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Developmental gene network analysis

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ABSTRACT The developmental process is controlled by the information processing functions executed by the *cis*-elements that regulate the expression of the participating genes. A model of the network of *cis*-regulatory interactions that underlies the specification of the endomesoderm of the sea urchin embryo is analyzed here. Although not all the relevant interactions have yet been uncovered, the model shows how the information processing functions executed by the *cis*-regulatory elements involved can control essential functions of the specification process, such as transforming the localization of maternal factors into a domain-specific program of gene expression; refining the specification pattern; and stabilizing states of specification. The analysis suggests that the progressivity of the developmental process is also controlled by the *cis*-regulatory interactions unraveled by the network model. Given that evolution occurs by changing the program for development of the body plan, we illustrate the potential of developmental gene network analysis in understanding the process by which morphological features are maintained and diversify. Comparison of the network of *cis*-regulatory interactions with a portion of that underlying the specification of the endomesoderm of the starfish illustrates how the similarities and differences provide insights into how the programs for development work and how they evolve.

KEY WORDS: *Gene network, genetic program, evolution and development, genomic regulatory system, sea urchin*

The genetic programs that control the processes by which the body plans of animals are built were invented, and shaped, by the evolutionary process. How these programs work, if nothing else, is a matter of great curiosity. Because gene networks constitute the control systems for development, analysis of such networks explains both the process of development and the process by which development has evolved (Davidson, 2001).

Ultimately, development is the process by which the body plans of animals are laid down. Distinct cell types are produced in particular spatial domains, each with particular structural properties given by the distinct programs of gene expression that the cells execute. Through the process of specification each domain in the embryo obtains its developmental identity. Once specified, each domain will run through a progression of states of regulatory gene expression, leading to the establishment and ultimately the stabilization of the terminal programs of gene expression that give each cell type its unique properties.

Spatial cues are always required in order to trigger specification in development. These spatial cues sometimes consist of localized maternal regulatory factors that are distributed to particular cells with the egg cytoplasm, and are partitioned during cleavage. Alternatively they can also consist of signaling ligands produced by other cells, in consequence of their own prior state of specification. Ultimately, these spatial cues affect the course of events in development by causing the activation (or repression), in a certain region of the embryo, of particular genes encoding transcription factors. Through this process, new, more refined, domains of specification are created, and the complexity of the embryo increases. But although it is the spatial cues that trigger the events of spatial specification, the locus of programmatic control for each developmental event is the sequence of the particular *cis*-regulatory elements that respond to the inputs presented (Davidson, 2001).

cis-Regulatory elements can recognize the presence or absence of those transcription factors for which they contain specific binding sites. According to the set of inputs presented in each cell, the *cis*-regulatory elements of given genes control the expression of the gene in each domain of the embryo. Of particular importance are genes encoding transcription factors, and their *cis*-regulatory elements. Spatial information is translated by the *cis*-regulatory elements of these genes into distinct states of regulatory gene expression. It is the network of all these *cis*-regulatory interactions that is ultimately responsible for driving the process of development. To fully understand how the process of development is programmed in the genomic DNA, it will be necessary to unravel the network of regulatory interactions, and to analyze the information processing functions executed by each *cis*-regulatory element (Davidson, 2001).

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The experiments reviewed here represent a step towards the goal of determining the complete network of DNA-based interactions that underlie one particular major process of development, namely, the specification of the endomesoderm of the sea urchin embryo. Given that evolution occurs by changing the program for development of the body plan, we also illustrate briefly how developmental gene network analysis sheds light on the process by which morphological features are maintained and diversify.

Unraveling the gene regulatory network that underlies the process of endomesoderm specification in the sea urchin embryo

The armature of the network

Figure 1 illustrates the process of endomesoderm specification in the sea urchin embryo (Fig. 1 A-D), and it shows a diagram (Fig. 1E) that describes the specification events and the genetic functions that underlie this process.

Ultimately, the endomesoderm consists of the endodermal gut, the skeletogenic mesenchyme and several other mesodermal cell types, including pigment cells (Fig. 1D).

By the seventh cleavage cycle (Fig. 1A), the cell lineages of typical sea urchin embryos have been segregated into a canonical set of territories, each of which is destined to give rise to certain distinct cell types (Hörstadius, 1939; Cameron *et al.*, 1987, 1991), and in each of which a distinct set of genes is already running (reviewed by Davidson *et al.*, 1998; Davidson, 2001). The upper or animal pole half of the embryo now consists of blastomeres that produce only the cell types ultimately found in the oral and aboral ectoderm. The lower half consists of the veg1 ring, their sister cells of the veg2 ring immediately below, and the large and small micromeres at the vegetal pole. In the undisturbed embryo, the large micromeres (the population of cells colored lavender in the diagram) will produce all the cells of the skeletogenic mesenchyme lineage, and the progeny of veg1 and veg2 will produce the rest of the endomesoderm. At the ciliated swimming-blastula stage (Fig. 1B), the veg2 lineage has been segregated into two distinct domains: the inner veg2 ring

