Evolutionary rewiring of gene regulatory network linkages at divergence of the echinoid subclasses

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Evolution of animal body plans occurs with changes in the encoded genomic programs that direct development, by alterations in the structure of encoded developmental gene-regulatory networks (GRNs). However, study of this most fundamental of evolutionary processes requires experimentally tractable, phylogenetically divergent organisms that differ morphologically while belonging to the same monophyletic clade, plus knowledge of the relevant GRNs operat- ing in at least one of the species. These conditions are met in the divergent embryogenesis of the two extant, morphologically distinct, echinoid (sea urchin) subclasses, Euechinoidea and Cidar- oidea, which diverged from a common late Paleozoic ancestor. Here we focus on striking differences in the mode of embryonic skeleto- genesis in a euechinoid, the well-known model Strongylocentrotus purpuratus (Sp), vs. the cidaroid Eucidaris tribuloides (Et). At the level of descriptive embryology, skeletogenesis in Sp and Et has long been known to occur by distinct means. The complete GRN controlling this process is known for Sp. We carried out targeted functional analyses on Et skeletogenesis to identify the presence, or demonstrate the absence, of specific regulatory linkages and sub- circuits key to the operation of the Sp skeletogenic GRN. Remark- ably, most of the canonical design features of the Sp skeletogenic GRN that we examined are either missing or operate differently in Et. This work directly implies a dramatic reorganization of genomic regulatory circuitry concomitant with the divergence of the euechi- noids, which began before the end-Permian extinction.

GRN evolution | network linkages | embryonic skeletogenesis | sea urchin embryogenesis

The mechanisms responsible for evolutionary divergence of ani- mal body plans, as so extensively documented in the Phaner- zoic fossil record, lie in alterations of the encoded genomic regulatory programs that direct development. This principle has long been evident a priori (1), and overwhelmingly, accumulating current evi- dence precludes any other general explanation (2). However, it still remains a challenge to adduce specific examples in which evolu- tionary rewiring of developmental gene-regulatory networks (GRNs) can be seen to account for observed differences in morphogenetic processes that distinguish descendants of a common ancestor. Knowledge of developmental GRNs remains insufficiently exten- sive, and it is not trivial to locate useful examples, which require comparison within a monophyletic clade at just sufficient distance so that the diverged morphology is clearly the output of homologous networks of developmental regulatory gene interactions.

In recent years, largely complete developmental GRN models have been solved that causally explain spatial specification in large domains of the embryo of the sea urchin Strongylocen- tortus purpuratus (Sp), up to gastrulation (3–5). The explanatory power of these networks was demonstrated, in these pages, by a predictive computational analysis that showed that they contain sufficient in- formation to regenerate the developmental course of events in silico, in automaton-like fashion (6). The present work stems from the almost irresistible opportunities that these same GRNs offer for approaching the basic evolutionary mechanisms of GRN divergence. Thus, here we focus on a sea urchin clade that diverges from a common ancestor with Sp, but in which embryonic structures are generated differently from those to which the known GRNs pertain.

Significance

This work provides direct evidence of evolutionary rewiring of gene-regulatory circuitry accompanying divergence of two subclasses of echinoderms, the cidaroid and euechinoid sea urchins. These forms descend from a known common Paleozoic ancestor, and their embryos develop differently, offering an opportunity to probe the basic evolutionary process by which clad divergence occurs at the gene-regulatory net- work (GRN) level. We carried out a systematic analysis of the use of particular genes participating in embryonic skeleto- genic cell specification, building on an established euechinoid developmental GRN. This study revealed that the well-known and elegantly configured regulatory circuitry that underlies skeletogenic specification in modern sea urchins is largely a novel evolutionary invention. The results dramatically display extensive regulatory changes in a specific developmental GRN, underlying an incidence of cladistic divergence at the subclass level.

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other fates near the very beginning of development, at fifth cleavage, and all descendants of these four vegetal pole cells exclusively execute skeletogenic specification and differentiation, according to a rigidly hierarchical, encoded network of regulatory gene interactions (4). In Sp embryos the cells of this lineage actively express skeletogenic genes during cleavage and blastulation (4). They divide exactly three times during this period, and then, well before gastrular invagination of the archenteron, they singly ingress into the blastocoel and divide one last time, and on the basis of internal ectodermal signal cues, they arrange themselves spatially within the blastocoel, form a syncytium, and progressively construct the skeleton during the remainder of embryogenesis (10–12). However, in cidaroid sea urchin embryos no precocious ingress of a skeletogenic micromere lineage occurs before gastrulation (13, 14). A variable number of micromeres, individual to individual, is formed at the vegetal pole early in cleavage. However, their ultimately skeletogenic descendants only emerge well after gastrulation is under way, together with a cloud of other mesodermal derivatives, by delamination from the tip of the midgastrular archenteron. As we see below, in cidaroid embryos specifically skeletogenic molecular functions are not transcriptionally executed in micromere descendants during cleavage. After emergence from the archenteron tip, the mesenchymal skeletogenic cells of cidaroid embryos migrate to the ectoderm and, late in embryonic development, proceed to construct the larval skeleton. We show here that the distinction in the mode of developmental origin of the larval skeleton in euechinoid vs. cidaroid embryogenesis is anything but a trivial heterochrony; rather, it is the morphological tip of an iceberg of fundamentally distinct GRN architecture.

