Activation of the skeletogenic gene regulatory network in the early sea urchin embryo

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SUMMARY

The gene regulatory network (GRN) that underlies the development of the embryonic skeleton in sea urchins is an important model for understanding the architecture and evolution of developmental GRNs. The initial deployment of the network is thought to be regulated by a derepression mechanism, which is mediated by the products of the *pmar1* and *hesC* genes. Here, we show that the activation of the skeletogenic network occurs by a mechanism that is distinct from the transcriptional repression of *hesC*. By means of quantitative, fluorescent whole-mount in situ hybridization, we find that two pivotal early genes in the network, *alx1* and *delta*, are activated in prospective skeletogenic cells prior to the downregulation of *hesC* expression. An analysis of the upstream regulation of *alx1* shows that this gene is regulated by MAPK signaling and by the transcription factor Ets1; however, these inputs influence only the maintenance of *alx1* expression and not its activation, which occurs by a distinct mechanism. By altering normal cleavage patterns, we show that the zygotic activation of *alx1* and *delta*, but not that of *pmar1*, is dependent upon the unequal division of vegetal blastomeres. Based on these findings, we conclude that the widely accepted double-repression model is insufficient to account for the localized activation of the skeletogenic GRN. We postulate the existence of additional, unidentified repressors that are controlled by *pmar1*, and propose that the ability of *pmar1* to derepress *alx1* and *delta* is regulated by the unequal division of vegetal blastomeres.

KEY WORDS: Gene regulatory network, Primary mesenchyme, Sea urchin, *alx1*

INTRODUCTION

A prominent feature of the embryogenesis of indirectly developing sea urchins is the formation of an elaborate, calcified cskeleton. Recent studies have revealed a gene regulatory network (GRN) that controls the development of the skeletogenic large micromere primary mesenchyme cell (PMC) lineage (reviewed by Oliveri et al., 2008; Ettensohn, 2009). The PMC gene network is currently one of the most complete developmental GRNs and is being used to elucidate GRN architecture, the evolution of developmental programs, and developmental plasticity. For example, recent studies suggest that the evolution of skeletal development in echinoderms involved the co-option by the embryo of an ancestral, adult skeletogenic GRN via the invention of new regulatory connections (Gao and Davidson, 2008; Erwin and Davidson, 2009; Ettensohn, 2009). The regulative deployment of the PMC GRN in non-micromere lineages during gastrulation is a striking example of developmental plasticity, and has been shown to involve novel upstream inputs (Ettensohn et al., 2007). Such studies have focused attention on the molecular mechanisms that activate the skeletogenic GRN and highlight the need to clarify the initial regulatory inputs into this network.

The activation of the PMC GRN in the large micromere territory is dependent upon the stabilization of β-catenin. One important target of β-catenin is the transcriptional repressor *pmar1*, which is transiently expressed in the micromere lineage beginning at the 16-cell stage (Kitamura et al., 2002; Oliveri et al., 2002). Ectopic expression of Pmar1 causes most cells of the embryo to adopt a

PMC-like fate (Oliveri et al., 2002; Oliveri et al., 2008). Because Pmar1 is a transcriptional repressor, it presumably activates the skeletogenic GRN indirectly, by blocking the expression of a second repressor. This second repressor is believed to be HesC, a member of the HES (hairy-enhancer-of-split) family (Revilla-i-Domingo et al., 2007). *hesC* transcripts are ubiquitous in the early embryo, but disappear from the vegetal region (including the presumptive PMCs) at the early blastula stage (Revilla-i-Domingo et al., 2007; Smith and Davidson, 2008). Overexpression of Pmar1 results in a decrease in the level of *hesC* transcripts, whereas morpholino (MO) knockdown of HesC leads to the ectopic expression of *delta* throughout the embryo and to an increase in the levels of *alx1*, *ets1*, and *tbr* mRNAs (Revilla-i-Domingo et al., 2007). These findings support the model that the skeletogenic GRN is activated by a Pmar1/HesC double-repression 'gate', a model that has been widely accepted (Davidson and Levine, 2008; Oliveri et al., 2008; Ettensohn, 2009).

