Synthetic in vivo validation of gene network circuitry

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Embryonic development is controlled by networks of interacting regulatory genes. The individual linkages of gene regulatory networks (GRNs) are customarily validated by functional *cis*-regulatory analysis, but an additional approach to validation is to rewire GRN circuitry to test experimentally predictions derived from network structure. Here we use this synthetic method to challenge specific predictions of the sea urchin embryo endomesoderm GRN. Expression vectors generated by in vitro recombination of exogenous sequences into BACs were used to cause elements of a nonskeletogenic mesoderm GRN to be deployed in skeletogenic cells and to detect their effects. The result of reengineering the regulatory circuitry in this way was to divert the developmental program of these cells from skeletogenesis to pigment cell formation, confirming a direct prediction of the GRN. In addition, the experiment revealed previously undetected cryptic repression functions.

reengineering development | *Strongylocentrotus purpuratus*

The sea urchin embryo gene regulatory network (GRN) provides a comprehensive causal framework for understanding the spatial regulatory functions leading to mesodermal and endodermal specification up to gastrulation (1–3). Portions of this network are relatively complete, in the sense that it appears to include most of the regulatory genes and the functional linkages among them that are required to explain the specification process. For these portions, the GRN affords predictions of the outcome that would be expected if the linkages in the network were deliberately altered. The sea urchin model system offers the opportunity of carrying out experimental tests of such predictions, as we report here.

In this embryo, two mesodermal lineages arise in cleavage. The sole fate of the first of these, the descendant from the skeletogenic micromeres (SM), is to produce the embryonic skeleton, although it also has the very important function of signaling to adjacent cells. The adjacent nonskeletogenic mesoderm territory is specified in response to a Delta signal from the skeletogenic lineage (4, 5). In normal development, the nonskeletogenic mesoderm gives rise to several differentiated cell types, one of which is the pigment cell type. According to the GRN shown in Fig. 1*B*, pigment cell specification is initiated by transcriptional activation of the regulatory gene *gcm* in direct response to Delta/Notch signaling (6). If our knowledge of the pigment cell specification network is complete, then there should be no other direct Notch targets required for pigment cell specification in addition to *gcm*, as shown in the GRN, and no additional transcriptional regulatory inputs upstream of *gcm* would be required for pigment cell fate other than those shown. A direct test of this is as follows: if we were to short-circuit the Notch input to *gcm* and instead place *gcm* transcription under direct control of a *cis*-regulatory system activated only in the cells of the skeletogenic lineage, then the predicted result would be the transformation of skeletogenic cells into pigment cells, or at least the expression of pigment cell genes in cells otherwise destined to become skeletogenic. By using recombineered BAC vectors (7) to rewire the GRN, we have carried out a test of this direct prediction. Of course, this project also challenges our understanding of the skeletogenic regulatory system, requiring that an effective strategy be devised to redeploy *gcm* expression to cells of the skeletogenic lineage. In the event, the results that we obtained prove the sufficiency of the portion of the GRN shown in Fig. 1 *A* and *B*. However, in addition, we learned something unexpected: the GRN was enriched by the discovery of a previously cryptic repressive linkage that had the interesting effect of sharpening the cell fate transformation.

Results

GRNs for Skeletogenic and Nonskeletogenic Mesoderm Specification. The relevant portions of the specification GRNs are shown in Fig. 1 *A* and *B* (an always current version of the complete endomesoderm GRN is publicly available at http://sugp.caltech.edu/endomes/). The essential features are as follows: (*i*) In the skeletogenic micromere domain (Fig. 1*A*), the GRN is activated by means of a double-negative logic gate, in which the *pmar1* gene, encoding a repressor, is activated by known localized inputs of maternal origin in the skeletogenic lineage founder cells (1, 2). *pmar1* transcription in turn precludes transcription of a second repressor that is encoded by the *hesC* gene. This gene is activated globally after *pmar1* is activated, except in the skeletogenic lineage where it is prevented from functioning by the Pmar1 repressor (8). The downstream target genes of the double-negative gate, which are activated exclusively in the skeletogenic lineage while being specifically repressed elsewhere, include *alx1*, *tbrain* (*tbr*), and *delta*, and the wiring connecting them to the double-negative gate in each case has been validated at the *cis*-regulatory level (8–11). Another double-negative-gate target gene, *ets1/2*, provides positive inputs into all three of these downstream targets, which all together generate the initial skeletogenic regulatory state. (*ii*) Downstream of these immediate target genes is a dynamic triple feedback loop linking the *hex*, *tgif*, and *erg* genes and activated by the double-negative-gate target genes *ets1/2* and *tbr*. This subcircuit determines the skeletogenic regulatory state, rendering it independent of the transient expression of *pmar1*. *erg* is transcribed first, and it contributes to activation of *hex* and *tgif*. These three genes cross-activate one another, constituting a positive feedback system, and, in a latch-like linkage, *tgif* also feeds back to the double-negative-gate target gene *alx1* (see wiring in Fig. 1*A*). The final tier of regulators of skeletogenesis, *foxO*, *foxB*, and *deadringer*, are activated by the inputs that have become available. Fig. 1*A* includes all of the feeds into the early activated effector genes of the skeletogenic differentiation gene batteries (1). (*iii*) The double-negative-gate target gene *delta* produces the signaling ligand received by the Notch receptor on the adjacent mesodermal precursor cells (Fig. 1*B*). In the pigment cell pathway activated by Delta reception in these cells, the direct *cis*-regulatory target of the activated Su(H), which results from Notch signal transduction, is the *gcm* gene (6). (*iv*) A small subnetwork downstream of *gcm* includes the *gatae* gene and the *six1/2* gene, which feeds back on *gcm*, as well as on an auto-regulatory feed from *gcm* onto itself (Fig. 1*B*) (12). The Gatae and Gcm regulators then directly activate pigment cell differentiation genes such as *polyketide synthase* (*pks*) (13). Thus, *gcm* is both a pigment cell specification gene and a driver of a pigment synthesis gene battery (14). Both Delta/Notch signaling and Gcm translation are absolutely required for pigment cell specification (6). The *gcm* gene was the primary focus of our rewiring strategy.