The extant echinoderm classes were established in the Ordovician, if not earlier, and in major aspects of their body plans they have exemplified evolutionary stasis of definitive character suites for the ensuing 430 million years (my) (2, 15). For echinoids as a whole, these features include the globular test form and developmentally rearrangements of the coeloms resulting in a stacked configuration in the juvenile (16, 17). Within these constraints, the fossil record displays a remarkable variety of early Paleozoic echinoid morphology. However, in the late Paleozoic, there arose an echinoid branch that is clearly ancestral to both the modern euechinoid and cidaroid subclasses, known as the archaeocidarid lineage (18). A new, more comprehensive paleontological analysis (19) indicates that the last common archaeocidarid ancestor of both modern echinoid subclasses existed at the latest ~268 my ago—i.e., at least 16 my before the Permian/Triassic extinction event, which terminated the Paleozoic and many of its canonical denizens. Since the Triassic, a curious and perhaps profound difference in evolutionary flexibility distinguishes euechinoid and cidaroid subclasses. The euechinoids have radiated prodigiously, diversifying into nearly 1,000 species of highly various morphology, whereas the cidaroids, comprised of only ~100 species, have retained extremely conservative morphologies seemingly not far removed from their ancestral forms (18, 20). For example, during the Mesozoic the echinoids evolved diverse clades displaying irregular morphology, such as sand dollars and heart urchins, whereas no such deviations from the ancestral symmetrical globular form have arisen in the cidaroid subclass. This fact generally biases the likelihood that novel features arising since divergence occurred in the euechinoid rather than the cidaroid lineage. Nonetheless, both subclasses display evolutionary innovations i.e., subclass-specific, shared derived characters (apomorphies) with respect to the (fossilized) skeletal characters of their archaeocidarid ancestor, just as both display plesiomorphic morphological characters (9, 18, 19, 21).

Our experimental object was to pry open the genomic program innovations that underlie observed phenomelogical distinctions in embryonic skeletogenesis between euechinoids and cidaroids. To approach this problem systematically, we carried out a large-scale investigation of developmental regulatory gene use in the embryonic endomesoderm of the cidaroid Euclidaris tribuloides (Et) (results from comparing development of endoderm and nonskeletogenic mesoderm in these embryos are reported separately). Et is the same species in which embryonic skeletogenic morphogenesis had been studied earlier (13, 14), and in which juvenile skeletogenesis was also investigated in our laboratory (9). We experimentally interrogated the Et skeletogenic specification system to determine the presence or absence of multiple distinct GRN circuit features that contribute decisively to embryonic skeletogenesis in Sp. Many relevant genes from the authenticated Sp skeletogenic specification GRN (4) were investigated, of which five essential participants are reported on in the following. These are the regulatory genes at the very top of the skeletogenic GRN hierarchy in Sp, the deployment of which our earlier work (22) predicted might have been the locus of the evolutionary changes that mobilized the skeletogenic network in the micromere lineage of the euechinoids. Experiments on another cidaroid species (23) have already cast doubt on the presence of one key component of this circuitry, the repressive paired box gene pmr1, which functions in a double negative transcriptional gate at the top of the skeletogenic GRN of Sp (4, 24). As described below, we show here that the pmr1 gene is indeed apparently not represented in the genome or in transcriptomes of Et. However, this turns out to be but one probably derivative feature of a very generally different regulatory architecture. The complete structure of the Et skeletogenic GRN is still a work in progress. The present study is more narrowly focused on evidence for evolutionary rewiring in this circuitry, which must have taken place following the separation of the surviving echinoid clades >260 my ago.

