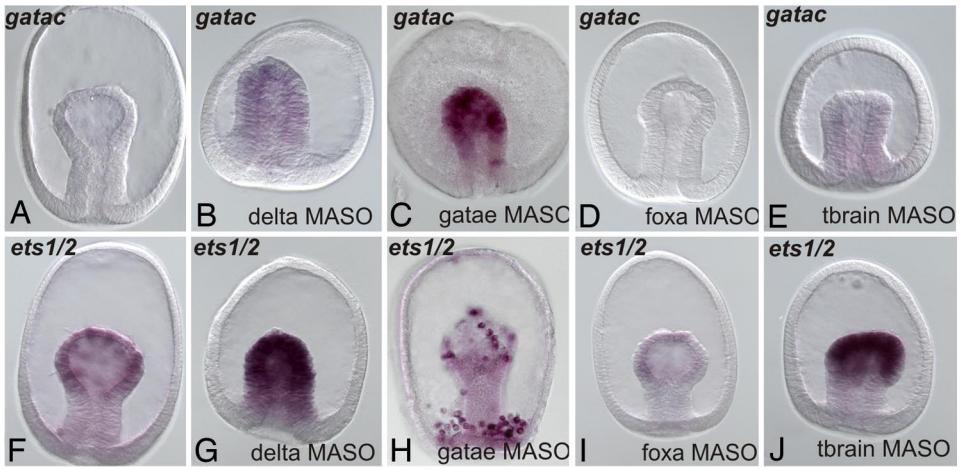

Fig. 3. Specification of mesoderm. (*A–E*) Expression of *AmGatac* in late gastrulae. (*A*) Control. (*B–E*) *AmGatac* perturbed with MASOs targeting Delta (*B*), Gatae (*C*), Foxa (*D*), or Tbr (*E*). (*F–J*) Expression of *AmEts1/2* in late gastrulae. (*F*) Control. (*G–J*) *AmEts1/2* perturbed with MASOs targeting Delta (*G*), Gatae (*H*), Foxa (*I*), or Tbr (*J*). (Magnification: ×200.)

mesodermal paralogue *gatac* [*gatae* is transcribed in mesoderm as well as endoderm until just before gastrulation (20)], and *gatac* expression is necessary for blastocoelar cell formation; we have already seen that *gatae* receives a Delta–N input. Second, the Delta–N target *gcm* also provides an input into *gatac*; third, there may be intramesodermal Delta–N signaling as well. In any case, in the absence of Delta–N signaling greatly reduced numbers of blastocoelar cells appear (10).

In direct contrast, when Delta expression is blocked in the sea star, excessive numbers of migratory blastocoelar cells are formed, as can be seen by comparing Fig. 2 *A* and *B*. We examined the expression of the mesodermally transcribed regulatory genes *ets1/2* (21) and *gatac* in Delta-deficient embryos. The orthologous *A. miniata* cDNAs were isolated. As in sea urchins, both are expressed in migrating blastocoelar cells and in their vegetal plate precursors. In *A. miniata*, *gatac* is expressed throughout the central vegetal plate fated to mesoderm (SI Fig. 7), but unlike its orthologue in *S. purpuratus* its expression is not restricted to the oral quadrant [in the sea urchin embryo the *gcm*-expressing pigment cell precursors are located in the aboral three-quarters of the mesodermal domain (14)]. Sea star *gatac* expression continues throughout the mesoderm at the top of the archenteron during early gastrulation but has almost disappeared by late gastrula stage (SI Fig. 7 and Fig. 3*A*). But in Delta-deficient embryos, *gatac* expression is up- regulated: it extends throughout the archenteron and persists during gastrulation and is more intense than in normal, similarly staged gastrulae (Fig. 3 *A* vs. *B*). Sea star *ets1/2* is normally expressed similarly to *gatac* in the vegetal plate and the mesoderm at the top of the archenteron in early gastrulation (SI Fig. 8). By late gastrulation this gene is expressed in some of the cells at the top of the archenteron (SI Fig. 8 and Fig. 3*F*) and is clearly seen in those migrating away from its tip by 72 h (SI Fig. 8). However, in Delta MASO embryos *ets1/2* expression is also up-regulated, extending throughout the archenteron and at greater than normal intensity (Fig. 3*G*). In sea stars the mesodermal regulatory state is thus spatially enhanced by interference with Delta–N signaling, rather than requiring Delta–N signaling.