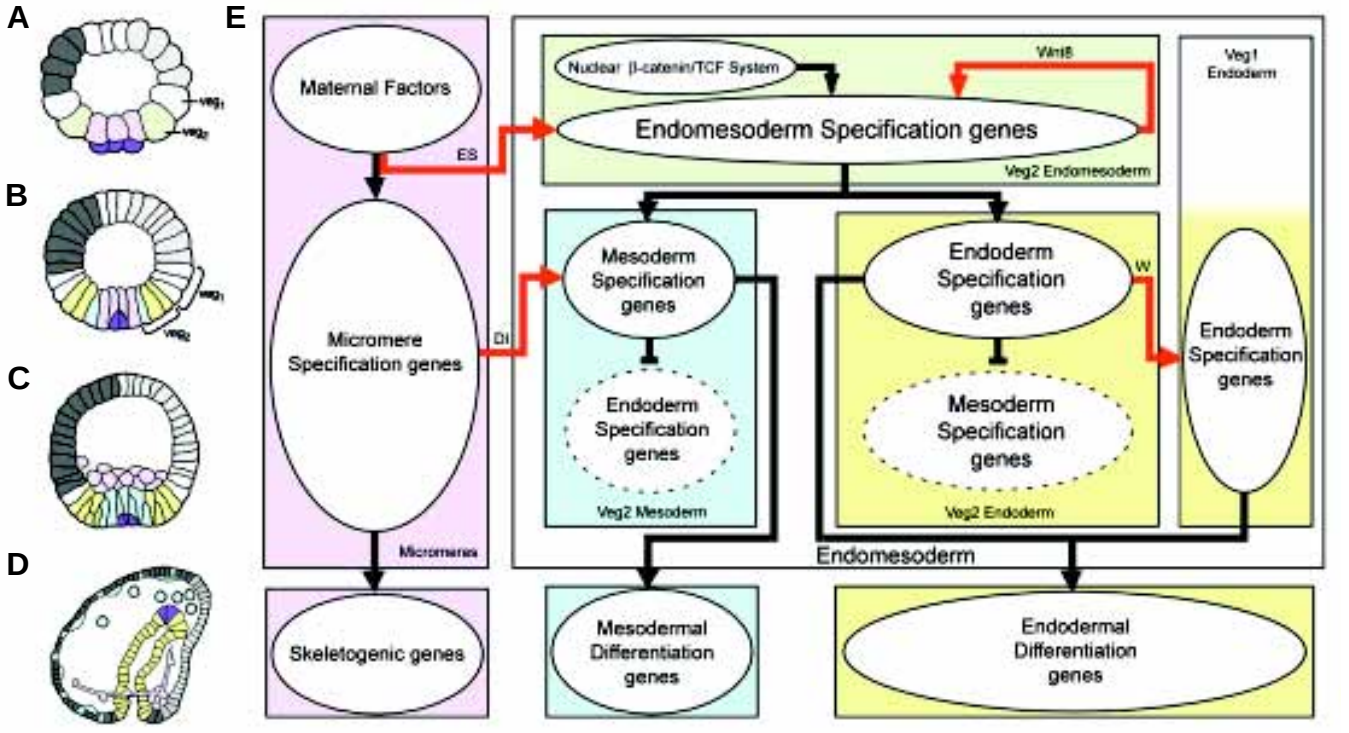

Fig. 1. Endomesoderm specification in the sea urchin embryo. (A-D) *Schematic diagrams of sea urchin embryos displaying specified domains, from Davidson* et al. *(2002b). The color coding shows the disposition of specified endomesoderm components: Lavender indicates skeletogenic lineage; dark purple indicates small micromere precursors of adult mesoderm; green indicates endomesoderm lineage that later gives rise to endoderm, yellow, and mesoderm, blue; light grey indicates oral ectoderm; dark grey indicates aboral ectoderm; white indicates regions yet to be specified at the stages shown.* **(A)** *7th cleavage embryo (about 10 h after fertilization).* **(B)** *Blastula stage embryo at about 9th cleavage (about 15 h after fertilization).* **(C)** *Mesenchyme blastula stage embryo (about 24 h after fertilization).* **(D)** *Late gastrula stage embryo (about 55 h after fertilization). The drawing shows the later disposition of all the endomesodermal cell types about midway through embryonic morphogenesis.* **(E)** *Process diagram describing endomesoderm specification events in the sea urchin embryo. Boxes represent domains of specification according to the color of their background. The color coding represents the same endomesoderm components as in the schematic diagrams A-D. Ovals in the boxes represent sets of genes that execute certain developmental functions. Arrows indicate that the set of genes in the oval where the arrow originates, triggers the developmental function executed by the genes in the oval where the arrow ends. In particular, red arrows represent signaling events. Barred lines indicate repression of the developmental function executed by the genes in the oval where the barred line ends. Developmental time in the process diagram runs from top to bottom in accordance with the stages represented by the schematic diagrams A-D. Abbreviations: ES, Early Signal; Dl, Delta; W, Wnt8. Evidence is reviewed in Davidson* et al. *(2002a), and from P. Oliveri, A. Ransick, D.R. McClay and E.H. Davidson, unpublished data.*

consists of cells that will give rise to mesodermal cell types, including pigment cells; and the rest of the veg2 domain will give rise to endodermal cells (Ruffins and Ettensohn, 1993, 1996). At the mesenchyme blastula stage (Fig. 1C), the skeletogenic mesenchyme cells have ingressed into the blastocoel, leaving behind a now fully specified central disc of prospective mesodermal cell types, and peripheral to them, the endodermal precursors (reviewed by Davidson *et al.*, 1998). After this, the adjacent veg1 progeny will become specified as endoderm as well (Logan and McClay, 1997), and gastrular invagination ensues.

The mechanisms that trigger each one of the specification events that are symbolized by the colors in Fig. 1 A-D are now reasonably well understood. The micromere lineage is autonomously specified as soon as these cells are formed at fourth cleavage (reviewed by Davidson *et al.*, 1998). The spatial cues that trigger their specification are maternally localized. As soon as they are born, the micromeres emit a signal that, together with spatial cues that are autonomously localized, triggers the specification of the surrounding veg2 cells to endomesodermal fate (Ransick and Davidson, 1993, 1995). The segregation of veg2 between mesodermal and endodermal domains depends on a second signaling event from the micromeres that takes place at 7^{th}-9^{th} cleavage, and is executed by the ligand Delta (Sherwood and McClay, 1999; Sweet *et al.*, 1999; McClay *et al.*, 2000; Sweet *et al.*, 2002). The cells in the inner veg2 ring, which are exposed to the Delta signal from the micromeres, are specified as mesoderm. The rest of the veg2 cells will acquire endodermal fate. The result is that the initial crude pattern of specification, which defines veg2 as endomesoderm, has now been refined into two distinct specification states. Finally, another signaling event from the veg2 endoderm triggers the specification of the surrounding veg1 also as endoderm (Logan and McClay, 1997; Ransick and Davidson, 1998).

The knowledge summarized in Fig. 1E provides us with the armature on which the network of gene interactions is subsequently built. It tells us what specification functions must be executed by the genes in each domain: for example we know that the genes in the lavender box (Fig. 1E) must be able to translate the maternally localized spatial cues into a skeletogenic program of differentiation, and they must also be able to cause expression of the ligand Delta; and that the genes in the blue box must be able to listen to the spatial information given by the Delta signal in order to create a state of specification on which the mesodermal differentiation program is then installed.

The process diagram of Fig. 1E also serves another purpose. It tells us how we can interfere specifically with a certain specification event or domain, which is an essential tool in the enterprise of building the regulatory network, as we see below.

