Supporting Information

Oliveri *et al*. 10.1073/pnas.0711220105

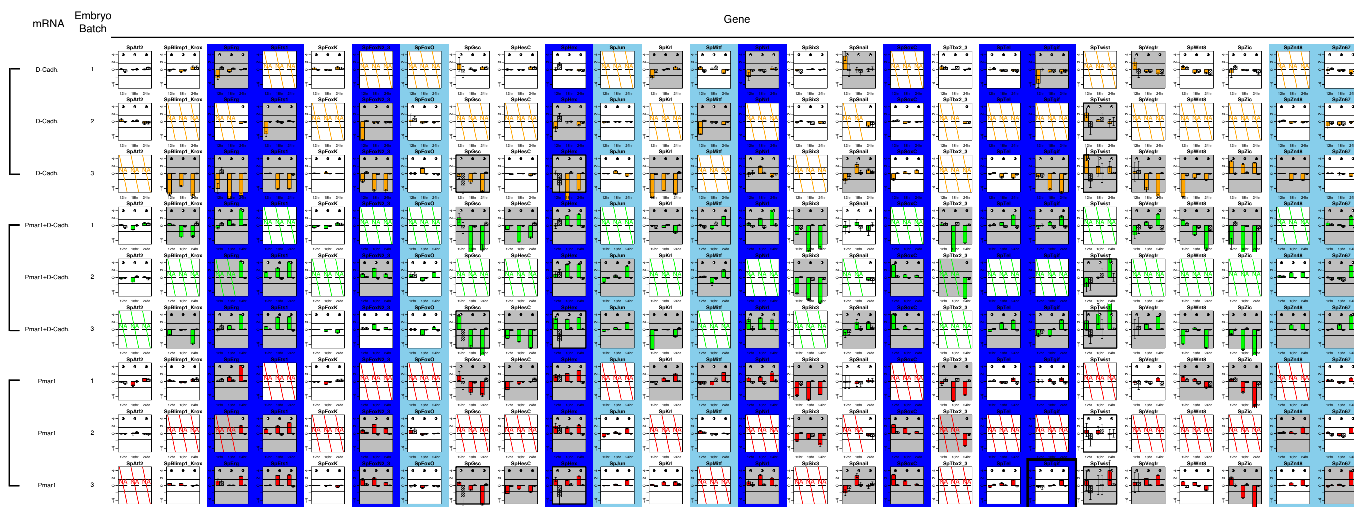

Fig. S1. Sensitivity of regulatory genes to *pmar1* expression and the β-catenin initial input. Quantitative measurements are shown for all genes expressed in the skeletogenic micromere lineage that had not been studied by similar means earlier. These genes had been identified by genome analysis (1–5). The QPCR measurements were conducted in embryos containing *pmar1* mRNA in all of the cells; embryos in which the nuclearization of β-catenin had been blocked by injection of Δ-cadherin mRNA; and embryos double perturbed with both *pmar1* and Δ-cadherin mRNA injection. Each plot shows the effects on a gene (column) in replicate batches of injected embryos (one batch per row) for the three experimental conditions, at three different developmental times: late cleavage (12 h), hatched blastula (18 h), and mesenchyme blastula (24 h). The measurements are expressed as $\Delta\Delta$Ct, which is the QPCR cycles at threshhold (Ct) normalized by the internal control ubiquitin (ΔCt), and then compared with normalized uninjected embryo values ($\Delta\Delta$Ct). A positive value means increased expression compared with the uninjected embryos, and a negative value means decreased expression. Columns highlighted by dark blue show the new genes included in the network; columns highlighted by light blue are genes specifically expressed in micromere lineage only after ingression (24 h), which are not relevant for early specification process. These genes are affected by *pmar1* overexpression indirectly. Colored bars are measurements in perturbed embryos; slashed bars in controls. In each plot the gray background indicates a measurement ($\Delta\Delta$Ct) over the cutoff ($\pm$1.6) at any time point, i.e., a significant result; black bordered boxes indicate that the injection control (GFP mRNA) also shows a $\Delta\Delta$Ct value relative to uninjected sample over the cutoff ($\pm$1.6) at any one of the time points analyzed, and thus a questionable result. An indication of level of expression for each gene at each developmental stage is given by the pie plots, which indicate the average Ct in the controls: full black pie is an average Ct of 20, white pie means an average Ct of 40. Generally speaking, a half blank (or less) pie indicates less reliable data. Cross NA lines mean the data are not available. Error bars are standard errors for the four QPCR replicas. Data were processed and illustrated by the software qpcrplot available on request.