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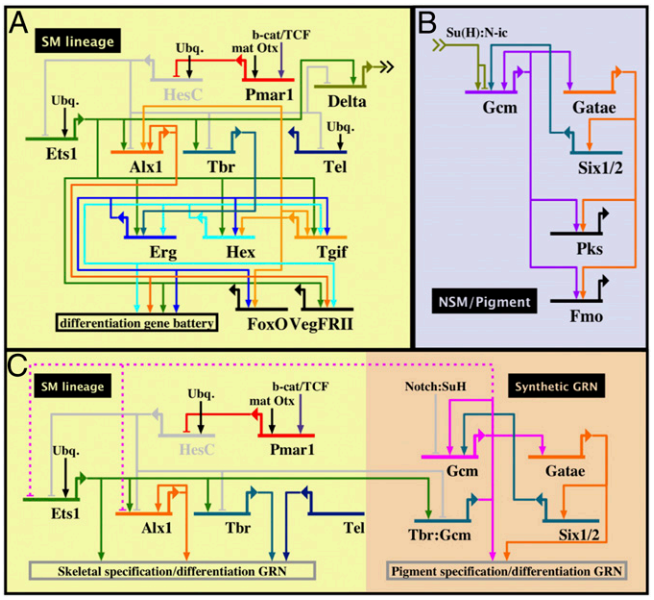

Fig. 1. Relevant portions of the GRNs for skeletogenic and nonskeletogenic mesoderm specification and differentiation in the sea urchin embryo. (*A*) Skeletogenic specification, as described in text. Output linkages for the genes *tbr* and *tel* are not shown as their targets are off the map. (*B*) Notch-signaling circuitry leading to activation of *gcm* and pigment cell differentiation. (*C*) Diagram of rewired skeletogenic specification network. Gcm expression is brought under control of the double-negative regulatory gate and is expressed in precursors to the skeletogenic mesenchyme. Because the endogenous *gcm* gene autoactivates, once expressed in skeletogenic cells, the Gcm factor may contribute to maintenance of expression of this factor. Because Gcm is a terminal regulator of the pigment cell differentiation battery, it would be expected to activate this subnetwork in cells originally destined to be skeletogenic. The pink dotted repression bars on Alx1 and Ets1 indicate the possibility, as discussed later in this paper in *Cryptic Exclusion of Skeletogenic Regulatory State*, of cross-repressive functions downstream of *gcm* expression that would lower expression of the skeletogenic regulatory state. A full diagram of the endomesodermal gene regulatory network can be found at http://sugp.caltech.edu/endomes/#EndoNetworkDiagrams.

Reengineering Mesodermal Regulatory Apparatus: Experimental Approach. A previously studied skeletogenic *cis*-regulatory system (10), which controls expression of the double-negative-gate target gene *tbr*, was selected for use as an in vivo driver of *gcm* expression in the skeletogenic cell lineage. This places *gcm* expression immediately downstream of the double-negative-gate circuitry (Fig. 1*C*). Normal zygotic *tbr* expression begins in the skeletogenic cell lineage quite early, just after the sixth cleavage with other double-negative-gate target genes [~8 h post fertilization (hpf)], and *tbr* expression is absolutely required for skeletogenesis to occur (1). It was important to choose as a regulatory driver a gene activated upstream of the triple feedback circuit in the skeletogenic GRN, so that it would initiate *gcm* expression before all of the subsequent skeletogenic regulatory apparatus had been brought into play. Additional advantages offered by the *tbr cis*-regulatory system are that it produces relatively high levels of expression (~2,000 molecules mRNA/embryo, or 250 mRNAs/skeletogenic cell at 20 hpf) and that at no time in embryogenesis is *tbr* expressed anywhere but in the skeletogenic lineage (10, 15) (Fig. S1*A*). The entire rewired circuit is shown in Fig. 1*C*.