Useful as the knowledge contained in Fig. 1E might be, it should be made clear that this knowledge by itself does not provide us with any real understanding of the developmental process. Figure 1E by itself fails to show us the explicit mechanisms of specification, the instructions followed by each cell on its way to becoming specified. These instructions are encoded in the genomic DNA. It is the goal of the following to unravel the network of DNA-based interactions from which the instructions for development can be read.

Building the network of cis*-regulatory interactions*

In order to clothe with real genes the armature of interactions indicated in Fig. 1E, a major gene discovery effort was undertaken, by performing several differential macroarray screens (Rast *et al.*, 2000). The goal of each of these screens was to isolate cDNA transcripts that are differentially expressed in a given domain of the endomesoderm. To this end, different specification events were interfered with so as to generate populations of RNA transcripts lacking given classes of endomesodermal sequence, and these populations were compared to normal embryo RNA or to RNA from embryos in which the RNA populations contained larger amount of endomesodermal sequences than normal. By using a very sensitive subtractive hybridization technology on these populations of transcripts, probes were created in which sequences differentially expressed in the chosen endomesodermal domain were greatly enriched. These probes were then used to screen large-scale arrays of ~10^5 clone cDNA libraries (macroarrays) (Rast *et al.*, 2000).

In order to determine the interactions among the different genes, a large-scale perturbation analysis was carried out, in which the expression of many genes was individually altered experimentally, and the effect on all other relevant genes in the network was then measured by quantitative polymerase chain reaction (QPCR) (Davidson *et al.*, 2002a). Given the *cis*-regulatory interactions predicted by the QPCR experiments, direct *cis*-regulatory analysis is used to test the predicted network linkages, and in certain instances to unravel the key information processing functions executed by the relevant *cis*-regulatory elements.

The *cis*-regulatory network: the control system for the specification process

A model for the process of endomesoderm specification is shown in Fig. 2 in the form of a network diagram that combines all significant perturbation data; information on time and place of gene expression, as determined by whole mount *in situ* hybridization (WMISH) and QPCR measurements; *cis*-regulatory data where available; and all the underlying information of experimental embryology.

At each *cis*-regulatory element in the model predicted regulatory interactions with the products of other genes in the network are indicated. Therefore each one of these predicted interactions can be experimentally tested by determining the presence and function of the relevant binding sites in the relevant *cis*-regulatory elements. The importance of this point is worth emphasizing. It means that eventually the *cis*-regulatory network can be turned into a solid, experimentally confirmed structure.

Even though not all the *cis*-regulatory interactions that underlie the specification of the endomesoderm of the sea urchin embryo have yet been identified, and even though not all the identified interactions have yet been tested, the model of Fig. 2 allows us to see how the network of *cis*-regulatory interactions controls the specification process. The model shows how the *cis*-regulatory interactions control the specification functions that need to be executed for the different domains of the endomesoderm of the sea urchin to become what they become.

Interpreting the spatial cues: specification of the micromeres

The network model of Fig. 2 indicates the mechanism by which maternal spatial cues in the micromeres are interpreted and translated into the specification state that is specific to the micromere lineage.

The genes *tbr, alx* and *ets*, are all known to activate a number of genes that are responsible for the differentiation of the micromere lineage into skeletogenic cells [Kurokawa *et al.*, 1999; Fuchikami *et al.*, 2002; Ettensohn *et al.*, 2003 and www.its.caltech.edu/~mirsky/qpcr.htm (End-mes Network QPCR Data)]. Early in development, these three skeletogenic regulators are all kept silent everywhere in the embryo by a repressor gene (*r of mic*). At this time, *delta*, which is responsible for executing one of the micromere-specific developmental functions, is also repressed everywhere in the embryo by the same repressor gene. Immediately after the micromeres are born at 4th cleavage, the *pmar1* gene is activated specifically in these cells. This gene has a repressor function that shuts down the expression of '*r of mic*'. Now, *delta*, and the skeletogenic regulators *tbr, alx* and *ets* are allowed to be expressed exclusively in the micromeres, and as a result the skeletogenic program is set in train (Oliveri *et al.*, 2002).

The mechanism just described ensures that once the *pmar1* is activated, the micromere specification program will be installed without the need for any further spatial cues. If *pmar1* is ectopically expressed everywhere in the embryo, the skeletogenic regulator *tbr*, the signaling ligand Delta, and the skeletogenic differentiation gene *sm50* are all also expressed everywhere, and the whole embryo is now expressing the functions normally executed only by the cells of the micromere lineage (Oliveri *et al.*, 2002, 2003). The fact that *pmar1* is sufficient to establish the skeletogenic program, together with the fact that *pmar1* is activated by factors that are all either maternally present or autonomously localized in the micromere nuclei, tells us why the micromeres are autonomously specified. The most important general point is that the explanation of this embryological phenomenon is now provided in terms of the genomically encoded map of *cis*-regulatory interactions.

Refining the specification pattern: specification of the pigment cells

The portion of the network in the diagram of Fig. 3 tells us the mechanism by which the pigment cells are specified and ultimately differentiated, according to the network model. The pigment cells arise specifically from the mesodermal cells of the veg2 domain (Ruffins and Ettensohn, 1993, 1996). The Delta signaling ligand produced by the micromeres between 7th and 9th cleavage serves as the spatial cue that triggers the segregation of the mesodermal and endodermal fates of veg2 descendant cells (Fig. 3 A,B). Expression of the ligand Delta in the micromere descendants activates a Notch (N) receptor in the adjacent veg2 cells, which is required for normal