The Mesoderm–Endoderm Balance. In sea urchins the cells of the vegetal plate that are exposed to the initial Delta–N signal become mesoderm, and the peripheral cells that are not become endoderm. In sea stars the balance between mesoderm and endoderm in the archenteron is set oppositely: the cells that receive the Delta–N signal become endoderm, and if they do not get this signal they become mesoderm. Thus, the domains of *ets1/2* and *gatac* expression in the Delta MASO experiments are expanded at the expense of the normally endodermal "barrel" of the archenteron. Another spatial reversal in regulatory state caused by the same treatment is *gatae* expression at the late gastrula stage. Normally in sea star embryos *gatae* is expressed in the archenteron barrel (future hindgut and midgut) and is expressed at very low levels in the mesoderm bulb (Fig. 2*D*), but in the absence of Delta–N signaling its expression is extinguished in the barrel as we have seen, and, for reasons not yet known, is dramatically up-regulated in the anterior bulb (Fig. 2 *E* and *F*, regions marked by *).

Direct evidence that mesodermal regulatory state expands when endodermal *gatae* expression is blocked is shown in Fig. 3 *C* and *H*. Here, interference with *gatae* expression in the archenteron barrel by introduction of *gatae* MASO causes a great up-regulation of *gatac* in this region (Fig. 3*C*; compare the control in Fig. 3*A*). This evidence is again directly contrary to the sea urchin relationship in which *gatae* expression provides a positive input into *gatac* expression, as noted above. *Gatae* MASO also causes delamination of excess *ets1/2*-positive cells later on, which can be seen ectopically collected around the hindgut in Fig. 3*H*. These results are similar to those of blocking Delta–N signaling. They are not just a general effect of disturbing endodermal regulatory state, however. Expression of *foxa* is a key component of the endodermal regulatory state and is necessary for endoderm development in both sea urchins and sea stars. But *foxa* expression can be blocked without causing up-regulation of either *gatac* or *ets1/2* (Fig. 3 *D* and *I*). *Gatae* regulation plays a special, pivotal role in organizing the mesoderm vs. endoderm allocation in the sea star. The data in Fig. 3 show that directly or indirectly *gatae* operates a regulatory "exclusion function" (22); that is, it is responsible for regulatory repression in the endodermal domain of mesodermal state controllers, including *ets1/2* and *gatac*.

We can now appreciate the true functional importance of what seemed a small difference in sea star and sea urchin GRN architectures when originally noted (6). In *S. purpuratus foxa* is expressed in endoderm and not mesoderm (23). It has no input to *gatae*, which is expressed in both until just before gut invagination. In the sea star *foxa* is initially expressed in mesoderm (as well as endoderm). But the *foxa* gene represses its own transcription in the mesoderm and also represses mesodermal *gatae* transcription, abolishing expression of both in that territory (6). The result is that once this repression has occurred, in the

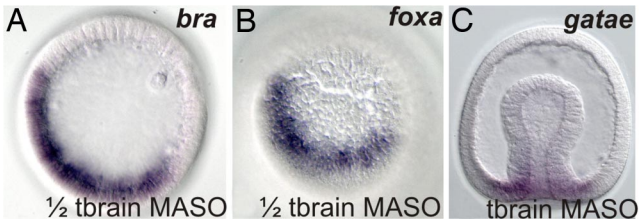

Fig. 4. Requirement of Tbr for activation of endoderm. (*A* and *B*) Vegetal views of blastula-stage embryos in which one of the first two blastomeres had been injected with a MASO targeted to Tbr. (*A*) Expression of *brachyury*. (*B*) Expression of *foxa*. (*C*) Expression of *gatae* in blastula bearing Tbr MASO. (Magnification: ×200.)