**Results**

**Spatial Expression of Five Key Genes of the Euechinoid Skeletogenic GRN in Et Embryos.** Initial observations indicated a surprising lack of congruence between Sp and Et in the spatial domains of expression of four regulatory genes (i.e., genes encoding transcription factors) and of an essential signaling gene. These genes are of particular interest because of the important roles they play in the skeletogenic specification GRN of Sp. Even though their embryonic behavior is completely different from those of the skeletogenic micromere precursors of euechinoids, it had been shown by Wray and McClay (13) that the micromeres appearing early in Et cleavage do ultimately give rise to the postgastrular skeletal cells of this embryo. Thus, we could directly study expression of genes of the euechinoid GRN in known skeletogenic precursors of the cidaroid Et. We note here that the behavior of early ingressing skeletogenic micromeres of Sp is typical of many euechinoids, as supported by numerous observations on several different echinoid species, both at morphological and molecular levels.

Detailed spatial expression of the genes reported on here had not previously been studied in Euclidaris embryos, and the whole-mount in situ hybridizations (WMISHs) of Fig. 1 provide an important baseline for consideration of their skeletogenic (or antiskeletogenic) functions. Each of the five genes was expressed differently in Et than would have been expected from the euclidean examples. 

**akt1.** The akt1 gene is a primary driver of skeletogenic specification and differentiation in sea urchin embryo and adult development, and it is a member of a family of homeodomain genes also used in vertebrate skeletogenesis (22, 25–27). In euechinoids akt1 is one of the initial set of positively acting transcriptional regulators that set up the skeletogenic regulatory state, and it is transcriptionally activated by a double-negative derepression subcircuit (24, 28), immediately upon segregation of the skeletogenic micromere founder cells early in cleavage (25). In Sp, this gene then participates in direct cross-regulation of the succeeding tiers of the skeletogenic specification GRN. However, it is immediately apparent that these features of akt1 regulation are not likely to exist in Et. Thus, in Et, akt1 is not even transcribed
significantly until 4–6 h after the earliest micromere-specific genes are activated, (delta and ets1; Fig. 1 and Fig. S1), although alx1 expression is thereafter confined to micromeres and their skeletogenic descendants. Expression of the Et alx1 gene is, however, ultimately required for postgastrular skeleton formation to occur (Fig. 2), just as it is required for postembryonic skeletogenesis in both sea urchins and sea stars (22).

**delta.** The euechinoid delta gene is also an immediate transcriptional activation target of the Sp micromere double-negative gate subcircuit, and it continues to be expressed in this lineage until blastula stage, when its expression is extinguished there and instead appears in the surrounding nonskeletogenic mesoderm cells (24, 29, 30). In Et, delta expression occurs early in micromeres, 4–6 h before that of alx1, suggesting a primary function unconnected to later skeletogenic specification (Fig. 1). Expression of Et delta does not become nonskeletogenic until much later, when a complex pattern of ectodermal expression is installed (Fig. S2).

**hesC.** The most dramatically different functional implications revealed by Fig. 1 are to be seen in the expression in Et of the hesC gene. In the Sp GRN, HesC is the repressor controlling the initial skeletogenic regulatory state (i.e., including expression of alx1, delta, and ets1), and this state is controlled spatially by the transcriptional activity of the hesC gene. In the Sp GRN, the skeletogenic regulatory state is installed in micromeres by specific repression of the repressive hesC gene, executed by the micromere-specific repressor Pmar1, thus opening the double-negative gate subcircuit. HesC is transcriptionally expressed throughout the whole Sp embryo, except where this gene is turned off by pmar1 expression in the micromeres; thus, in Sp, hesC transcription and skeletogenic function are Boolean exclusives (4, 24, 31). However, in Et, hesC is expressed in micromeres at the same time as are ets1 and delta (by 10 h), in direct contrast to its double-negative gate function in Sp. Furthermore, the hesC gene is never vigorously expressed throughout the whole Et embryo as it is in Sp, and instead is strongly expressed (by blastula stage) only in the immediately surrounding nonskeletogenic mesoderm, as we see in more detail below.

**ets1.** Zygotic transcription of ets1 is turned on as the double-negative gate is unlocked in early cleavage in the Sp GRN, and thereafter this gene provides powerful positive inputs to both regulatory and effector genes in skeletogenic specification, far into development (26, 28, 29). In Sp, there is also a prevalent store of maternal ets1 mRNA, but this is entirely missing in Et. As in Sp, the ets1 gene is activated as early in the micromeres as is the delta gene, but, strikingly, by 12–14 h (blastula stage), Et ets1 expression spreads to the nonskeletogenic mesoderm and is then extinguished in the micromere descendants altogether (Fig. 1). Thus, neither is this gene likely to function similarly in the cidaroid as in the euechinoid skeletogenic lineage.