In addition to the programmed imposition of *gcm* expression in early skeletogenic cell development, the rewired circuit accomplishes several other changes of regulatory significance. First, it removes *gcm* expression in the cells originally destined to be skeletogenic from any dependence on Delta/Notch signaling (Fig. 1*C*). The need for the activated Su(H) normally required for initiation of *gcm* expression (as in Fig. 1*B*) is short-circuited. However, the repressive function normally exerted on the endogenous *gcm* gene by Su(H) in the absence of the Delta ligand (6), including in skeletogenic cells, is also avoided; both the positive and the negative effects of Su(H) are mediated by known *gcm cis*-regulatory target sites (6), which are of course absent from the re-engineered construct. Second, the skeletogenic regulatory gene *alx1* also represses *gcm* expression in the skeletogenic cells (1, 16), but because this function also requires the *gcm cis*-regulatory system, the ectopic expression system that we have introduced escapes that level of control as well.

Recombinant BACs Used for Synthetic Rewiring. The starting point for these experiments was a recombineered *tbr*:GFP BAC, the expression of which had been shown to recreate the authentic endogenous skeletogenic expression pattern (10), which is illustrated in Fig. S1*A*. The *tbr*:GFP BAC expressed with excellent spatial accuracy and exclusively in skeletogenic arrays in 86 and 89% of embryos at 48 and 72 hpf, respectively (Table 1). There was virtually no ectopic expression of this BAC construct in pigment cells, and no endoderm or ectoderm expression; the ectopic expression noted in Table 1 was confined to detached fluorescent cells in the blastocoel, which are an occasional artifact of injection in sea urchin embryos. Injected constructs incorporate in a stable but randomly mosaic fashion in sea urchin embryo nuclei during the first few cleavage cycles (17), and thus, initially, *tbr*:GFP reporter expression is expressed in only a fraction of skeletogenic cells (Fig. S1*A*, *A1*). After the onset of gastrulation, the skeletogenic cells fuse to form a syncytium, whereupon GFP protein distributes evenly to all of these cells (Fig. S1*A*, *A2* and *A3*). The *tbr*:GFP BAC also expressed quantitatively over time, as did the endogenous gene (Fig. S1*B*), and it was activated at the same time at ~8–10 h. To construct a vector that would express *gcm* under control of the complete *tbr* regulatory system, the *gcm*-coding sequence and a functional 3′-UTR were inserted into the parental *tbr* BAC just before the start codon in the first exon of the *tbr* gene, i.e., in the same site as the GFP sequence in *tbr*:GFP BAC (Fig. 2*A*). As detectors of change in regulatory state in individual cells, other reporter BACs in addition to *tbr*:GFP BAC (10) were also made by in vitro recombination, namely *alx1*:GFP BAC and *ets1*:GFP BAC, and several small constructs expressing GFP or Red Fluorescent Protein (RFP) were built as well (Fig. 2). A key aspect of the sea urchin gene transfer system, which we exploited in these experiments, is that coinjected constructs concatenate together and are invariably incorporated into the same cells (17), so that reporters can be used to identify all transgenic cells.

Fate Transformation Effects of Reengineered *gcm* Expression in Skeletogenic Cells. Coinjection of the *tbr*:GCM BAC with the *tbr*:GFP BAC detector showed that cells carrying the transgenes were diverted from skeletogenic to pigment-cell fate with remarkable efficiency (Table 1). Thus, forced *gcm* expression causes skeletogenic cells to fail to participate in skeletogenic syncytium formation. These cells fall into two classes. In the first class, the more completely transformed cells migrate into the aboral ectoderm and embed themselves there exactly as the native pigment cells do; in this experiment, the result was observed in 10 and 58% of fluorescing embryos at 48 and 72, respectively. As Table 1 shows, in the absence of *tbr*:GCM BAC expression, this behavior almost never occurs. The second class represents cells that are incompletely transformed at the times of observation. Their skeletogenic functions are evidently impaired, so they are unable to join in syncytium formation as the bona fide skeletogenic cells do; nor do they proceed to the normal pigment cell destination, the aboral ectoderm, and so they remain in the blastocoel, expressing GFP (and GCM). At 48 h this class was observed in 54% of fluorescing embryos, compared with the 4% of embryos displaying injection background, but by 72 h this class was observed in only 17% of fluorescing embryos, suggesting that with time some of the cells of this class move into the more completely

Table 1. Expression of *tbr*::GFP and *tbr*::GCM constructs at mid/late gastrula stage (48 and 72 hpf)

Injected constructs	hpf	Total expressing (%)	Total scored	SM lineage* (%)	SM + pigment lineage† (%)	SM lineage + blastocoelar expression (%)	Blastocoelar‡ expression (%)	Pigment lineage (%)	Ectoderm or endoderm (%)
tbr:GFP BAC	48	63	329	84	0	0	4	4	7
	72	28	101	89	0	0	7	4	0
tbr:GCM BAC + *tbr*:GFP BAC	48	53	179	32	7	14	40	3	4
	72	32	76	21	4	0	17	54	4

Three categories of GFP expression patterns were scored. Skeletogenic cell expression includes complete expression in the ring of fused skeletal mesenchyme. Blastocoel expression includes any morphologically round fluorescent cells seen in the blastocoel. Pigment/aboral ectoderm includes any cells expressing in the aboral ectoderm (some of which express pigment), as well as any pigmented and unpigmented cells abutting the aboral ectoderm in the blastocoel.