1. Howard-Ashby M, *et al.* (2006) Identification and characterization of homeobox transcription factor genes in *Strongylocentrotus purpuratus*, and their expression in embryonic development. *Dev Biol* 300:74–89.
2. Howard-Ashby M, *et al.* (2006) Gene families encoding transcription factors expressed in early development of *Strongylocentrotus purpuratus*. *Dev Biol* 300:90–107.
3. Materna SC, Howard-Ashby M, Gray RF, Davidson EH (2006) The C2H2 zinc finger genes of *Strongylocentrotus purpuratus* and their expression in embryonic development. *Dev Biol* 300:108–120.
4. Rizzo F, Fernandez-Serra M, Squarzoni P, Archimandritis A, Arnone MI (2006) Identification and developmental expression of the ets gene family in the sea urchin (*Strongylocentrotus purpuratus*). *Dev Biol* 300:35–48.
5. Tu Q, Brown CT, Davidson EH, Oliveri P (2006) Sea urchin Forkhead gene family: Phylogeny and embryonic expression. *Dev Biol* 300:49–62.

Expression Levels of skeletogenic lineage transcription factors

Gene	0hr	5hr	7hr	9.5hr	12hr	15hr	18hr	21hr	24hr	27hr	30hr	33hr	40hr	45hr	48hr	52hr	70hr
pmar1	3	8	585	399	430	134	102	86	69	73	119	101	129	107	92	66	115
alx1	28	62	316	988	6091	2826	1985	1481	2371	2412	959	873	913	1025	941	562	1063
ets1	60623	49209	25927	39015	39519	10761	6593	5403	6852	4013	1695	2371	1870	2233	2296	1549	3013
tbr	3463	2975	3411	2351	2874	2937	6453	6052	7448	4189	1716	2778	2725	2417	1910	877	444
soxC	586	577	506	1688	4004	5760	9735	6448	11032	8259	13920	6565	8823	4866	9435	6431	13551
tel	3715	5173	4439	3516	1336	1551	1589	1137	1453	1049	1411	871	1224	929	1334	1087	1754
foxN2_3	161	49	49	125	408	1131	2406	1461	1638	2284	2373	1488	2847	1758	1695	1779	2400
erg	3	1	2	16	37	145	792	786	837	675	1257	936	773	765	648	608	687
hex	2	1	8	4	51	249	511	261	502	525	1014	551	682	920	1304	951	1641
tgif	188	59	48	52	146	75	361	759	1323	1699	3229	2487	6889	4635	7952	6414	7625
dri	3	8	11	16	74	446	1058	734	705	805	3340	3109	16336	10054	15020	8843	13327
foxB	198	8	28	41	27	349	250	311	904	1197	676	281	336	357	509	462	1051
foxO	147	244	216	369	247	520	876	680	1466	1767	4678	2027	4941	2984	5532	4667	9505
jun	10761	2688	6734	2766	2038	1916	2298	1062	1740	1576	2800	762	1538	1219	1852	1469	3406
vegfr	15	142	10	185	11	64	134	366	637	1185	509	674	1081	1093	782	594	1627
gsc	21	176	2	61	59	342	1114	1927	3912	4814	2924	3240	3275	5155	3560	2336	2205
snail	4	1	2	4	2	2	2	3	26	37	81	24	127	162	173	180	176