**tbr.** Finally, the tbr gene, which is required for and coopted to skeletogenic function in euechinoids (22, 24, 32, 33), is again expressed very differently in Et. Although the tbr gene is first activated in the micromeres, its expression quickly spreads to the entire nonskeletogenic mesodermal domain, where by double in situ hybridization, it can be seen to totally overlap that of ets1, and, in direct contrast to Sp, there is no evidence from its expression pattern that it ever plays a skeletogenic role.

Descriptive patterns of gene expression can never demonstrate the existence of given regulatory linkages, but they can certainly exclude their existence. Fig. 1 alone implies a very different cidaroid regulatory configuration than used in euechinoid skeletogenic specification.
Experimental Tests for Specific Linkages of the Euechinoid Skeletogenic GRN. We now set about challenging *Eucidaris* regulatory linkages among the above and additional genes, with the specific intent of determining whether these linkages could be the same, or must be exclusive, of the linkages among these same genes in the *Sp* skeletogenic GRN.

**Test for global confinement to skeletogenic lineage by HesC repression, of *axl1*, *tbr*, and *ets1* transcription.** A dramatic demonstration of the function of the skeletogenic double-negative gate in *Sp* is afforded by either overexpression of the repressor Pmar1 or introduction into the egg of *hesC* morpholino antisense oligonucleotide (MASO), either of which results in global transformation of embryonic cells to skeletogenic fate, and in global expression of the double-negative target genes *delta*, *axl1*, and *tbr* (4, 24, 28, 31, 32). In Fig. 3 *A, Left,* and *B, Left,* we see the spatial effect of *hesC* MASO on *axl1* expression in *Et* embryos. At blastula stage (Fig. 3B) expression of *axl1* indeed expands but (reasonably enough) only to the extent of significant *hesC* expression, which, as evident from Fig. 1, is confined to the immediately surrounding nonskeletogenic mesoderm. At gastrula stage (Fig. 3A), *axl1* expression expands to the immediately surrounding archenteron tip (mesoderm) cells. Thus, although *hesC* does repress *axl1*, it is not responsible for preventing *axl1* expression throughout the embryo as in *Sp*, but only in the nonskeletogenic mesoderm. In Fig. 3B the effects of *hesC* MASO on spatial expression of *tbr* and *ets1* are shown. Because Fig. 1 demonstrates the overlap of expression domains of *hesC* expression with those of *ets1* and *tbr* genes, they are unlikely to be subject to HesC repression, and indeed, *hesC* MASO has no effect on their spatial expression, again in direct contrast with the case in *Sp*. In Fig. 3C these results are substantiated quantitatively in a quantitative PCR (QPCR) experiment, which shows that the only significant effects (i.e., >1.5× cycle number change, a log₂ metric) are the modest increase in *axl1* expression seen spatially above and in *hesC* transcript level itself; this gene apparently depresses its own transcription.

These experiments preclude the global control of skeletogenesis by *hesC* repression in *Et*, which is its prominent role in the *Sp* skeletogenic GRN. They also preclude any control in *Et* of either *ets1* or *tbr* by HesC repression. We have already seen that neither of these genes is likely to have anything to do with skeletogenesis after cleavage in *Et* in any case.

**Lack of evidence for existence of the pmar1 gene in *Et*.** A complete genomic sequence has been obtained for *Et*, although it is not annotated and has been assembled only to contigs of several kilobase median length. In addition, a mixed embryonic transcriptome has been sequenced and analyzed (data from Human Genome Sequence Center). Despite the unfinished genomics analyses, these genomics resources sufficed for identification of >95% of a large set of *Sp* protein-coding genes. However, we were unable to find any sequence whatsoever in either the *Et* genome or transcriptome datasets indicating the existence of any genes resembling *Sp* pmar1. The *Sp* genome includes at least six clustered paralogues of this divergent paired box gene, and two of these genes, for which cis-regulatory evidence has also been obtained, are directly similar to the *pmar1* transcripts that we functionally characterized earlier (31, 34). Because failure to identify *pmar1* genes in the *Et* genome or embryo transcriptomes is not an entirely convincing result, we embarked on an additional, although indirect, approach and asked whether the regulatory state of *Et* micromeres (or indeed of any polar early cleavage *Et* cells) would support transcription of an *Sp* pmar1 gene. An accurately expressing, recombinered pmar1 BAC construct bearing a knocked-in GFP marker had previously been constructed and authenticated in gene-transfer experiments (34). It responds at known cis-regulatory sites to the two transcription factors that in *Sp* constitute the localized input responsible for endogenous pmar1 expression as soon as micromeres form (4). These are a Tcf input, which uses for its spatial activation function maternally localized β-catenin (35), and Otxa transcription factor, which is also transiently localized to the micromeres in *Sp* (36).