late blastula period, in sea stars *gatae* can have no effect on mesodermal specification, whereas in sea urchins it can assist in driving mesodermal specification. But conversely, in sea stars *gatae* can be used strictly as an endoderm specifier and as an excluder of mesodermal state. In other words, the origin of the mesoderm/endoderm boundary in sea stars is ultimately the repression of *gatae* in the mesoderm by *foxa*, whereas in sea urchins it is the initial phase of Delta–N signaling. In sea stars Delta–N signaling is used to potentiate *gatae* expression in the endoderm, where this gene is not subject to *foxa* repression. It does this in sea urchins, too, in the second phase of Delta–N signaling, and in neither does N input into *gatae* determine the endoderm/mesoderm boundary.

The Diverse Roles of *tbr*. In sea urchins the *tbr* regulatory gene is a controller of the skeletogenic specification and differentiation pathway and is expressed exclusively in the skeletogenic micromere lineage (9, 12, 24, 25). This gene is required for normal skeletogenesis. The cooption of *tbr* to skeletogenic function is a derived feature of the echinoids: endomesodermal expression of *tbr* is the pleisiomorphic state in deuterostomes. This pleisiomorphy is shown in the endomesodermal transcription pattern of *tbrain* orthologs in the sea star and in a holothurian, as well as in a hemichordate, amphioxus, and many vertebrates (6, 26–28).

Expression of the sea star *tbr* gene is in fact necessary for expression of many other endomesodermal and endodermal regulatory genes. The evidence comes from qPCR assessment of quantitative effects on the individual expression levels of these genes after introduction of MASOs targeted to *tbr* mRNA (SI Fig. 9). From >65% to ≈80% of expression of *otxβ1/2*, *foxa* and *bra*, *gatae*, *tbr* itself, and *otxα* is abolished by this treatment, whereas control genes not expressed in endomesoderm at this stage are unaffected. The loss of expression of the endodermal markers *bra*, *gatae* and *foxa* in Tbr-deficient embryos is confirmed visually by using WMISH (Fig. 4). The Tbr effect on *otxβ1/2* gene expression was demonstrated earlier to be the result of a direct *cis*-regulatory input (29). The effect of *tbr* MASO on *gatae* is at least in part caused by its strong depression of the *delta* transcript level (SI Fig. 9); as we saw above, without *delta* expression *gatae* expression fades out, and the archenteron expresses mainly mesodermal regulatory genes. Because *bra* and *foxa* are downstream of both *gatae* and *otxβ1/2* (6), the positive input of *tbr* to these endodermal genes can easily be accounted for as an indirect consequence of Tbr inputs into *delta* (direct or indirect) plus its direct inputs into *otxβ1/2*. The prediction that *tbr* MASO would elevate *ets1* and *gatac* expression in the endodermal barrel as does *delta* MASO is confirmed in Fig. 3 *E* and *J*.

In summary, the requirement of *tbr* for endomesodermal specification could be explained at minimum by GRN inputs into *delta* and *otxβ1/2* genes, although there, of course, may be other upstream endomesodermal targets as well. These linkages have been unhooked or substituted for during the evolution of the sea urchin GRN. Thus, as we show elsewhere (29), the endomesodermal *otxβ1/2 cis*-regulatory module of the sea urchin indeed lacks a Tbr site and instead another positive regulator interacts with it to drive its early expression; something similar would be predicted for the relevant *cis*-regulatory module of the *delta* gene.