Fig. S2. High-resolution temporal profiles of transcription factors expressed in the skeletogenic lineage. The levels of expression for each gene are given as number of transcripts per embryo. Measurements are obtained by QPCR using ubiquitin as internal standard (1) on unperturbed embryos. Samples were collected roughly every 3 h from unfertilized egg (0 h) to larval stage (70 h) as indicated. One hundred-fifty molecules per embryo is used as cutoff for a significant level of expression. Color keys are: dark blue, 0–149; light blue, 150–1,000; green, 1,000–10,000, and yellow >10,000 transcripts per embryo. Data show the common logarithms of the transcript numbers. *Ets1*, *tbr*, *soxC* and *tel*, are maternally expressed in all of the cells of the embryo (striped colors) but by cleavage stage (7–12 h) they have become restricted to the skeletogenic micromere lineage (2–4). *Jun* and *foxO* are initially expressed ubiquitously and become localized to the skeletogenic lineage only after ingression [24 h (3, 5)]. In *S. purpuratus snail* is not expressed at significant levels until gastrulation. Thus it is not relevant for the specification of this lineage, contrary to the case in another species, *L. variegatus* (6). Spatial expression pattern of these genes was confirmed at high resolution by *in situ* hybridization (data not shown). The usual sensitivity of this procedure is 20–40 transcripts per cell.

1. Oliveri P, Carrick DM, Davidson EH (2002) A regulatory gene network that directs micromere specification in the sea urchin embryo. *Dev Biol* 246:209–228.
2 Howard-Ashby M, *et al.* (2006) Gene families encoding transcription factors expressed in early development of *Strongylocentrotus purpuratus*. *Dev Biol* 300:90–107.
3. Rizzo F, Fernandez-Serra M, Squarzoni P, Archimandritis A, Arnone MI (2006) Identification and developmental expression of the ets gene family in the sea urchin (*Strongylocentrotus purpuratus*). *Dev Biol* 300:35–48.
4. Croce J, Lhomond G, Lozano JC, Gache C (2001) ske-T, a T-box gene expressed in the skeletogenic mesenchyme lineage of the sea urchin embryo. *Mech Dev* 107:159–162.
5. Tu Q, Brown CT, Davidson EH, Oliveri P (2006) Sea urchin Forkhead gene family: Phylogeny and embryonic expression. *Dev Biol* 300:49–62.
6. Wu SY, McClay DR (2007) The Snail repressor is required for PMC ingression in the sea urchin embryo. *Development* 134:1061–1070.