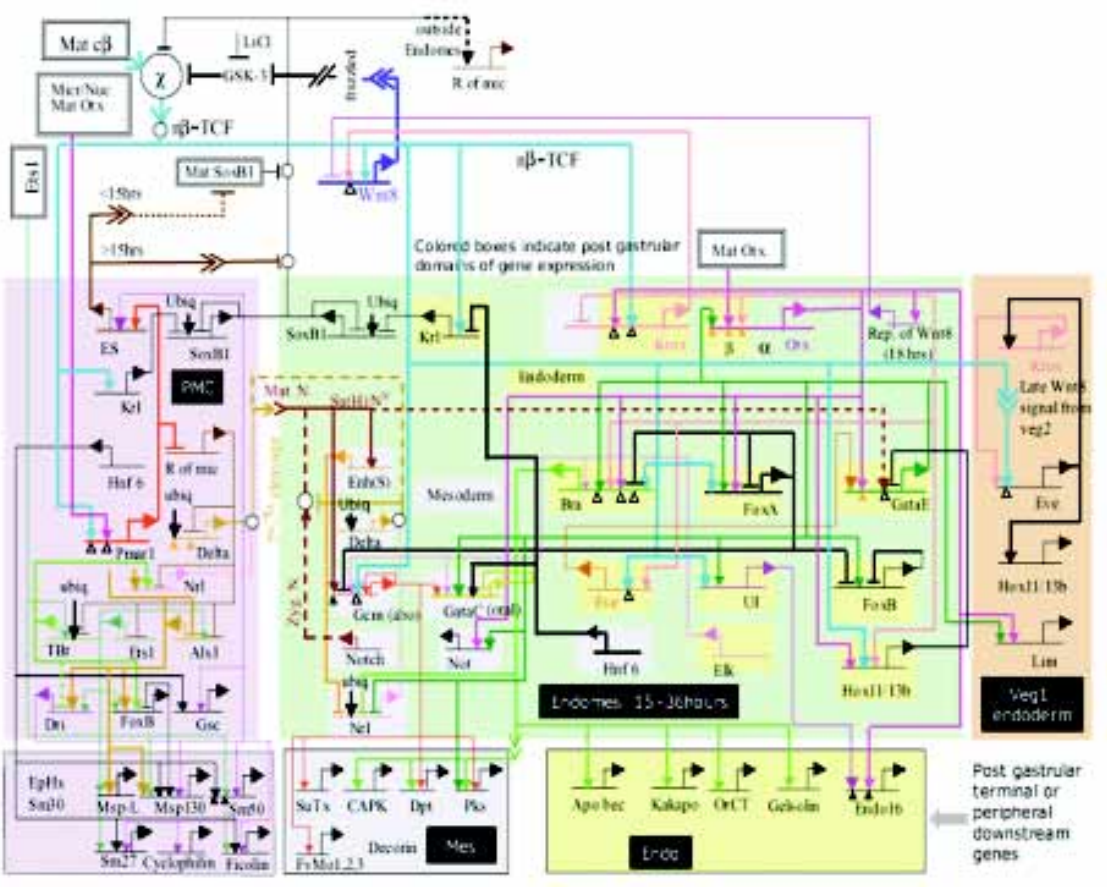

Fig. 2. Regulatory gene network model for endomesoderm specification from fertilization to just before gastrulation. *This is a recent version of the model originally presented by Davidson* et al. *(2002a, 2002b). The current version of the model and the perturbation data on which it is based are available at www.its.caltech.edu/~mirsky/endomes.htm (End-mes Gene Network Update) and www.its.caltech.edu/~mirsky/qpcr.htm (End-mes Network QPCR Data), respectively. Short horizontal lines from which bent arrows extend represent* cis*-regulatory elements responsible for expression of the genes named beneath the line. The arrows and barred lines indicate the inferred normal function of the input (activation or repression), as deduced from changes in transcript levels due to the perturbations. Each input arrow constitutes a prediction of specific transcription factor target site sequence(s) in the* cis*-regulatory control element. Dotted lines indicate inferred but indirect relationships. Arrows inserted in arrow tails indicate intercellular signaling interactions. Large open ovals represent cytoplasmic biochemical interactions at the protein level. The spatial domains are color coded as in Fig. 1, and genes are placed therein according to their loci of expression. The interactions at the top of the diagram, with no background color, are very early interactions. The rectangles in the lower tier of the diagram show downstream differentiation genes. "Ubiq" indicates an inferred ubiquitously active positive input. Abbreviations: Mat cβ, maternal cytoplasmic β-catenin; nβ/TCF, nuclear β-catenin complexed with TCF. For further details see Davidson* et al. *(2002a, 2002b) and www.its.caltech./~mirsky/endomes.htm. For evidence see text, Davidson* et al. *(2002a, 2002b), Oliveri* et al. *(2002), Ransick* et al., *(2002), Rast* et al., *(2002), www.its.caltech./~mirsky/endomes.htm.*