*SM lineage includes complete expression in the ring of fused skeletal mesenchyme at 48 hpf and in the skeletal cells at 72 hpf.

†Pigment lineage includes any cells expressing in the aboral ectoderm (some of which express pigment), as well as any pigmented and unpigmented cells abutting the aboral ectoderm in the blastocoel.

‡Blastocoelar expression includes any morphologically round fluorescent cells seen in the blastocoel.

transformed category. Concomitantly, only 32 and 21% of embryos display only normal syncytial skeletogenic chains, which express *tbr*:GFP at 48 and 72 h, compared with 84 and 89% of expressing embryos in the absence of *tbr*:GCM BAC (Table 1).

A majority of both the more completely and the incompletely transformed cells, whether embedded in the aboral ectoderm or remaining in the blastocoel, have produced pigment granules by late embryogenesis (72 hpf). This is an indication that these cells are expressing the terminal pigment differentiation gene battery. Fluorescent cells expressing *tbr*:GFP BAC plus *tbr*:GCM BAC in late embryos, and also bearing prominent pigment granules, are illustrated in Fig. 3*A*. However, the variable extent of developmental alteration seen in these respecified skeletogenic cells reveals that activation of pigment-generating differentiation genes is not the only molecular change required for total transformation. The timing and character of their ingression, their loss of ability to form skeletogenic syncytia, and their ability to embed themselves singly in the aboral ectoderm, together indicate alterations in a suite of signal responses and other cell biological properties. The following experiments were designed to provide additional details of the sequence of events in this synthetic transformation and to explore the extent to which the regulatory state of the affected cells has been altered.

To determine how early the fates of originally skeletogenic cells are diverted toward pigment cell fate, embryos were injected with *tbr*:GCM BAC, and two-color whole-mount in situ hybridization (WMISH) was used to detect expression of endogenous pigment differentiation genes that had been up-regulated (Fig. 3*B*). Cells that are wholly or partially skeletogenic in function can be identified at mesenchyme blastula stage (24 h) by their ingressed positions within the blastocoel. In control 24-h embryos, endogenous skeletogenic genes such as *tbr* are expressed only in these ingressed cells, and pigment cell genes such as *gcm* are expressed only in cells still resident within the vegetal wall of the embryo (6, 10), as seen in, respectively, the first two panels of the first row of Fig. 3*B*. However, the second and third rows of panels show that, in embryos bearing *tbr*:GCM BAC, the synthetic *gcm* transcription occurs in cells (of skeletogenic origin) that fail to ingress, an indication of skeletogenic loss of function and pigment cell gain of function. Here the cells expressing the *tbr*:GCM BAC construct were identified by use of a probe that recognized the SV40 3′-UTR sequence of its transcript. Furthermore, the endogenous downstream chromogenic pigment cell genes *pks* and *fmo* (13) have already been up-regulated in the newly ingressed cells of some 24-h embryos, an indication of pigment cell gain of function (endogenous pigment cell precursors in the wall of the embryo also continue to express *pks* and *fmo*). Thus, the transformation of fate has begun as early as ~14 h after the initial activation of *gcm* in cells normally of skeletogenic lineage.

To explore further the occurrence of incompletely transformed phenotypes, even if temporary, a small *tbr*:GCM construct (Fig. 2) was coinjected together with *tbr*:RFP and *pks*:GFP detector constructs, and the embryos were examined at 48 h. As expected, in control embryos, these detector constructs express in nonoverlapping patterns in, respectively, skeletogenic and pigment cell lineages at all times in embryogenesis (Fig. S3 *A–F*). When coinjected with the *tbr*:GCM construct (Fig. S3 *G–L*), however, the embryos fell into two large classes (Table S1). About 67% of the embryos displaying construct activity contained some normal skeletogenic syncytia, which expressed RFP from the *tbr*:RFP construct, even though they were also expressing Gcm from the coincorporated *tbr*:GCM construct. In these embryos, the expression in the skeletogenic cells of *gcm* was evidently too weak to have affected their skeletogenic identity or to drive expression of the *pks*:GFP construct that they also contained. The skeletogenic

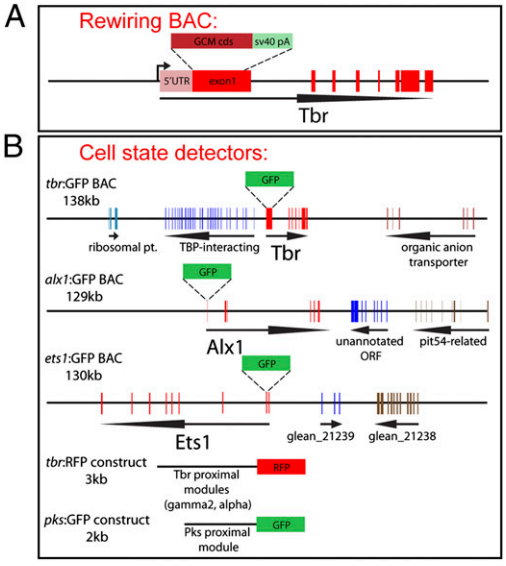

Fig. 2. Diagram of BAC constructs used in the synthetic rewiring experiment. (*A*) *gcm*-coding sequence was inserted using homologous recombination into the first exon of the *tbr* gene in a 140-kb BAC that contains the entire *tbr* regulatory architecture. (*B*) A similar knock-in strategy was used to generate BAC-GFP reporters that were used as detectors for measuring cell state. BAC-GFP constructs were made for the *tbr*, *alx1*, and *ets1/2* genes. Short-construct GFP reporters were made for detecting *tbr* and *pks* expression. These constructs faithfully recapitulate the spatial expression patterns of their corresponding endogenous genes.