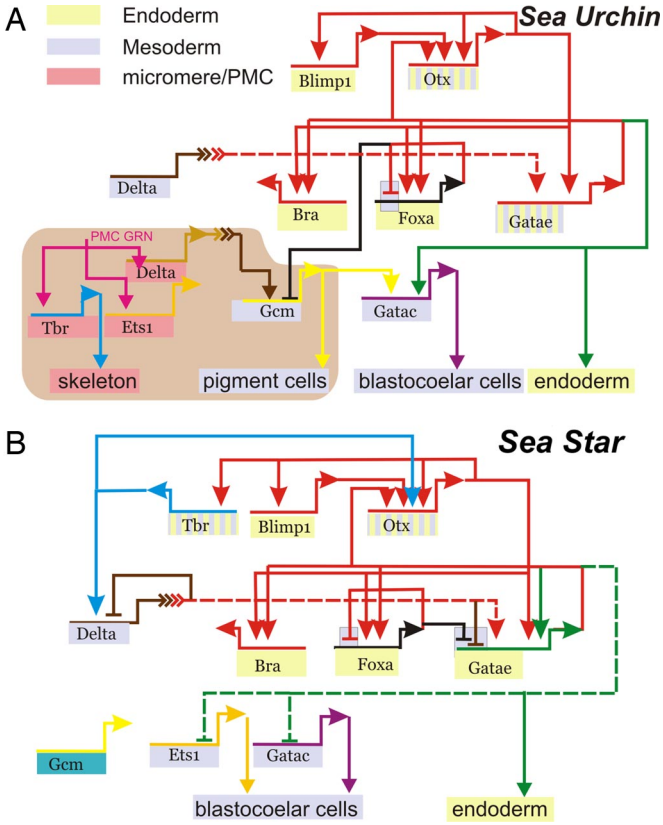

Fig. 5. GRN for endomesodermal specification in sea urchins and sea stars. Notation is described in ref. 4. All genes encode transcription factors, except for the *delta* gene. Yellow background indicates expression in the endoderm at blastula stage; blue indicates expression in mesoderm. Yellow and blue stripes indicate expression in endoderm and mesoderm, and pink indicates expression in micromere/primary mesenchyme cell lineage. Lines ending in arrowheads indicate positive transcriptional regulation of the target gene; lines ending in bars indicate repression of the target gene. Derived echinoid GRN subcircuit of micromere lineage and Delta response subcircuit for mesodermal pigment cell specification are boxed in light brown. Dashed lines are regulatory interaction, which evidence suggests are indirect. Solid lines indicate interactions that are highly likely to be direct (details are in *SI Text*).

Discussion

The sea star GRN for endomesoderm specification, as it emerges from these and our previous studies (6, 29) is compared with the relevant regions of the sea urchin GRN in Fig. 5. The conserved five-gene kernel discovered earlier is shown in red in Fig. 5, to which we have now added the equally conserved *delta* to *gatae* input demonstrated in this work in the sea star. Those components reproduced here that belong to the derived sea urchin-specific subnetworks for skeletogenesis and pigment cells are boxed in Fig. 5. Of course, none of the linkages within that box are to be found in the sea star endomesoderm GRN. Of the boxed linkages the most important for us is the entirely different mechanism the sea urchin uses for initial specification of mesoderm, beginning with the Delta–N dependant signal of which the *cis*-regulatory target is the *gcm* gene (11). But Fig. 5 shows

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that that there are other linkages involving orthologous genes that are also different in the two GRNs, even though these genes (*foxa*, *gatae*, *ets1*, *gatac*) are used for the same general purpose, endomesoderm specification. Fig. 5 also includes the remarkable case of the *tbr* cooption, providing an image of the role of this gene in what is probably its pleisiomorphic regulatory context. The bottom line is the surprising GRN plasticity revealed by the comparison. There are still many missing linkages in the sea star GRN. For example, we do not yet know what causes initial mesodermal expression of *gatac*, but it is certainly not what turns on this gene in the sea urchin (i.e., positive inputs from *gcm* after the Delta–N signal and from *gatae*). Thus, when the repertoire of regulatory genes in the sea urchin endomesodermal specification GRN is complete, and when the corresponding genes of the sea star GRN have all been studied as well, the differences shown in Fig. 5 will not go away; much more likely, the distinctions in GRN structure will appear even greater.