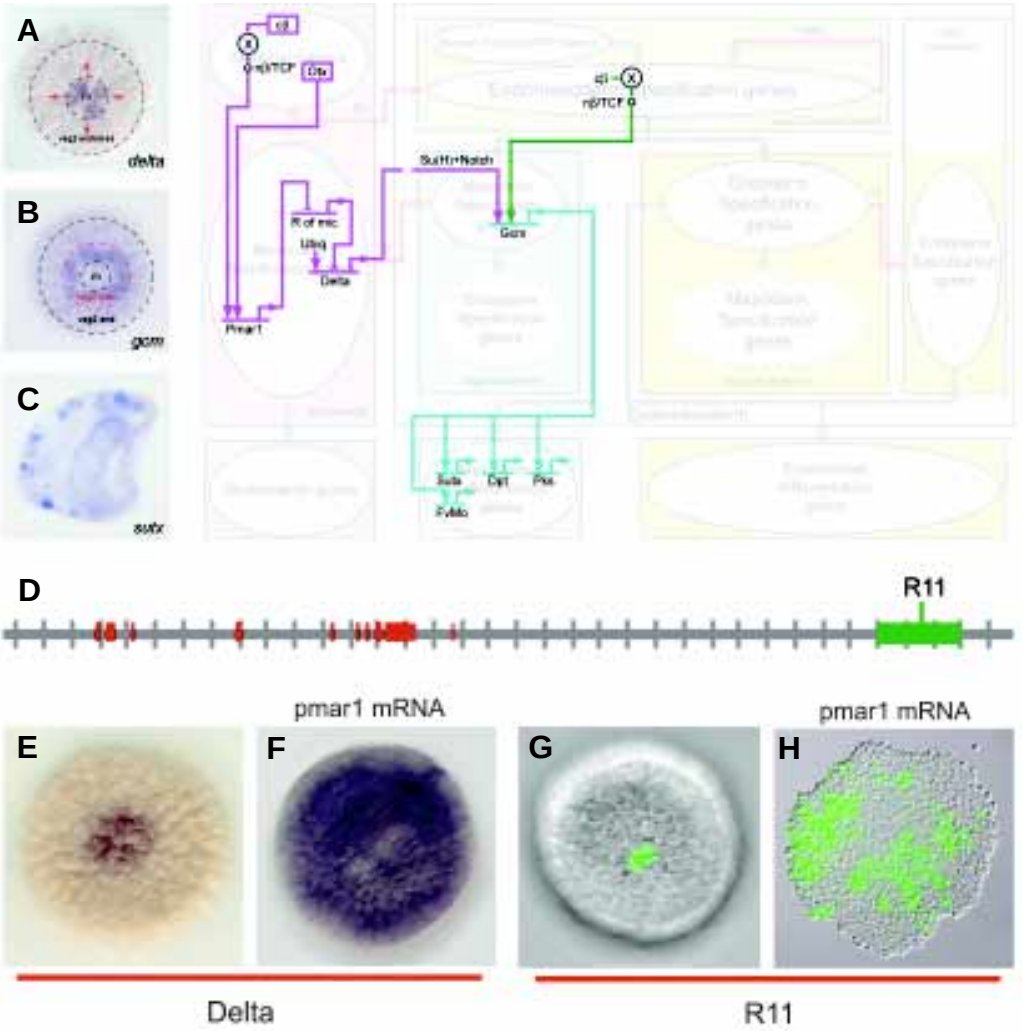

Fig. 3. Segregation of the veg2 domain into mesodermal and endodermal territories and installation of the pigment cell differentiation program. *The diagram shows key interactions, extracted from the model of Fig. 2, that control the segregation of the veg2 domain and the installation of the pigment cell differentiation program. The dimmed background shows the process diagram of Fig. 1E to indicate the domains where the interactions shown happen, and the developmental functions that the genes shown execute.* **(A)** *Between 7th and 9th cleavage the micromeres express the signaling ligand Delta (Oliveri* et al., *2002; Sweet* et al., *2002). The figure shows a whole mount* in situ *hybridization photomicrograph, from P. Oliveri, displaying the expression of delta gene 12 h after fertilization (around 8th cleavage). "m" indicates micromeres domain. Red arrows indicate the signaling event from the micromeres to the surrounding veg2 endomesodermal cells.* **(B)** *The veg2 endomesodermal cells that receive the Delta signal from the micromeres become specified as mesoderm, and express the gene* gcm*; the rest of the veg2 endomesodermal cells become specified as endoderm (Sherwood and McClay, 1999; Sweet* et al., *1999; McClay* et al., *2000; Ransick* et al., *2002; Sweet* et al., *2002). The figure shows a whole mount* in situ *hybridization photomicrograph, modified from Ransick* et al. *(2002), displaying the expression of* gcm *gene 12 h after fertilization (around 8th cleavage). The red dotted circle indicates the newly formed border that segregates the veg2 domain into mesodermal and endodermal territories.* **(C)** *Ultimately, a subset of the veg2 mesodermal cells differentiate into pigment cells, and express the gene* sutx *(Calestani* et al., *2003), among other pigment cell differentiation genes. The figure shows a whole mount* in situ *hybridization photomicrograph, modified from Calestani* et al. *(2003), displaying the expression of* sutx *gene in a gastrula stage embryo.* **(D-H)** *The* cis*-regulatory element R11 controls the localization of* delta *gene expression in the micromeres.* **(D)** *The R11 element consists of a sequence of genomic DNA near the coding sequence of the Delta gene. Each tic on the horizontal grey line representing genomic sequence demarcates 1 kb from the previous tic. 5' direction is to the left. Red blocks on the sequence indicate positions of the* delta *gene coding sequence. The green box on the sequence indicates the position of the R11 element.* **(E-F)** pmar1 *mRNA injection results in* delta *expression everywhere in the embryo. The figures show whole mount* in situ *hybridization photomicrographs, modified from Oliveri* et al. *(2002), comparing the expression of* delta *gene in normal blastula stage embryos* **(E)**, *and embryos that have been injected with* pmar1 *mRNA* **(F)**. **(G,H)** *The R11 element is responsible for localizing the expression of* delta *gene in the micromeres of normal embryos, and for driving the expression of the gene in every cell of embryos that have been injected with* pmar1 *mRNA (R. Revilla-i-Domingo and E. Davidson, unpublished data). The photomicrographs compare the expression of the GFP reporter gene in blastula stage embryos that have been injected with R11 reporter construct* **(G)**, *and embryos that have been injected with* pmar1 *mRNA in addition to the R11 reporter construct* **(H)**.

specification of mesodermal fate in these cells (Sweet *et al.*, 1999; McClay *et al.*, 2000; Sweet *et al.*, 2002). Localization of the Delta signal in the micromere descendants depends on the operation of the *pmar1* repression system, as explained above and illustrated in the diagram of Fig. 3. The response of Delta to the *pmar1* repression system depends on the *cis*-regulatory element named R11 (Fig. 3 D-H) (R. Revilla-i-Domingo and E. Davidson, unpublished data). In normal embryos R11 drives expression of a reporter construct in the micromere descendants. When '*r of mic*' is repressed everywhere in the embryo by ectopic expression of *pmar1*, the *delta* gene is activated in every cell (Fig. 3 E,F), and in the same embryos R11 also drives expression of the reporter construct everywhere (Fig. 3 G,H) (R. Revilla-i-Domingo and E. Davidson, unpublished data).

Expression of the *gcm* gene begins in the single ring of mesoderm progenitor cells that directly receive the Delta micromere signal (Fig. 3B). As shown in the diagram of Fig. 3, activation of this gene depends on inputs from both the Notch signal transduction pathway, activated by the Delta signal, and (directly or indirectly) the nuclear β-catenin/TCF system (see diagram of Fig. 3), which is active in the whole of veg2 (Davidson *et al.*, 2002a and A. Wikramanayake, unpublished data). The expression of *gcm*, therefore, reflects the creation of the new mesoderm-endoderm border, which did not exist

before the Delta signal was received from the micromeres. The *cis*-regulatory element of *gcm* is responsible for integrating the spatial information provided by the inputs from the Notch transduction pathway, and the β-catenin/TCF system. In normal embryos this element drives the expression of a reporter construct in a localized region in the vegetal plate. But if a portion of this element, containing binding sites for the Notch transduction pathway, is eliminated, expression of the reporter construct is expanded to a broader region that includes the whole of the veg2 domain (A. Ransick and E. Davidson, unpublished data). In other words, now the *cis*-regulatory element that controls *gcm* expression is 'blind' to the mesoderm-endoderm border established by the activation of the Notch transduction pathway.