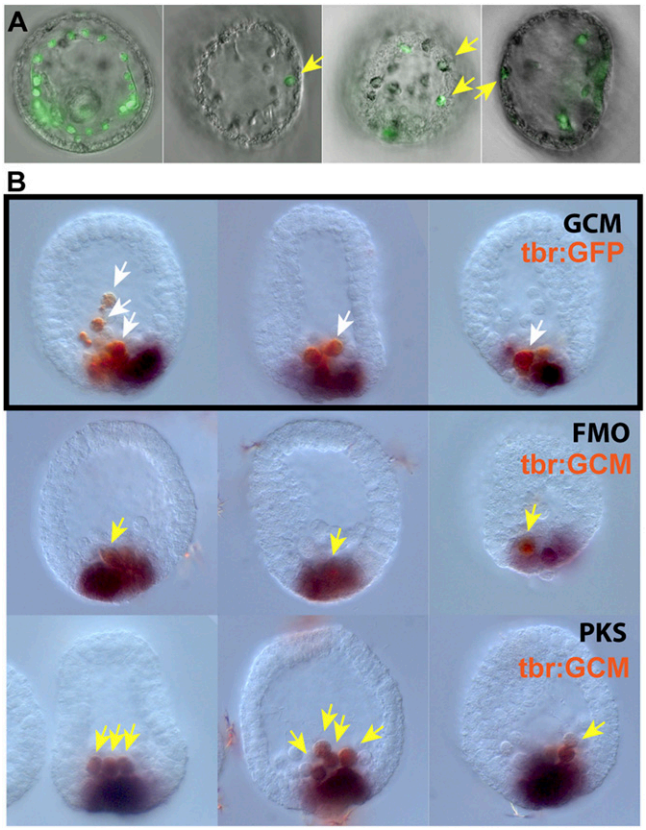

Fig. 3. Fate transformation in *gcm*-expressing SM cells. (*A*) Late respecification of *gcm*-expressing SM cells. Leftmost image is a control injection of Tbr:GFP BAC at the late gastrula stage. The remaining three images show *tbr*:GFP and *tbr*:GCM BAC coinjections at the late gastrula stage. Yellow arrows mark pigment granules in GFP-positive cells. (*B*) Two-color WMISH of embryos at mesenchyme blastula stage. Probes used for detection are indicated at the right of each row and are colored according to their stain. (*Top* row) As a control, *tbr*:GFP BAC-injected embryos were stained for *gfp* and *gcm* mRNA. (*Middle* and *Bottom* rows) *tbr*:GCM BAC-injected embryos were costained to detect the synthetically expressed *gcm* and either the pigment-cell–specific *fmo* or *pks* genes. *fmo* and *pks* are direct regulatory targets of *gcm*, and their expression overlaps perfectly with *gcm* expression (Fig. S2). A single probe matching the 3′-UTR SV40 polyadenylation sequence was used to detect the products of both *tbr*:GFP and *tbr*:GCM BACs. White arrows indicate wild-type SM cells expressing *tbr*:GFP, and yellow arrows indicate converted cells with coexpression of a BAC reporter and pigment-cell marker.

program may dominate once syncytia form because of the dilution of Gcm protein among the cojoined cells. However, the large majority of embryos either wholly transformed or became a variety of intermediately transformed cellular phenotypes (Fig. S3). At one extreme are functionally transformed cells embedded in the aboral ectoderm, which express *pks*:GFP and generate pigment granules, although they also continue to express *tbr*:RFP, as seen in 28% of embryos; other phenotypes seen together in 48% of embryos range from blastocoelar cells that are also functionally transformed, to syncytial, apparently skeletogenic cells that nonetheless express *pks*:GFP as well as *tbr*:RFP. These observations were made at 48 h, and very possibly the experiment would have appeared more dichotomous by 72 h, judging by the comparison at these times in Table 1. Different degrees of transformation were observed within the same embryo in many cases (Table S1), which precludes the simple possibility that the results reflect only the load of exogenous *tbr*:GCM because all transgenic cells in each embryo should contain replicates of the same initially incorporated concatenate (17).

As we have seen (Fig. 3*A* and Table 1), by late development, most cells expressing *gcm* under *tbr*:GCM BAC regulatory system control exclude themselves from syncytial skeletogenic formations, produce pigment granules, and array themselves singly either in the blastocoel or in the aboral ectoderm. However, at high magnification we noted that the morphology of these functionally transformed cells is different from that of bona fide pigment cells (Fig. S4). These cells lack the pseudopodia of normal pigment cells embedded in the ectodermal wall and retain more of a spherical shape despite, in some cases, their ectodermal intercalation and their prominent accumulations of pigment. Thus, even though these cells have become functionally differentiated as pigment cells, close observation shows that their cell biological transformation is not 100% complete.