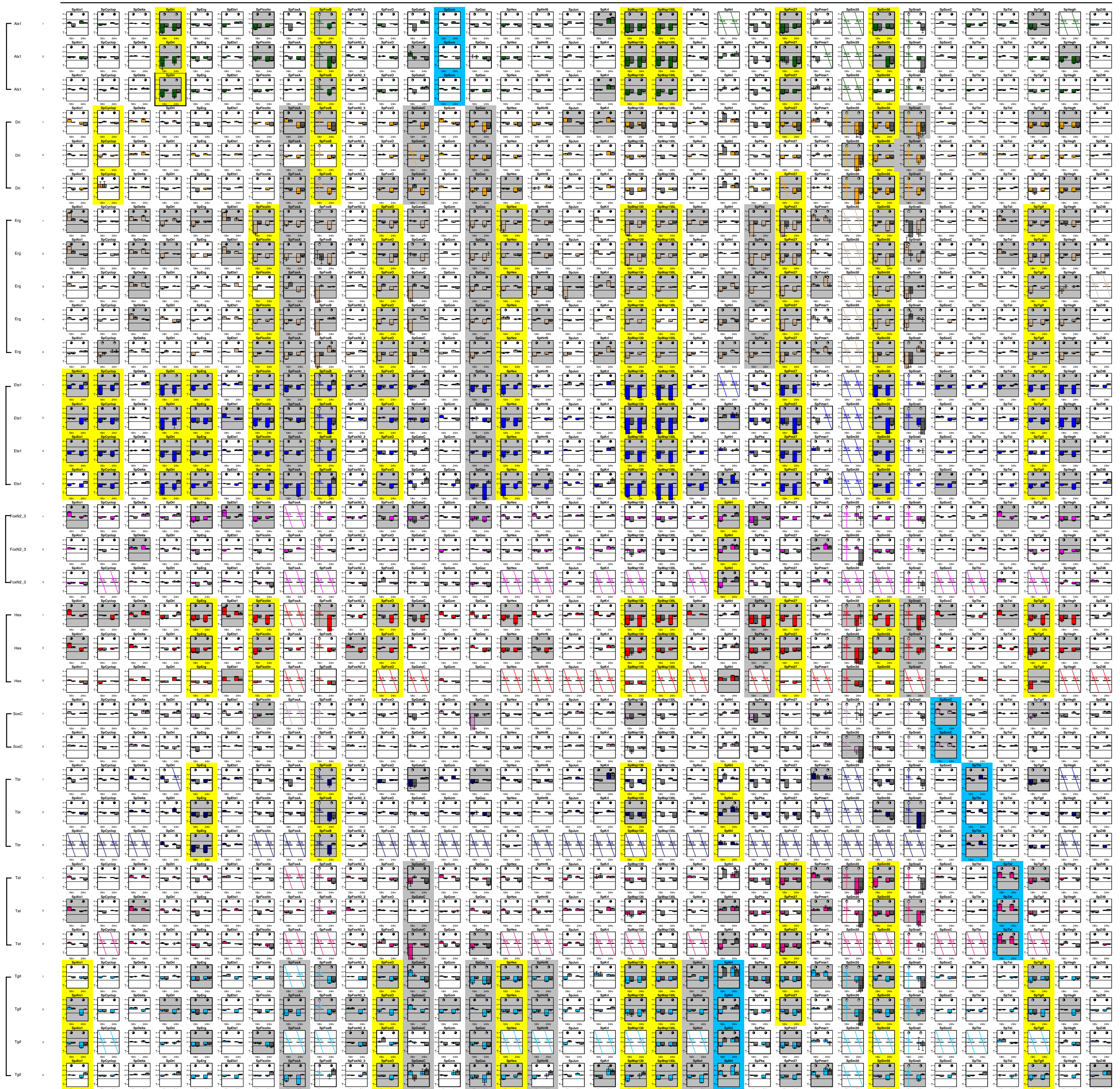

Fig. S3. Functional linkage analysis for the regulatory genes expressed in the skeletogenic micromere lineage. Expression of regulatory genes *alx1*, *dri*, *erg*, *ets1*, *foxN2/3*, *hex*, *soxC*, *tbr*, *tgif*, and *tel* was blocked with specific morpholinos (see *Materials and Methods*). The effects of those perturbations were quantified on all of the other genes of the skeletogenic micromere lineage and some mesodermal and endodermal genes. For explanation of diagrams see Fig. S1. In yellow are highlighted positive (activation) linkages occurring in the micromere domain, in blue are negative (repression) linkages in the same domain, and in gray are positive and negative linkages occurring in domains other than micromeres, which may indicate indirect linkages.