Ultimately, the gene *gcm* is expressed in the pigment cells (a prominent subset of the veg2 mesodermal cell types), where it activates a number of differentiation genes (see diagram of Fig. 3), the products of some of which are likely to be required for synthesis of the red quinone pigment that these cells produce (Davidson *et al.*, 2002b; Ransick *et al.*, 2002; Calestani *et al.*, 2003). If translation of *gcm* transcripts is blocked experimentally, the perturbed embryos show a perfectly normal morphology, except that they have no pigment cells (A. Ransick and E. Davidson, unpublished data).

The portion of the network depicted in Fig. 3 is a piece of the genetic program encoded in the *cis*-regulatory genomic sequence. It consists of a transcriptional apparatus, including R11 element, that localizes the Delta signal, and another transcriptional apparatus, including the Notch responsive element of the *gcm* gene, that interprets the signal. It explains why the cells in the inner ring of the veg2, and no others, give rise to pigment cells. And it also explains why elimination of expression of a single player in the program, *gcm*, results in the absence of the pigment cells. The overall function of this portion of the network is, first, to create a new domain of specification in the embryo (the veg2 mesoderm), by setting a new border in the specification pattern; and then to install the program for pigment cell differentiation in the cells of the new domain. Other similar network subelements not yet resolved are undoubtedly responsible for differentiation of additional mesodermal cell types.

Stabilizing states of specification: the endoderm

Figure 4 illustrates the process by which the veg2 endoderm is specified. The veg2 lineage is born at 6th cleavage. By this time, the two spatial cues that trigger the specification of veg2 as endomesoderm are already operating. These initial cues consist of the autonomous nuclearization of β-catenin, which is a cofactor of the Tcf transcription regulator required for Tcf to function as a gene activator, and the early micromere signal (Ransick and Davidson, 1993, 1995; Logan *et al.*, 1999). Two regulatory subcircuits execute the process by which the zygotic transcriptional apparatus interprets these initial cues, and by which it establishes an endomesodermal state of specification (Fig. 4A). The β-catenin/Tcf input activates the *krox* gene (Davidson *et al.*, 2002b). This gene stimulates expression of *wnt8* gene and one of the transcription units of the *otx* gene. Wnt8 is a ligand which activates the β-catenin/Tcf system, and is itself a target of the β-catenin/Tcf input. This implies an autoreinforcing Tcf control loop, which is set up within the endomesodermal domain once this is defined (Davidson *et al.*, 2002a). So, the result of the stimulation of *wnt8* expression, first by the β-catenin/Tcf system and later by *krox*, is to transfer control of the β-catenin/Tcf system from the autonomous cytoplasmic mechanism by which its activity was initiated to a zygotically controlled, intercellular signaling mechanism operating among the cells of the endomesoderm. The "community effect" (as defined by Gurdon, 1988; Gurdon *et al.*, 1993) established by this regulatory subcircuit (dark blue connections in Fig. 4A) takes the cells out of a condition of alternative transcriptional possibility that is

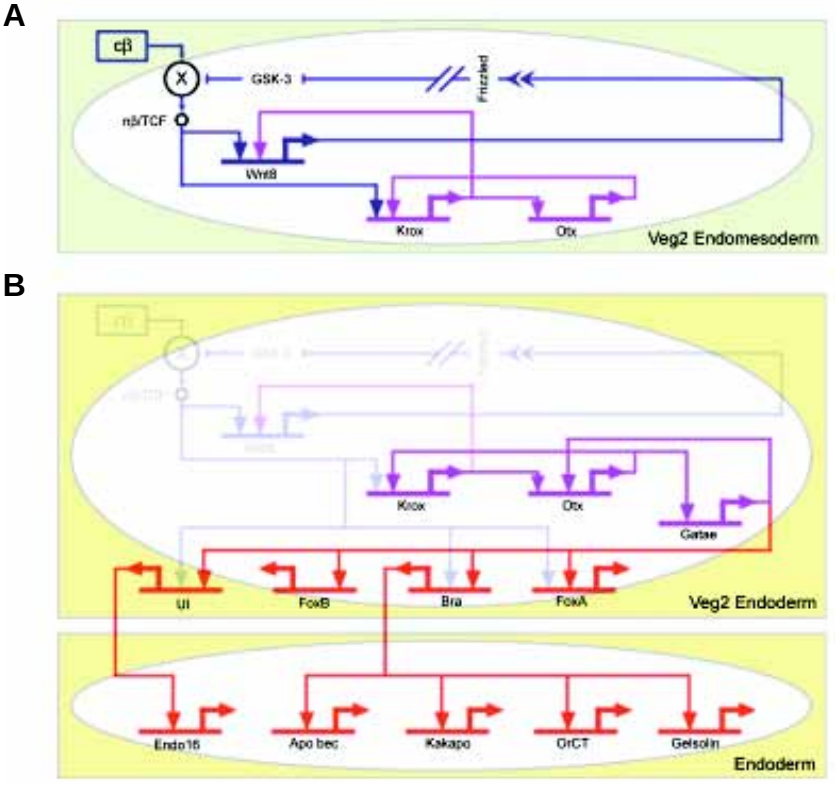

Fig. 4. Stabilization of the endomesoderm specification state and installation of the endoderm differentiation program. *The diagram shows key interactions, extracted from the model of Fig. 2, that control the stabilization of the endomesoderm state of specification and the installation of the endoderm differentiation program.* **(A)** *The box with green background shows the interactions that operate in the veg2 endomesoderm domain up to about 9th cleavage. Nuclearization of β-catenin is autonomous, and results in the activation of two regulatory subcircuits. Dark blue subcircuit: Wnt8 intercellular signaling among cells of the veg2 domain stimulates the nuclearization of β-catenin and establishes a "community effect," which defines and locks the endomesodermal state of specification in the veg2 cells. Purple subcircuit:* krox *and* otx *cross-regulate, which results in a reinforcing loop that renders the endomesoderm state of specification independent of the initial inputs.* **(B)** *The box labeled "Veg2 Endoderm" shows the interactions that operate in the veg2 endoderm domain, from about 9th cleavage to mesenchyme blastula stage. The* gatae *gene is added to the* krox-otx *feedback loop (purple interactions), and together with β-catenin/TCF system, installs the endoderm specification program (red interactions). When β-catenin/TCF/Wnt8 inputs disappear, the stabilization loop maintains the endodermal specification program active, which eventually results in the activation of endodermal differentiation genes (lower box in the diagram labeled "Endoderm").*

their initial condition, and locks them into a stable state of gene expression.