Alx1 is one of the earliest regulatory genes to be activated specifically in the large micromere lineage, and this gene plays a pivotal role in PMC specification (Ettensohn et al., 2003). Delta is a signaling molecule that mediates an interaction between the large micromere progeny and adjacent, non-skeletogenic mesoderm (NSM) cells (Sweet et al., 2002). The cis-regulatory control of *delta* has been analyzed in considerable detail (Revilla-i-Domingo et al., 2004; Smith and Davidson, 2008). By contrast, little is known concerning the regulation of the *alx1* gene, other than a proposed input from the *pmar1*/*hesC* double-repression system. A positive regulatory input from *ets1* has been demonstrated by MO knockdown experiments and quantitative PCR (qPCR) studies (Oliveri et al., 2008). Perturbation of Ets1 function by MO knockdown or by overexpression of a dominant-negative form of the protein blocks PMC ingression and skeletogenesis, whereas overexpression of Ets1 transforms most cells of the embryo to a mesenchymal fate (Kurokawa et al., 1999; Rottinger et al., 2004; Oliveri et al., 2008). Rottinger et al. (Rottinger et al., 2004) provided

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evidence that the phosphorylation of Ets1 by MAP kinase is essential for its function and showed that inhibition of MAP kinase signaling downregulates the expression of *alx1*. Together, these findings suggest that the MAP kinase pathway upregulates *alx1* expression via the phosphorylation of Ets1.

The micromeres arise as a consequence of an unequal cell division at the vegetal pole. This stereotypical pattern of cleavage plays an important role in PMC specification. The unequal fourth cleavage division is a consequence of the displacement of the nuclei and mitotic spindles of the four vegetal blastomeres of the 8-cell stage embryo toward the vegetal pole (Dan and Tanaka, 1990). These cytological changes might be dependent upon a specialized microtubule attachment site in the vegetal cortex. Pharmacological agents have been used to inhibit the displacement of the mitotic spindles toward the vegetal pole, thereby equalizing cleavage and producing micromere-less embryos (Tanaka, 1976; Dan, 1979; Tanaka, 1979; Langelan and Whiteley, 1985). Micromere-less embryos show a striking reduction in the development of the skeleton. These studies preceded the recent elucidation of the skeletogenic GRN, and no linkage between unequal cleavage and specific steps in the molecular specification of PMCs has been established.

Here, we focus on the initial deployment of the large micromere PMC GRN. Our findings lead to a significant revision of the current model of the activation of this GRN. We show that the lineage-specific expression of at least two early genes in the network, *alx1* and *delta*, occurs by a mechanism that is distinct from the transcriptional repression of *hesC*. We confirm that *alx1* is regulated by MAPK signaling and by the transcription factor Ets1, but show that these inputs influence only the maintenance of *alx1* expression, and not its activation, which occurs by a distinct mechanism. By experimentally altering normal cell division patterns, we show that the initial expression of *alx1* and *delta* is linked tightly to the unequal cleavage of vegetal blastomeres. Surprisingly, the activation of *pmar1* is not dependent upon unequal cleavage.

MATERIALS AND METHODS
Animals
Adult *Lytechinus variegatus* were obtained from the Duke University Marine Laboratory (Beaufort, NC, USA) and from Reeftopia Inc. (Key West, FL, USA). Adult *Strongylocentrotus purpuratus* were obtained from Patrick Leahy (California Institute of Technology, USA). Embryos were cultured at 23°C (*L. variegatus*) or 15.5°C (*S. purpuratus*) in temperature-controlled incubators.

Constructs and mRNA injections
Capped mRNAs were synthesized using the SP6 mMessage mMachine RNA Transcription Kit (Ambion) and were microinjected into fertilized eggs as described by Cheers and Ettensohn (Cheers and Ettensohn, 2004). A C-terminal, GFP-tagged form of Pmar1 (coding region only) was cloned into the *Bam*HI and *Xba*I sites of the pCS2+ vector.

Equalization of cleavage
The fourth and fifth cleavage divisions were equalized by treating *L. variegatus* embryos for 1 hour with sodium dodecyl sulfate (SDS) at a concentration of 30 μg/ml, beginning at the 4-cell stage (Langelan and Whiteley, 1985). The embryos were then transferred to artificial sea water (ASW) without SDS and were allowed to continue development.