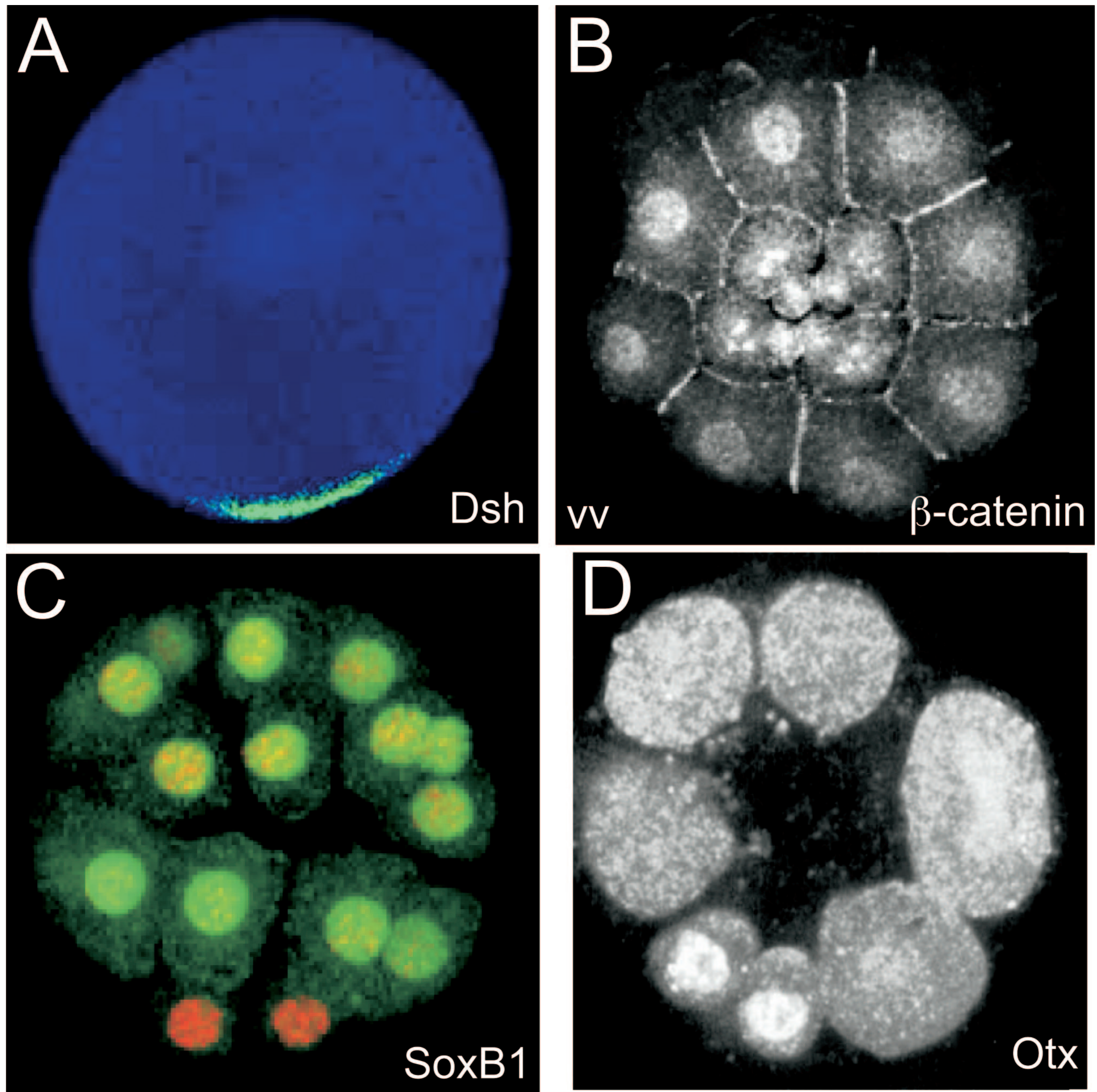

Fig. S4. Initial regulatory state. Immunocytological demonstrations of primordial anisotropies of regulatory significance in micromeres. (*A*) Localization of Dsh in an unfertilized egg [modified from Weitzel *et al.* (1)]. (*B*) Nuclear localization of β-catenin in the eight veg2 cells and large and small micromeres of a sixth-cleavage embryo, vegetal view (vv). This is a cell-autonomous, maternally loaded early function of the sea urchin embryo. [Reproduced with permission from ref. 2 (Copyright 1999, The Company of Biologists]. (*C*) Nuclear localization of the SoxB1 transcription factor in all of the cells of a fourth-cleavage embryo except the micromeres; DNA is stained in orange and Soxb1 in green [modified from Kenny *et al.* (3)]. (*D*) Nuclearization of Otx factor in micromeres of a fourth-cleavage embryo [Reproduced with permission from ref. 4 (Copyright 1996, Wiley-Liss).]