The *otx* gene stimulates expression of the *krox* gene. A regulatory subcircuit consisting of *otx* and *krox* cross-regulation produces a transcription-level stabilization of the endomesodermal regulatory state (purple connections in Fig. 4A; see Davidson *et al.*, 2002a). The *otx* gene also provides an input into the *gatae* gene, which in turn has an input back into *otx* gene. This is a further positive feedback that links the *gatae* gene into the stabilization circuitry (purple connections in Fig. 4B). The *gatae* gene plays an important role in endoderm specification (red connections in Fig. 4B), since, together with the β-catenin/Tcf system, it is responsible for the activation of many of the known endodermal regulators, including the *bra, foxA* and *ui* genes (Davidson *et al.*, 2002a and P.Y. Lee and E. Davidson, unpublished data). The FoxA transcription factor is a repressor that has multiple roles in the spatial control of gene expression patterns in the endoderm; Bra results in the activation of endodermal differentiation genes which are involved in cell motility and are needed for gastrulation and invagination to occur (Gross and McClay, 2001; Rast *et al.*, 2002); the UI factor directly controls expression of *endo-16* (Yuh *et al.*, 2001), which encodes a differentiation protein that is secreted in the lumen of the midgut. The crucial role that *gatae* plays in the specification of the endoderm explains the phenotype shown by embryos in which translation of the *gatae* transcripts has been blocked. This treatment produces a severe interference with endoderm specification and gut development (P.Y. Lee and E. Davidson, unpublished data).

During the late blastula stage, β-catenin disappears from the nuclei of the veg2 endodermal domain (Logan *et al.*, 1999). But by this time, a network of stable intergenic interactions has been installed, so that the β-catenin inputs used earlier to set up transcriptional specification are no longer needed (Fig. 4B).

We see here that the *cis*-regulatory interactions control the operation of at least three different regulatory devices that are directly responsible for establishing at least part of the endoderm differentiation program. The first device consists of the "community effect," which first defines and then locks on the endomesodermal specification state in the veg2 domain (dark blue connections in Fig. 4A). The second device depends on a feedback loop, including *krox* and *otx* (purple connections in Fig. 4A), which generates a robust and resilient regulatory structure in the already defined endomesoderm domain. The third device consists of the addition of *gatae* to the *krox-otx* feedback loop (purple connections in Fig. 4B), which ensures the operation of many endodermal regulatory genes in the endoderm. The result is a control system that drives the specification process forward as a progression of states, and it prevents it from reversing direction when the initial cues that trigger the specification process disappear. Progressivity and stability are fundamental properties of the developmental process. They derive from regulatory devices consisting of assemblages of *cis*-regulatory interactions.

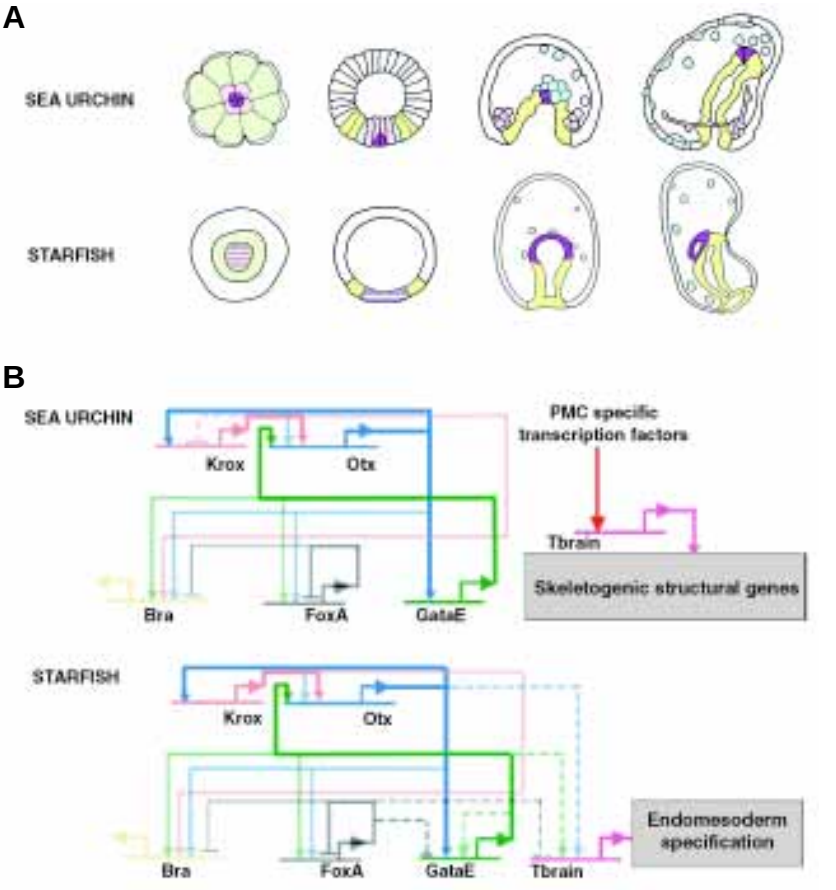

Fig. 5. Comparison of sea urchin and starfish gene regulatory networks. *The figure compares portions of the gene regulatory networks underlying the specification of the endomesoderm in the sea urchin and the starfish embryos.* **(A)** *Comparison of the fate maps. Schematic diagrams of sea urchin embryos (top row) and starfish embryos [lower row, modified from Hinman* et al. *(2003)] at selected stages. Stages are (from left to right): cleavage/early blastula stage; blastula stage; gastrula stage; and early larval stage. Color coding indicates the fate of domains of cells through development: lavender indicates cells that will become skeletogenic; green indicates cells that will contribute to mesoderm and endoderm; blue indicates cells that will become mesodermal; purple indicates cells of the mesoderm that specifically will become coelomic cells; purple stripes indicate domains that might contain a subset of cells that will contribute exclusively to coelomic cells; yellow indicates cells that will become endodermal.* **(B)** *Comparison of portions of the underlying gene regulatory networks. The top diagram, corresponding to the sea urchin, is extracted from Fig. 2. The bottom diagram, corresponding to the starfish, is from Hinman* et al. *(2003). Regulatory connections are represented as described in Fig. 2. In this figure dashed lines indicate a regulatory connection observed in sea urchin not present in starfish, or* vice versa. *The positive feedback loops between* krox, otx *and* gatae *that are present in both echinoderms are highlighted in bold.*