However, when this pmar1 reporter construct was injected into *Et* eggs, no localized expression could be seen, and instead the construct expressed more or less equivalently in all domains of the embryo. This result is shown in Fig. 3 D and E. Additionally, we checked whether a localized Otxa factor might be used for early control of the skeletogenic regulatory state in *Et*, even if this effect were not mediated by a pmar1 gene (or a recognizable pmar1 gene). A sequence encoding the maternal Otxa factor was truncated to produce a dominant-negative form, which was shown to be functional by its effect on endoderm genes when the mRNA was injected into *Et* eggs. However, injection of this mRNA into *Et* eggs had no effect whatsoever on expression levels of any of the micromere genes, such as *axl1* or *ets1*, as assessed by OPCR.

The minimum conclusion from these experiments is that the combinatorial localization system that in *Sp* provides the β-catenin/Tcf and Otxa transcriptional inputs causing micromere pmar1 expression does not exist in *Et*. In all probability, neither does pmar1, the lynchpin upstream gene of the double-negative gate, even exist in the *Eucidaris* genome. Together with the foregoing *hesC* MASO experiments, it can be concluded that the double-negative gate circuitry of the euechinoid micromere lineage does not control the skeletogenic regulatory state in *Et*. Absence of this circuit feature was also inferred for another cidaroid embryo (23).

**What Does Specify the Ultimate Skeletogenic Fate of Micromeres in *Et***? The *axl1* gene is clearly not an initial regulatory mediator of skeletogenic specification in *Et* micromeres as it is in the *Sp* skeletogenic micromere GRN, because, as we show here, it is not even transcribed during the period of activation of the initial set of micromere-specific genes. However, *axl1* is ultimately just as clearly a canonical driver of later skeletogenic differentiation in *Et* (Fig. 2), as it is also in euechinoids (25). Thus, we can use its expression as a faithful indicator of skeletogenic fate, unlike genes such as *tbr* and *ets1*, which, although expressed early in *Et* micromeres, apparently end up having little to do with skeletogenesis.
A not entirely unexpected clue as to the nature of the initial molecular input specifying skeletogenic fate evolves from the experiments in Fig. 4A, although they raise as well as answer a mechanistic question. As shown there, when mRNA encoding GFP-tagged β-catenin is injected into Et eggs, it is ubiquitously translated, but then over the next few cleavages, this protein is asymmetrically degraded (37), leaving it concentrated, dramatically and exclusively, in the micromeres. This negative cytoplasmic localization system is mediated by the β-catenin protein sequence per se, and the behavior of the tagged construct perfectly reflects the early highly localized retention of native β-catenin in euechinoid sea urchin micromeres, as observed immunocytoologically (38) (we cannot of course be certain whether the kinetics of asymmetric clearance in Et are affected by the GFP tag). The responsible localization system does not depend on asymmetric Wnt signaling in the cleavage stage embryo; thus, the same localization of GFP-tagged β-catenin occurs in the presence of a potent antagonist of all canonical and noncanonical Wnt signaling, “CS9” (Fig. 4B). CS9 works by inhibiting Porcupine-dependent Wnt mobilization and secretion, and is both effective and specific in sea urchin embryos (39); detailed evidence for sea urchin embryos and references to its specificity and mode of action in other bilaterian systems are to be found in this reference. It follows from the results of experiments such as those reproduced in Fig. 4A and B, that the β-catenin localization system of early Et embryos is a property of the oocyte/egg cytoplasmic localization system, which falls into the category of anisotropic deposition of molecules of gene-regulatory significance, a general feature of very early animal eggs (2). The main import of Fig. 4, however, is in the QPCR experiment of Fig. 4D. Here we see that there is virtually no expression of alx1 in micromeres (<8% of control values), if maternal β-catenin is sequestered by introduction of excess cadherin fragment, even though alx1 transcription is a late cleavage event. Furthermore, these effects depend not at all on Wnt signaling, even as late as 15 h. Thus, we are confronted with a missing link: β-catenin construct localization is complete in Et by the seventh cleavage (Fig. 4A), and a significant time gap of several hours separates this event from activation of the alx1 gene. The actual transcriptional mediator of alx1 activation that responds to the localized β-catenin/Tcf cue therefore remains unknown. We cannot yet experimentally either exclude or support the possibility that the initial transducer of the β-catenin/Tcf input is the cis-regulatory system of the Et alx1 gene, which is activated hours earlier than alx1 at approximately the right time. It may be significant that cis-regulatory analysis of alx1 expression in Sp showed it to be subject initially to obligatory ets1 activation (28). This independence, however, remains to be demonstrated for Et alx1.