The surprising fact is that outside of the kernel not one of the genes included in Fig. 5 has all of the same regulatory inputs in the two GRNs. Even within the kernel, the structure of which remains the same, there is the difference that as discussed above *foxa* represses *gatae* in the sea star. In addition, there is a compensatory *cis*-regulatory change upstream of the kernel, in the substitution of *blimp1* in the sea urchin for *tbr* as an early *cis*-regulatory input initiating *otx* expression (29, 30). Against this background of very extensive "rewiring" outside of the kernel the extreme conservation of the kernel itself is cast into high relief. The result shown in Fig. 5 certainly fulfills the prediction (5) that the diverse regions of a developmental GRN evolve at very different rates.

Types of Evolutionary Change in GRN Structure. The most dramatic difference in the two GRNs is in the deployment of the Delta–N signaling system. Davidson and Erwin (5) highlighted deployment of signaling systems as a prominent type of GRN change, and this comparison provides a startling example: in sea stars Delta–N signaling has the effect of preventing mesoderm specification (in the archenteron barrel), whereas in sea urchins it is required to produce mesoderm specification. In evolutionary terms the deployment of this signaling system to the embryological address of the micromere lineage is a major aspect of the echinoid-specific functionality of this lineage. However, there is also a perfectly conserved aspect of Delta–N signal deployment (so far as is yet evident), i.e., the requirement in both systems of this input into the *gatae* gene.

A second type of change in GRN structure seen here is regulatory gene cooption. The *tbr* gene provides the best-studied example, and another surprising example is the *gcm* gene, which is not used at all in sea star endomesoderm specification. As discussed above, *tbr* plays totally different roles in the two systems, and we know enough from observations in outgroups that it is clear that the echinoid use of this gene is the cooption and the sea star use the pleisiomorphic state. Cooption requires multiple *cis*-regulatory changes. The control system determining the redeployment of the gene, in this case to the micromere lineage must be novel, and its target *cis*-regulatory modules, one of which is apparently in a skeletogenic gene has been altered. In addition, old linkages must be destroyed, and in other work (29, 30) we have shown that a target of the Tbr transcription factor in sea stars, the *otx cis*-regulatory module, is not a target of Tbr in sea urchins. Furthermore the feedback of *otx* into *tbr* in sea stars has also been lost in the echinoid cooption.

A third type of change in these developmental GRNs is in the genomic code controlling location of the boundary between mesoderm and endodermal territories. In sea urchins this boundary depends originally on the juxtaposition of the future mesoderm cells to the cell-bound Delta ligand produced by the micromere lineage cells, and the existence of a Delta—N-responsive *cis*-regulatory module (11) in a mesoderm regulatory gene, *gcm*. In the sea star the boundary depends on Foxa repression of *gatae* in the mesoderm (6), a function that requires a Foxa-responsive *gatae cis*-regulatory module. An additional part of the mesoderm/mesoderm boundary setting mechanism in the sea star is the unknown *cis*-regulatory apparatus that prevents the Foxa repressor from interfering with *gatae* transcription in endoderm. In the sea urchin *gatae* runs in both the mesoderm and endoderm almost up to gastrulation. The consequence is that this gene can be used to provide input into mesoderm genes in sea urchin and does so to *gatac*, a role that would be impossible in the sea star.

In general it is clear that the major mechanism of GRN architecture change is redeployment of regulatory gene expression, i.e., change in the *cis*-regulatory modules controlling regulatory gene expression. Each such change is propagated and amplified by losses and gains in the downstream targets of the factors encoded by these genes.

Most of the altered regulatory connections in this comparison are responsible for processes relating to the specification of mesoderm. These changes underlie the prominent biological differences between sea stars and sea urchins in the mesodermal cell types of the embryo, for instance, the presence of pigment and skeletogenic cells in sea urchins but not in sea stars.