1. Weitzel HE, *et al.* (2004) Differential stability of β-catenin along the animal-vegetal axis of the sea urchin embryo mediated by dishevelled. *Development* 131:2947–2956.
2. Logan CY, Miller JR, Ferkowicz MJ, McClay DR (1999) Nuclear β-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* 126:345–357.
3. Kenny AP, Kozlowski D, Oleksyn DW, Angerer LM, Angerer RC (1999) SpSoxB1, a maternally encoded transcription factor asymmetrically distributed among early sea urchin blastomeres. *Development* 126:5473–5483.
4. Chuang CK, Wikramanayake AH, Mao CA, Li X, Klein WH (1996) Transient appearance of *Strongylocentrotus purpuratus* Otx in micromere nuclei: Cytoplasmic retention of SpOtx possibly mediated through an alpha-actinin interaction. *Dev Genet* 19:231–237.

Fig. S5. Repression of nonskeletogenic mesoderm genes by Alx1. (*A*) QPCR measurements at two different developmental stages made on two key mesodermal regulatory genes, *gcm* and *gatae*, and a differentiation gene, *pks*, after injection of *alx1* mRNA into the egg. A dramatic reduction of expression is seen for all three genes. Levels of expression are indicated as ΔΔCt relative to the control (GFP mRNA). A positive value means increased expression compared with the control embryos, whereas a negative value refers to decreased expression. For explanation see Fig. S1. Embryos injected with *alx1* mRNA have increased numbers of skeletogenic mesenchyme cells (1). (*B–I*) In the absence of *alx1* the micromere skeletogenic lineage is specified as, a consequence of releasing *gcm* from *alx1* repression. In *alx1* morpholino-injected embryos, the micromere descendants do not ingress in the blastocoel nonskeletogenic mesoderm (compare *B* and *C*). (*D* and *E*) A single micromere was injected with rhodamine dextran (10,000 kDa; 1:5 dilution of a 50-mg/ml stock) after initial injection of *alx1* morpholino into fertilized eggs. Its descendants display nonskeletogenic mesoderm characters. They remain associated with the tip of the archenteron (white arrowhead) or integrate in the ectoderm (black arrowhead). Embryos lacking *alx1* do not form larval skeleton for several days (*I*) and show extra nonskeletogenic mesoderm, i.e., pigment and blastocoelar cells (*G* and *I* compared with *F* and *J*). The experiment in *D* and *E* was conducted in collaboration with Takuya Minokawa (Tohoku University, Tohoku, Japan).

1. Ettensohn CA, Kitazawa C, Cheers MS, Leonard JD, Sharma T (2007) Gene regulatory networks and developmental plasticity in the early sea urchin embryo: Alternative deployment of the skeletogenic gene regulatory network. *Development* 134:3077–3087.

PNAS PNAS PNAS PNAS

Expression levels of skeletogenic lineage differentiation genes

Gene	0hr	5hr	7hr	9.5hr	12hr	15hr	18hr	21hr	24hr	27hr	30hr	33hr	40hr	45hr	48hr	52hr	70hr
SpCycloph	3	1	1	97	223	829	1770	1689	1950	2883	7889	1509	4960	3149	3241	3679	1243
SpFicolin	3	1	8	16	9	58	679	968	1132	1203	3207	471	1204	727	772	1001	1975
SpMsp130	2	1	4	11	26	400	1585	2728	4373	6217	8708	8287	9267	7024	6875	6305	4565
SpMsp130L	5	3	4	38	286	2385	5542	7652	9445	8688	12337	9483	6362	4855	5339	5590	9444
SpPm27	1	1	1	17	145	139	392	1390	4186	4919	12017	6909	5643	3994	4656	5344	7453
SpSm30	1	1	1	3	11	1	1	2	7	33	268	637	3239	2537	3187	4120	3058
SpSm50	1	1	1	1	9	99	221	327	856	1225	4044	1866	1654	929	957	1052	1426