Understanding development and evolution

Developmental and evolutionary processes both have their root in the heritable genomic regulatory programs that determine how the body plan of each species is built (Davidson, 2001). It has been clear for a long time that the evolution of body plans has occurred by change in the genomic programs for the development of these body plans (Britten and Davidson, 1971), and it is now clear that we need to consider this in terms of change in the regulatory devices that execute these programs. The bilaterians all rely on essentially the same repertoire of regulatory genes to

control the developmental organization of their body plans. Analysis of *cis*-regulatory networks affords the means to focus on the significance of preserved uses of these genes, and on the exact consequences of differences in their use (Davidson, 2001).

Figure 5 compares the way certain genes are utilized in the specification of the endomesoderm of two different bilaterians, namely, the sea urchin and the starfish. All genes in Fig. 5, except for *tbr*, are central elements that control the specification of the endoderm in the sea urchin (see Figs. 2 and 4). The *tbr* gene, on the other hand, is activated exclusively in the micromere derived skeletogenic cells (see Fig. 2) (Croce *et al.*, 2001; Fuchikami *et al.*, 2002; Oliveri *et al.*, 2002). Its regulation depends on other genes specifically expressed in the micromere lineage (Oliveri *et al.*, 2002), and in turn, it drives expression of larval skeletogenic differentiation genes (Davidson *et al.*, 2002a; Oliveri *et al.*, 2002 and www.its.caltech.edu/~mirsky/endomes.htm). While the formation of the endoderm is at least superficially similar in the two species (Fig. 5A), starfish embryos do not have a micromere lineage, nor do they produce a larval skeleton (Fig. 5A).

Figure 5B shows that the *cis*-regulatory interactions that constitute the endodermal three-gene stabilizing loop in the sea urchin (see Fig. 4B), is found in identical form in the starfish (connections in bold in Fig. 5B) (Hinman *et al.*, 2003). This set of identical *cis*-regulatory interactions must serve conserved evolutionary roles, since the possibility of convergence is ruled out by the number of similar functional starfish and sea urchin *cis*-regulatory interactions.

Sea urchins and starfish have diverged for at least 500 million years (Sprinkle and Kier, 1987; Smith, 1988; Bowring and Erwin, 1998). The reinforcing loop is therefore a regulatory device that was invented at least about 500 million years ago, and that has been conserved in at least two independently evolving lineages during all this time. 500 million years represents a very long genomic divergence, in the sense that comparisons of starfish and sea urchin DNA sequences around orthologous regions do not show any conservation distinguishable from random occurrence between the *cis*-regulatory elements, even when the genes are similarly regulated (V. Hinman and E. Davidson, unpublished data). The preservation of this regulatory device suggests that the function it serves in the specification process must be essential. As we have already seen, in the sea urchin the regulatory feedback loop between *krox* and *otx* genes generates a robust regulatory structure in the endomesoderm domain, and the addition of the *gatae* gene to this feedback loop ensures and maintains the operation of many endodermal regulatory genes after the initial transient inputs have disappeared (Davidson *et al.*, 2002a and P.Y. Lee and E. Davidson, unpublished data). In the starfish, *gatae* also drives the expression of many endodermal regulatory genes (Hinman *et al.*, 2003), and in many other bilaterians, members of the Gata family of transcription regulatory genes are required for gut development (Reuter, 1994; Maduro *et al.*, 2002; Patient and McGhee, 2002). What makes the reinforcing loop especially useful, and hence likely to be preserved during evolution, may therefore be that it controls the installation and stabilization of the expression of the *gatae* gene in the endoderm (Hinman *et al.*, 2003). Other intergenic feedback loops are used across the Bilateria to serve similar functions. For example a reinforcing feedback loop is found in the *hox* gene network that controls rhombomere specification in the mouse hindbrain (Nonchev *et al.*, 1996; Barrow *et al.*, 2000), in the regulatory network for tracheal placode specification in *Drosophila* (Zelzer and Shilo, 2000), and in the specification of the oral ectoderm in sea urchin embryos (Amore *et al.*, 2003), among others. It seems a general property of the developmental process to use feedback loops as a mechanism to achieve the progressivity of the process.

The *tbr* gene, on the other hand, is used in completely different ways in the starfish and sea urchin embryos (Fig. 5B). It is required for the formation of the archenteron in the starfish embryo, and its expression is under the control of endodermal regulators (Otx, Gatae) (Hinman *et al.*, 2003), whereas it is involved solely in skeletogenic functions in the sea urchin embryo (Croce *et al.*, 2001; Oliveri *et al.*, 2002 and www.its.caltech.edu/~mirsky/endomes.htm). The skeletogenic micromere lineage is a relatively recent echinoid invention (Wray and McClay, 1988; Tagawa *et al.*, 2000). This suggests that in the sea urchin the skeletogenic use of *tbr* may have been coopted from an adult skeletogenic regulatory system, while an original embryonic endomesodermal regulatory element was lost (Hinman *et al.*, 2003).

If indeed the larval skeletogenic lineage is the result of a cooption from the adult skeletogenic regulatory system, it represents an example of how a regulatory subroutine can be "wired" into the specification system as the result of evolutionary change. How the intrinsic behavior of the subroutine is preserved in the new context, and how the rest of the developmental control system can cope with this change without disrupting its workability, speaks directly to the intrinsic robustness of the subroutine, and the robustness of the developmental process in general. Regulatory networks serve as the link between development and evolution. They provide a new means to address specific questions about the robustness of the developmental process, and about the preservation of aspects of the process through evolutionary time. Questions such as these can only be answered by considering evolution and development together.

Conclusions

Gene network analysis identifies the mechanisms that control and operate the program for the developmental process. This will be true for all aspects of the developmental process that are required to generate the species-specific body plan. To address some of the general and fundamental questions about the process of development, though, will require understanding evolution. Because gene regulatory networks underlie the processes of both development and evolution, unraveling their architecture in appropriately chosen species will be the key to understanding how genomes control development and how they evolve.

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