Reengineering Reveals Cryptic Exclusion Functions. An implication of these results is that the forced expression of the pigment cell specification gene *gcm* results in repression of the skeletogenic regulatory state in the same cells, as well as institution of an ectopic pigment cell differentiation program. It has already been shown that expression of the skeletogenic *alx1* gene results in repression of the pigment cell regulatory gene *gcm*, so that if *alx1* expression is prevented, *gcm* expression spreads to the skeletogenic domain in preingression embryos (1). Here we sought evidence of a reciprocal exclusion, such that forced expression of *gcm* would specifically down-regulate skeletogenic regulatory genes. To examine this directly, embryos bearing *tbr*:GCM BAC were fixed at mesenchyme blastula stage (22–24 h), and expression of various endogenous skeletogenic regulatory genes was monitored by double WMISH (Fig. 4). These were the skeletogenic regulatory genes *alx1*, *ets1*, and *jun*, and the downstream skeletogenic differentiation gene *msp130*. This experiment exploited the mosaic incorporation of the exogenous construct in that it divided the originally skeletogenic lineage into two components: those cells that contain *tbr*:GCM BAC and those that do not, each providing a control for the other. As noted earlier, when present by itself, *tbr*:GFP BAC expresses accurately in ingressed skeletogenic cells—in this experiment in 80% of the control embryos (Fig. 4*A*). However, we found that 65% of embryos bearing *tbr*:GCM BAC expressed the exogenous *gcm* gene under *tbr* regulatory control in cells that remained in the vegetal wall of the embryos (Fig. 4 *B–E*), and 30% also expressed it in ingressed cells. The striking result in Fig. 4 *B–E* is that the transgenic cells remaining in the vegetal wall appear not to be expressing *alx1* (Fig. 4*B*), *ets1* (with one exception, Fig. 4*C*, *Right*), *msp130* (Fig. 4*D*), or *jun* (Fig. 4*E*), whereas the remaining nontransgenic ingressed cells all do. Additional examples of embryos expressing endogenous *alx1* and exogenous *tbr*:GCM BAC are seen in Fig. S5, which also illustrates the occurrence in some embryos of cells of mixed fate that behave like skeletogenic cells and ingress but also express exogenous *gcm* (the 30% class noted above). In the 65% class, however, forced *gcm* expression does appear to produce cells that autonomously exclude the skeletogenic regulatory state.

A quantitative analysis by quantitative PCR using the skeletogenic detector constructs (Fig. 2) demonstrates this effect directly at the regulatory level. Here the outputs of the detector constructs were measured in the absence and the presence of *tbr*:GCM BAC. In interpreting these experiments, it is necessary to recall that in sea urchin embryos all exogenous constructs are stably incorporated together into the same cells and their descendants. Table S2 shows that, when *tbr*:GCM BAC is coinjected with the *alx*:GFP BAC, *ets*:GFP BAC, or *tbr*:GFP BAC detector constructs, a strong quantitative down-regulation in the activity of all three skeletogenic *cis*-regulatory systems is observed.

To test directly for a repressive effect of Gcm on transcription of skeletogenic genes, *gcm* mRNA was injected into fertilized eggs (Fig. S6 *A* and *B*). As expected, because it is a direct *cis*-regulatory target of Gcm (13), expression of the *pks* gene is sharply up-regulated in this experiment, whereas all of the skeletogenic genes