**The Basal Role of hesC in Mesoderm Specification.** The relation between hesC and delta expression is a well-known constant of Notch signaling systems (40, 41). Although there are countless variations, in simple form, the Delta ligand promotes Notch receptor activation with the consequence that the immediate transcriptional effector, Su(H), activates Notch signal transduction target genes. Among these are very often genes encoding bHLH repressors of the same family as hesC. The expression of these repressors enforces the distinction between Delta signal-sending and Notch signal-receiving genes by transcriptional exclusion of delta transcription in the Notch signal-receiving cells. A beautiful illustration of this relationship can be seen in Fig. 5A. As we report elsewhere, Notch signaling is taking place in the Et embryo, but aside from the following negative relationship, it plays no role whatsoever in specification or differentiation of skeletogenic cells per se.

Fig. 5A shows that hesC expression, by this time in the surrounding nonskeletogenic mesodermal cells, is entirely dependent on Delta.

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**Fig. 3.** Functional tests for presence in Et of known regulatory linkages of the Sp skeletogenic GRN. Test for global HesC repression of skeletogenic regulatory state. (A) The 20-h embryos injected with hesC MASO exhibit an expanded domain of the skeletogenic lineage marker alx1. (B) The 20-h embryos injected with hesC MASO showing no global expression of dominant-negative gate genes. Alx1 expands locally only, whereas ets1 and tbrai are unaffected. (C) Quantitative effects of hesC MASO on mRNA abundance at 18 h on expression of skeletogenic genes alx1, delta, ets1, hesC, and tbrai. Alx1 and hesC mRNAs are significantly up-regulated. The difference in cycle number (ddCt) with respect to an uninjected control group is shown on the ordinate. Error bars represent the SD of two independent experiments. (D) Spatially nonrestricted expression of Sp pmn1 expression construct on injection into Et eggs. (E) Quantification of multiple expression domains observed in (D).
expression in the immediately adjacent skeletogenic micromeres. That is, as in the canonical case, Notch signal transduction of the micromere Delta signal results in hesc expression in these nonskeletogenic mesodermal cells. Because, as shown above, HesC is the repressor excluding alx1 expression from the nonskeletogenic mesoderm, if Delta expression is prevented, alx1 expression spreads to the nonskeletogenic mesoderm, now involving twice as many cells (Fig. 5 B–D). We perhaps see here the original role of micromere delta expression in sea urchin embryos, the spatial separation of skeletogenic from nonskeletogenic mesodermal specification. This result capsulizes the depth of the differences between the echinoid and cidaroid specification systems; whereas Sp uses its double-negative gate circuitry to position skeletogenic function in the micromere lineage, Et uses Delta/Notch signaling for this purpose.

Discussion
Our main and specific objective was to assess at least the minimum evolutionary divergence that took place within a thoroughly known
The evolutionary novelty (see below), each of these regulatory inputs represents the appearance of a new GRN linkage that had to be encoded in cis-regulatory DNA of genes in the euechinoid lineage, a linkage that is lacking in the cis-regulatory sequences of the same genes in the cidaroid lineage. Perforce a minimum estimate, we see here something of the scale of genomic regulatory change required for architectural network evolution, even in a small, confined GRN dedicated to specification of one cell lineage. Canonically, this type of evolutionary process is far removed from the single cis-regulatory module divergences easily accessed in studies of intra- and interspecific adaptive variation (2).