Fig. S6. Temporal expression profiles of skeletogenic differentiation genes. Data are presented as in Fig. S2.

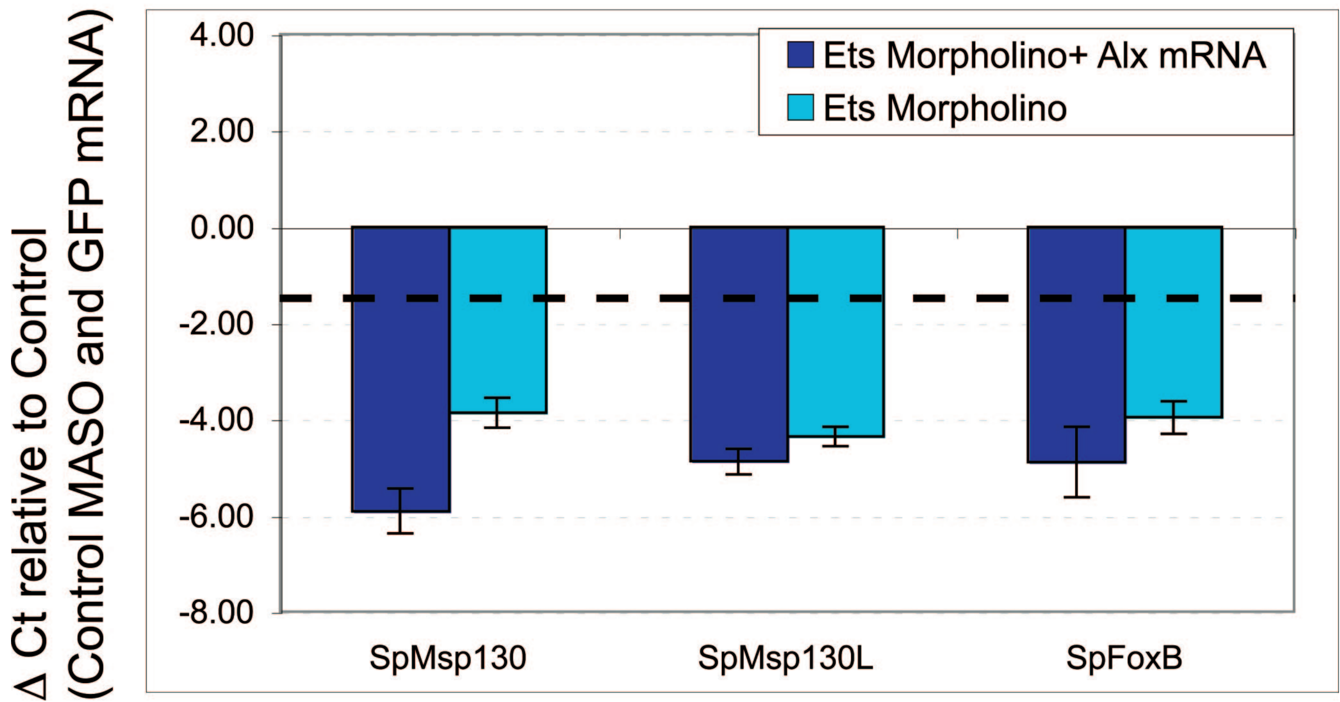

Fig. S7. Confirmation of the feed-forward motif between *ets1*, *alx*, and the downstream genes. The embryos were injected with *ets1* morpholino and *alx1* mRNA. The effects were measured on differentiation genes that show positive inputs from both of these regulatory genes, *msp130*, and *msp-L* (*msp130-L*), and *foxb*. The absence of even minimal rescue by the *alx1* mRNA confirms the feed-forward architecture of the network motif and shows that both inputs are required for the expression of these downstream genes. The quantitative data also suggest the operation of AND logic in the *cis*-regulatory systems of *msp130*, *msp-L*, and *foxb*, such that the Ets1 and Alx1 inputs must be simultaneously present to drive expression. A positive value means increased expression compared with the uninjected embryos, and a negative value means decreased expression.

Table 1. Feed-forward circuit motifs in the regulation of PMC differentiation genes

Regulatory gene A	Regulatory gene B	Differentiation gene C
Ets1	Alx1	Sm27, Sm 50, Msp130†, Msp-L†, Ficolin
Ets1*	Dri*	Sm27, Sm50*, Cyclophillin*
Ets1*	Hex	Sm27, Sm 50*, Msp130, Msp-L, Ficolin
Ets1*	Erg*	Sm27, Sm 50*, Msp130, Msp-L, Ficolin
Alx1	Dri*	Sm27, Sm50*, Cyclophillin*
Tbr	Erg	Sm27, Sm 50, Msp130, Msp-L, Ficolin
Erg	Hex	Sm27, Sm 50, Msp130, Msp-L, Ficolin

See Fig. 6 for the architecture of these feed-forward loops.

*These inputs have been validated by *cis*-regulatory analysis.

†The AND logic of the Ets1 and Alx1 inputs in the promoters of these genes has been demonstrated by Ets1 morpholino and Alx1 mRNA double perturbation (Fig. S7).