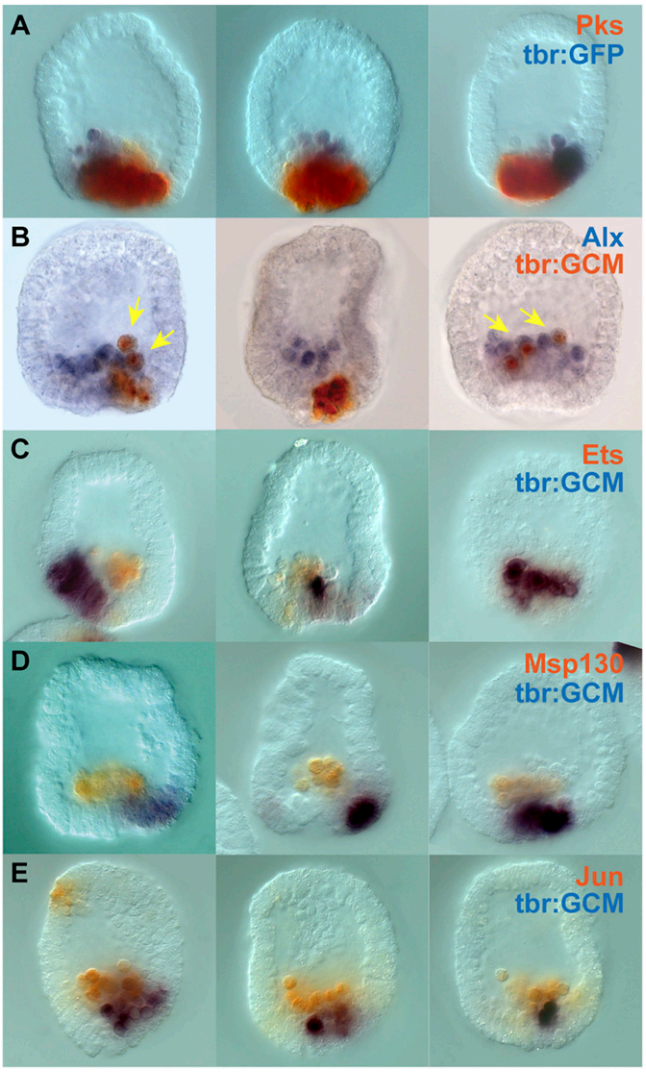

Fig. 4. Two-color WMISH of *gcm*-expressing SM cells in mesenchyme blastula-stage embryos. (*A*) As a control, *tbr*:GFP BAC-injected embryos were probed for reporter expression and endogenous *pks*. (*B–E*) *tbr*:GCM BAC-injected embryos were probed for synthetic *gcm* expression and one of several SM-specific genes: *alx1* (*B*), *ets1* (*C*), *msp130* (*D*), and *jun* (*E*). Yellow arrows indicate SM cells that are coexpressing *alx1* and the *tbr*:GCM BAC. Probes used for detection are indicated at the right of each row and are colored according to their stain. A single probe matching the 3′-UTR SV40 polyadenylation sequence was used to detect the products of both *tbr*:GFP and *tbr*:GCM BACs.

tested—*alx1*, *msp130*, *tbr*, *vegfrII*, and *foxO*—were very sharply down-regulated. The various positions of these genes in the skeletogenic GRN (Fig. 1*A*) indicates that forced *gcm* expression represses the skeletogenic network somewhere high up in the network hierarchy. Examination of the GRN architecture (Fig. 1*A*) focuses suspicion on the *ets1* gene, which provides inputs to multiple genes at the top of the skeletogenic GRN, such as *tbr* (10) and *alx1* (11, 18); to other regulatory genes further down, such as *erg*, *tgif*, and *deadringer* (1); and to differentiation genes at the lower terminus of the GRN, such as *sm50* (19) and *cyclophilin* (20). As shown in Table S2, forced expression of *gcm* in primordially skeletogenic cells causes a very sharp repression of *ets*:GFP BAC expression. This is most likely the explanation for the general down-regulation of the skeletogenic regulatory state, and of skeletogenic function, caused by the reengineered expression of *gcm*. However, this must remain a supposition until the specific inputs into the *ets cis*-regulatory system (19) are determined.

In addition, there could be a direct repressive link from *gcm* to *alx1*, which, like *ets1*, is required for skeletogenic specification and differentiation (18). To test whether this regulatory link exists, the activity of an *alx1*:GFP reporter construct was measured in the presence of *gcm* mRNA overexpression. This construct contained 400 bp of genomic DNA located upstream of the start site of transcription, and it expresses GFP only in skeletogenic lineage cells (11). The overexpression of *gcm* mRNA caused a twofold depression of the GFP transcript generated by the construct. However, a series of 5′ deletions of the minimal reporter failed to reveal a *gcm*-responsive subelement; neither did the regulatory DNA in this construct contain a consensus *gcm*-binding site [$^{G}/_{A}$CCCGCAT (21)]. Thus, the down-regulation of the *alx1* reporter by *gcm* mRNA is probably explained as the indirect effect of *ets* repression.

Discussion

A GRN that explains the causal genomic code for an embryonic specification function in principle also offers the opportunity of rationally predicting the outcome of changes in its topology. Here we challenged this precept, using as a test bed the GRN underlying specification of the skeletogenic and pigment cell lineages in the sea urchin embryo. The essential parts of the GRN for this study, and the manner in which we experimentally rewired it, are summarized in Fig. 1. Although the change that we made was apparently simple—placing a pigment cell regulatory gene under skeletogenic *cis*-regulatory control—close consideration of the GRN topology shows that it would be expected to produce multiple effects, and the network topology explains in detail why respecification actually occurred.

There are at least five different downstream consequences of the network rewiring that contributed to the institution of pigment cell functionality in cells originally destined to become exclusively skeletogenic. Four of these were directly predictable from the GRN topology, and indeed they are the reason why we chose the strategy we did. The fifth, the import of which we discuss later, emerged unexpectedly.

Short Circuit of Delta/Notch Signaling. In the normal development of sea urchin embryos, Delta/Notch signaling is used to position mesodermal specification in the veg2 cell lineage. This signaling input is directly responsible for *cis*-regulatory activation of *gcm* in these cells (6), and hence it is indirectly responsible for pigment cell differentiation (Fig. 1*B*). The Su(H) target sites of the *gcm* early *cis*-regulatory module are also required for repression of *gcm* outside of the veg2 ring of cells receiving the Delta signal (6). Therefore, to effect the cell fate transformation, it was required to turn on the *gcm* gene by a *N*-independent mechanism that would function only in skeletogenic cells. Placing *gcm* under control of the *tbr cis*-regulatory system is a gain-of-function, regulatory addition that did not destroy the embryonic process as would have been the case had we interfered with endogenous Delta/Notch signaling.