Plesiomorphy and Polarity in the Echinoderm Regulatory Linkages. All of the changes enumerated above are gains of function with respect to the regulatory configuration of the Et system, with most of them involving multiple different inputs per cis-regulatory module. Although it is conventional to note that all such changes could also represent loss-of-function changes in the cidaroid lineage—meaning that the euechinoid regulatory system could equally be plesiomorphic—the evidence is no longer balanced; it is much more likely [just as intuitively assumed by past observers (13, 14)] that the euechinoid skeletogenic GRN is the derived, novel character shared among descendants of the common euechinoid ancestor. A crucial argument that now comes into view is that the gains of function are sequentially and logically nested. That is, a given change requires particular sets of sequential changes, which impose polarity on the process. For example, acquisition of cis-regulatory response to a global regulator in the hesC gene introduces the possibility of release of control of genes such as act1 from a strictly mesodermal activator to control by a general global activator, and of the delta gene from its strictly Notch-dependent control also to that of a general activator. However, such relocations of domain-specific positive regulatory constraint in turn make it necessary to control micromere expression by negative rather than positive means, as executed by the euechinoid double-negative gate. This is not to propose a specific pathway, but to point out that, whatever the pathway, we are dealing here with an internally sequential logic train, rather than a series of independent changes that indeed individually might be considered equally likely to be gain as loss of function. A second argument concerns the cooption of the tbr gene to skeletogenic function. This work shows that cooption to be a euechinoid novelty, because in Et tbr is not skeletogenic in function, and because we know from developmental GRN, during or soon after the last major cladistic split in the evolution of the echinoids. This divergence occurred in a late Paleozoic time interval that is constrained in real time by the fossil record. One uncertainty that could affect dynamic interpretation of the results is the possibility that the differences we observe between the test species of this work, Sp and Et, are actually in part the sum of changes that occurred only gradually—that is, during the Mesozoic (Triassic, Jurassic, and Cretaceous), subsequently to the split from which emerged the modern euechinoid and cidaroid subclasses. This would require, however, that the specific circuitry features we investigated vary among modern euechinoid orders that arose during the Mesozoic (18). However, although indeed incomplete, the evidence so far limits this possibility. Thus, a euechinoid belonging to an irregular euechinoid group (the Spatangoids), far removed from typical euechinoids such as Sp, also contains a pmar1 gene and also zygotically expresses the hesC gene all over the embryo except for the skeletogenic micromeres (42), exactly as in Sp. This global hesC expression, as we have seen, is in direct contrast to Et (we refer here only to the key shared linkages of interest, irrespective of the many and various other intraeuechinoid divergences that are also observed, but are irrelevant to skeletogenesis) (42). Therefore, key diagnostic features of the modern euechinoid (i.e., Sp) GRN are found in descendants of a euechinoid clade, the last common ancestor of which with Sp arose anciently, perhaps at the beginning of the Jurassic (18). This result leaves untested only the most basal orders of euechinoids, but because those clades emerged directly from the subclass split per se, they limit the temporal argument pertaining to postdivergence events. Similarly, on the cidaroid side, as noted above, the orders composing this Subclass have displayed remarkably invariant and conservative morphology ever since their appearance (18). Consistent with this, as we have seen, Et indeed shares with a distant cidarid the key property of lacking the double-negative skeletogenic specification gate (23). Therefore, with the caveat of the yet-unexamined most basal euechinoid orders, we can tentatively assume that we are here assaying genomic wiring features typical of almost the whole euechinoid subclass vs. those typical of the whole cidaroid subclass. These must be differences that indeed arose during the late Paleozoic at the divergence between these clades and/or in the earliest subsequent phases of euechinoid divergence—differences that have ever since been inherited by descendants of the crown group ancestors of each branch.

GRN Linkages of the Embryonic Sp Skeletogenic GRN Shown Here to be Specifically Absent from the Embryonic Et Skeletogenic Specification System. We can now list specific regulatory features encoded in Sp cis-regulatory sequence that contribute decisively to the architecture of the Sp skeletogenic GRN (6), but that do not operate at all or operate differently in Et (reference citations below all refer to cis-regulatory studies or other decisive studies in Sp). This provides a minimum but hard estimate of regulatory differences between the embryonic skeletogenic specification circuitries that have arisen since the last common ancestor from which these two genomes descend. The hesC cis-regulatory system. First, in Sp, the hesC gene responds to a powerful global euechinoid activator (24), a feature totally lacking in Et. In Et, hesC transcription is spatially controlled by Delta/Notch signaling from the micromeres and hence is expressed only in mesoderm immediately adjacent to the micromeres [Delta/Notch signaling does still provide an additional cis-regulatory input to hesC in Sp (34)]. Second, in Sp, the hesC gene is negatively controlled at the transcriptional level by Pmar1 repression (24, 34). In Et, no pmar1 gene or similarly functioning gene appears to exist. The tbr cis-regulatory system. First, in Sp, this gene is negatively controlled by HesC and positively controlled by a ubiquitous activator (32). Second, in Sp, tbr is expressed in skeletogenic cells. In Et, none of these three inputs operates on tbr transcription.

The ets1/2 cis-regulatory system. First, in Sp, this gene is expressed maternally. Second, in later development, it is expressed in differentiating skeletogenic cells (where it plays a major role in activating skeletogenic effector genes). However, in Et, neither is true.

The delta cis-regulatory system. In Sp, early embryonic spatial expression of delta is negatively controlled by HesC, and Ets1 serves as a positive driver (24, 29). However, in Et, HesC provides no spatial input into delta expression (although for unknown reasons, hesC MASO somewhat increases delta mRNA levels); in Et, Ets1 does not provide any positive input into delta expression.