PNAS PNAS PNAS PNAS

Table 2. Morpholinos used in this article

Name	Sequence (5′->3′)	Block type	Working concentration, μM	Efficiency validation
alx1	ATATTGAGTTAAGTCTCGGCACGAC	Translation	400	
dri	CTGTCTTCGCTGGTTCTTCAAC	Translation	200	
*erg**	GGCTGCTCAATCTCTTGTTTCATGC	Translation	200	GFP construct†
erg-trans*	GCATATAACAAATTGAGGAACACTG	Translation	200	GFP construct†
ets1	GAACAGTGCATAGACGCCATGATTG	Translation	400	
foxN2/3	CGGGCAAATCTGTATCCTCCATCTC	Translation	200	GFP construct†
hex-E3I3	AGTGGTGAAATTACCTGTTTTAATC	Splicing	400	QPCR‡
soxC	GAACCATCTTGAAGTCAGCATTCAC	Translation	400	GFP construct†
tbr	TGTAATTCTTCTCCCATCATGTCTC	Translation	400	
tel	CCTGTCTGGTAGAGGCCGGGTCCAT	Translation	400	GFP construct†
tgif	ATCTTTCTTGTGGTAAATCCGCATC	Translation	400	GFP construct†

All morpholino-substituted antisense oligonucleotides were produced by Gene Tools.

*Perturbations of *erg* were done by the mixture of two morpholinos as 200 μM each, because we have identified different alternative splicing forms that are targeted by different morpholinos.

†Controls of morpholino knock-down efficiency was conducted on GFP construct in which specific DNA fragments around the morpholino target site (≈300 bp) was cloned in frame with the GFP sequence (1). The mRNA *in vitro*-transcribed (2) from these constructs was injected along with specific and nonspecific morpholinos.

‡The presence of unspliced form was quantified by QPCR.

1. Oliveri P, Davidson EH, McClay DR (2003) Activation of pmar1 controls specification of micromeres in the sea urchin embryo. *Dev Biol* 258:32–43.

2. Oliveri P, Carrick DM, Davidson EH (2002) A regulatory gene network that directs micromere specification in the sea urchin embryo. *Dev Biol* 246:209–228.