GRN Subcircuits and Linkage Plasticity. The GRN components that execute developmental jobs are its individual subcircuits (4), and the effects of change in linkage must be considered in terms of how and whether they affect the subcircuit composition of the GRN. Some subcircuits are wired totally differently in the two systems, as in the echinoid subcircuits boxed in brown in Fig. 5. However, a significant fraction of the changes we observe in regulatory inputs to given genes, each of which represents a change in a GRN linkage, do not result in complete rewiring or creation of novel subcircuits. What has happened since divergence of these two echinoderms is that some of the same regulatory tasks have come to be accomplished by different means; it is the task that is conserved, the subcircuit logic abstracted from its biochemical components. An illustrative example is the endoderm to mesoderm exclusion subcircuits of these two organisms. In sea urchins endoderm cells are prevented from expressing mesoderm genes by *foxa* repression of *gcm* (22, 23), whereas in sea stars endoderm cells are also prevented from expressing mesoderm genes, but not by *foxa*: instead it is *gatae* that executes the exclusion function (possibly indirectly), by blocking expression of *gatac* and *ets1*. The players are all different, but the function is the same. The substitution of *blimp1* for *tbr* as the early activator of the *otx* gene as the kernel begins to be deployed in the sea urchin and sea star, respectively, can be viewed in the same way. So also can the mechanism of activation of *gatac*, probably a key regulator of blastocoelar cells in both systems: the sea urchin activates this gene initially with the aid of mesoderm-specific inputs from *gcm* and *gatae*; it is activated in the equivalent cells in the sea star, but neither of these inputs can be used for that purpose because they are not expressed there. But from a bird's eye view, the two systems basically work in a similar fashion. In both, the same endomesodermal kernel is deployed; *delta* is expressed in mesoderm; endoderm specification is driven by the kernel effectors *gatae* and *foxa*; endoderm specification occurs in vegetal cells circumferential to the central plate of mesodermal precursors; the mesoderm expresses *gatac* and *ets1*; in both systems a mesoderm/endoderm boundary is formed; and this boundary is enforced by a regulatory exclusion function.

The fundamental result of this study, then, is that the genomic regulatory code underlying these similar developmental tasks differs very significantly in the two animals at the *cis*-regulatory level, but much less so at the circuit design level. We note that

in echinoderms regulatory genes are generally not 2- or 4-fold paralogous (31), as in mammals, so the process of *cis*-regulatory changes in input cannot be explained by functional diversification of duplicated genes. Where the biology is different, of course, the regulatory design is totally nonhomologous. But the degree of redeployment of regulatory genes, and of the Delta–N signaling system, justifies the term "plug-in" used in discussing evolutionarily pliable GRN components (5). The actual amount of GRN change is grossly underestimated by external metrics such as expression pattern. Regulatory change in the control systems of regulatory genes is the raw driver of developmental evolution. This is a surprisingly active evolutionary process, the magnitude of which is partially concealed by its frequently compensatory nature.

Materials and Methods

Cloning of *A. miniata* Orthologs of *delta*, *ets1/2*, and *gatac*. *S. purpuratus* partial cDNAs for *delta*, *ets1/2*, and *gatac* were radiolabeled and used as probes in low-stringency hybridization on *A. miniata* late-gastrula stage arrayed cDNA library following standard protocols. Positive clones were sequenced, and predicted amino acid alignments were used to confirm orthology. WMISH using digoxigenin-labeled antisense RNA probes was used to examine spatial expression following previously published protocols (32). Alkaline phosphatase expression was assayed by using standard protocols (33).

Perturbations of Gene Expression to Determine Epistatic Relations. Normal gene function was determined by blocking normal translation with sequence-specific MASOs following previously published protocols (6). MASO sequences for Tbr, Foxa, and Gatae were as published (6, 29) and for Delta was CCAGGAGAGCCGTAACCGACCCATG. Quantitative measures of gene expressions in MASO-treated versus control MASOs (provided by GeneTools) were performed by using qPCR as described (6).

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