The pmar1 gene. This key gene of the Sp skeletogenic specification system is almost certainly absent altogether from the Et genome. The initial combinatorial Otxα:Tcf1/-catenin cis-regulatory micromere input. In Sp, this combinatorial input is used to trigger pmar1 transcription in micromeres (4, 34), whereas in Et this combination is not functional in skeletogenic micromere specification by direct test, and Otxα is not used at all in skeletogenic specification. Although this transcriptional regulator is encoded maternally in Et as in Sp, its function remains undemonstrated.

In sum, here there are nine specific cis-regulatory inputs into genes operating in both species that function in Sp and are absent in Et, plus a key gene missing in Et (or small subfamily of genes), plus a key localized combinatorial cis-regulatory transcriptional input used in Sp by the gene that is absent in Et. Assuming the euechinoid network is the evolutionary novelty (see below), each of these regulatory inputs represents the appearance of a new GRN linkage that had to be encoded in cis-regulatory DNA of genes in the euechinoid lineage, a linkage that is lacking in the cis-regulatory sequences of the same genes in the cidaroid lineage. Perforce a minimum estimate, we see here something of the scale of genomic regulatory change required for architectural network evolution, even in a small, confined GRN dedicated to specification of one cell lineage. Canonically, this type of evolutionary process is far removed from the single cis-regulatory module divergences easily accessed in studies of intra- and interspecific adaptive variation (2).
comparative studies that the plesiomorphic role of tbr in echinoderms is not skeletogenic either (33). Therefore, this cooption is a derived euechinoid character, and in Sp, tbr is driven by a ubiquitous activator (32) so that its expression is made skeletogenesis-specific only by the double-negative gate. Third, and similarly, control of delta gene expression is executed by the Notch response system in Et, whereas addition of a global positive control input in the delta gene in Sp (24) is therefore also a euechinoid derivation. We conclude that all of the linkages of the skeletogenic control GRN that are found in Sp but are absent from Et are probably shared derived characters of the euechinoids.

However, if this is the case, there must also remain plesiomorphic aspects of the skeletogenic program that would have been identified in this work as shared features present in both Et and Sp.
Indeed this logical expectation is fulfilled. The most prominent plesiomorphic GRN character is of course the dominant role of alx1 as a driver of skeletogenic differentiation. The role of alx1 is plesiomorphic for echinoderm skeletogenesis in general (22, 25). A second major plesiomorphic in circuit wiring is indicated by the retention in both systems of negative spatial control of alx1 by HesC repression. Similarly, a third plesiomorphic linkage is retained for the polarity of the evolutionary train of events. The presumably plesiomorphic cidaroid skeletogenesis system has a fundamental, key feature that would have allowed the accumulation of the novel GRN linkages without at the same time destroying its needed function of programming embryo/larval skeletogenesis. This feature is that development of the cidaroid micromere cell lineage is in functional terms essentially a dual process. In Et, cleavage-stage micromere functions per se and skeletogenic functions per se are separate. The cleavage-stage micromeres do not execute skeletogenic specification, and instead their role is to emit Delta signals, which are used negatively in late cleavage to prevent the nonskeletogenic mesoderm from skeletogenic differentiation fate. Skeletogenic specification occurs only subsequently (in micromere descendants), when and after alx1 is belatedly turned on. Skeletogenic differentiation takes place even later, mainly at the tip of the archenteron and subsequently in the blastocoele. Thus, the precocious skeletogenic functions controlled by the novel euechinoid skeletogenic GRN could have assembled over evolutionary time due to the loss of the micromere lineage, during or soon after the period the cladistic cidaroid/euechinoid split was taking place, without interrupting any of the developmentally later skeletogenic functions on which the embryo of the euechinoid stem lineage would still have depended. In other words, in the plesiomorphic state the micromere lineage executed signaling but not skeletogenic functions during cleavage and blastulation, but during euechinoid divergence novel skeletogenic circuitry executed in the micromere lineage during early development could have been superimposed, without necessarily interfering with gastrular skeletogenesis until the latter became redundant.

**Materials and Methods**

Detailed materials and methods are available in SI Materials and Methods. We thank Dr. Isabelle Peter for excellent discussions and for her creative suggestion of injecting the minimal Sp-pmar1 reporter construct; Ryan Range for providing plasmid containing j-catenin:GFP insert; and Miaoa Cui for providing C59 inhibitor and educating us in its use. We extend a special thanks to Andy Cameron and Parul Kuttukat at the Beckman Institute’s Center for Computational Regulatory Genomics for providing support with transcriptions in Euclidaris. This work was supported by a National Science Foundation CREATE Grant 1240626.

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