Hierarchical Position of *tbr* in the Skeletogenic GRN. As can be seen in Fig. 1*A*, the *tbr* gene operates high in the skeletogenic hierarchy, in that it is activated as a direct target (10) of the double-negative gate that initiates the skeletogenic regulatory state. Its output (i.e., the Tbr transcription factor) provides one of the inputs into the triple feedback subcircuit (including the *hex*, *erg*, and *tgif* genes), which renders the progressively augmented skeletogenic regulatory state impervious to the transient initiating events that initially unlock the double-negative gate. It was thus essential to install the diversion from skeletogenic fate upstream of this skeletogenic feedback circuit, which continues to operate permanently in skeletogenic cells and even apparently in adult skeletogenic tissues (22). It would be interesting, for example, to test the prediction that

expressing *gcm* in skeletogenic cells under *hex cis*-regulatory control, instead of under *tbr* control, might result in bona fide skeletogenic cells that also express the pigment differentiation program.

Transcription of the *gcm* Sequence by the Recombineered BAC Closely Mimicked *tbr* Transcriptional Dynamics and Spatial Expression. Skeletogenic cells bearing the *tbr*:GCM BAC construct generated the *gcm* transcript very early in their development, and almost exclusively in former skeletogenic cells (Fig. 3 and Table 1) and at about the same total rate as the *tbr* gene normally generates *tbr* transcript. We can compare the recombineered BAC expression to the ~250 *tbr* mRNAs generated by the two endogenous *tbr* genes per skeletogenic cell at the peak expression point around 20 h, or 125 copies per *tbr* allele (10, 15). The *tbr*:GCM BAC produces ~40 copies per construct, but multiple copies of the construct are integrated per nucleus, and our measurements indicate >200 *gcm* mRNAs per transgenic cell at 11.5 hpf, soon after activation, and >800/cell at 20 h. We know from the *gcm* mRNA injection experiments (Fig. S6*A*) that 280 *gcm* mRNAs per cell suffice to repress skeletogenic functions and to drive strong expression of the downstream pigment cell gene *pks*. These results indicate that the *tbr cis*-regulatory architecture provided more than the sufficient dosage of *gcm* mRNA to mediate its cross-repressive functions on skeletogenesis and to deploy the pigment cell differentiation program. Furthermore, the *tbr cis*-regulatory system continues to be expressed actively through development, lacking the auto-repression device of *alx1*, for example (11).

"Latch" Wiring of the Pigment Cell Specification Subcircuit. In the normal veg2 aboral mesoderm pigment cell lineage, after the initial period of *gcm* activation by Delta/Notch signaling, control of *gcm* activity shifts to other inputs (6) (Fig. 1*B*). This renders *gcm* expression independent of further Delta/Notch input. The *gcm* mRNA overexpression data of Fig. S6*B* shows that the level of *gcm* mRNA produced in the transgenic cells expressing *tbr*:GCM BAC is capable of activating the endogenous *gcm* gene. The result is to free continuing *gcm* expression in transformed cells from control by the exogenous *tbr cis*-regulatory architecture. This is ultimately important because the *tbr* regulatory system depends in turn on *ets1* expression, which eventually becomes repressed, as we have seen (Table S2). Thus, the *tbr cis*-regulatory architecture operates basically as a switch for throwing open the *gcm* transcriptional latch, but once open, expression of the subnetwork of genes linked to *gcm* (*gatae*, *six1/2*; cf. Fig. 1), which together turn on the pigment cell differentiation gene battery, will continue to operate autonomously and permanently.

Cryptic Exclusion of Skeletogenic Regulatory State. The function that the extant GRN topology did not predict was the repression of the skeletogenic regulatory state once the *gcm* latch had been thrown. The evidence for this (Fig. 4; Table S2; Figs. S5 and S6*A*) shows clearly that many different skeletogenic genes are similarly affected. For many different skeletogenic genes to be affected, *gcm* expression must directly or indirectly affect a skeletogenic gene very high in the GRN hierarchy, and as pointed out above, the *ets1* gene is the most likely target; repression of this gene would produce all of the observed effects, and it is indeed quantitatively very sharply responsive to ectopic *gcm* expression (Table S2). As already shown, there is a reciprocal exclusion of expression of the canonical skeletogenic regulatory gene *alx1* in the pigment cell lineage (1). Thus, each of these mesodermal cell populations possesses a mechanism for locking down its own fate choice, once this has been made, by specifically excluding the alternative. This is a very general feature of developmental GRNs (16). It has a potentially powerful implication, which is that it operates to sharpen regulatory state differences and in reengineering experiments to decrease the incidence of cells of "mixed" regulatory state. Nonetheless, as we show throughout this work, there were various degrees of incomplete transformation observed, particularly early on, although much less often at late developmental stages. The exclusion function, together with the latch function just considered, are what locks in the changed regulatory state. The existence of this particular exclusion linkage would have remained undiscovered, if it were not for the context generated by the rewiring experiment. This illustrates the additional return that may accrue from the experimental reengineering of development.

Materials and Methods

Details pertaining to the cloning of BAC and plasmid constructs, microinjection into sea urchin eggs, and whole-mount in situ hybridization are given in *SI Materials and Methods*.

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