Polymerase chain reaction (PCR)
Total RNA was isolated using the Nucleospin RNA II Kit (Clontech). cDNA synthesis was carried out using the RETROscript Kit (Ambion) and HiFi Taq polymerase (Invitrogen). Quantitative PCR (qPCR) was performed using an ABI 7300 real-time PCR system and SYBR-Green/ROX Master Mix (Bio-Rad). PCR primers are shown in Table S1 in the supplementary material.

Whole-mount in situ hybridization (WMISH)
Conventional WMISH was carried out as described previously (Ettensohn et al., 2007). For single-color, fluorescent WMISH (F-WMISH), embryos were incubated in a blocking buffer that comprised 5% lamb serum in phosphate buffered saline containing 0.1% Tween-20 (PBST) for 30 minutes at room temperature (RT), followed by incubation in a 1:1500 dilution of horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibody (Roche) in blocking buffer for 30 minutes at RT. The embryos were then incubated in a 1:100 dilution of FITC-Tyramide Signal Amplification Solution (Fluorescein-TSA Plus Fluorescence System, PerkinElmer) in the diluting buffer provided with the kit for 4 minutes at RT. The embryos were counterstained with Hoechst 33342 (0.5 μg/ml) in PBST for 5 minutes, followed by several washes with PBST. For two-color F-WMISH, embryos were incubated overnight with a mixture of a (DIG)-11-UTP-labeled probe and a fluorescein-labeled probe. After incubating the embryos in blocking buffer as described above, the embryos were incubated in a 1:750 dilution of HRP-conjugated anti-fluorescein antibody in PBST for 2 hours, followed by a 4-minute incubation (RT) with a 1:100 dilution of Cy3-TSA in the diluting buffer provided with the kit (Cy3-TSA Plus Fluorescence System, PerkinElmer). Peroxidase activity was quenched by incubating the embryos in 5% (v/v) H_2O_2 in PBST for 30 minutes. The transcripts for the second gene were then detected using the HRP-conjugated anti-digoxigenin antibody and the fluorescein-TSA Plus Fluorescence System, as described above.

Microscopy and quantitative image analysis
Embryos labeled by F-WMISH were examined using a Zeiss LSM 510 metal/UV DuoScan spectral confocal microscope and a 40× oil immersion lens. Embryos that had been double-labeled with *hesC* and *alx1* probes were used to measure the levels of *hesC* mRNA in the large micromere territory and in the remainder of the embryo. ImageJ was used to generate two-dimensional projections of small stacks (3-5 images with a 1 μm spacing) of confocal sections that approximately bisected the region of *alx1* expression (the large micromere territory). For each projection, we used ImageJ to calculate the average pixel intensity of *hesC* signal in the cells that also expressed *alx1* and in the remainder of the embryo. These two values were obtained from a total of 6-12 confocal image stacks, each of which was collected from a different embryo. We then calculated the mean and standard deviations of the values for the two regions and compared them using a paired, two-sided *t*-test. In control experiments using single probes, we confirmed that there was no detectable spill-over between the *alx1* and *hesC* channels. In addition, controls processed in the absence of probe indicated that there was no detectable background signal in either channel. In all experiments, pixel intensities were below saturation.

RESULTS
The activation of *alx1* and *delta* precedes the clearing of *hesC* transcripts from the large micromere territory
Several early genes in the skeletogenic GRN, including *alx1*, *tbr*, *delta* and *ets*, are thought to be activated via the transcriptional repression of *hesC* (Oliveri et al., 2008). *Tbr* and *ets* mRNAs are abundant maternally and are ubiquitous in the early embryo, making it difficult to pinpoint the time at which these genes are first activated in the large micromere territory. *alx1* and *delta*, by contrast, are expressed only zygotically. We reported previously that *Spalx1* mRNA accumulates in the four large micromeres in the first cell cycle after these cells are born (Ettensohn et al., 2003). This early onset of expression suggested to us that the activation of *alx1* transcription might precede the loss of *hesC* mRNA from vegetal blastomeres.

To compare directly the dynamic patterns of *Spalx1* and *SphesC* expression in the same embryo, we used two-color F-WMISH. The domain of *Spalx1* expression served as an unambiguous marker of the large micromere territory. Quantification of the F-WMISH signals confirmed that when *Spalx1* mRNA was first detectable at

the 56-cell stage (8 hours post-fertilization, or hpf), the level of *SphesC* mRNA in the large micromere territory was equivalent to that in the other cells of the embryo (Fig. 1A,A′,F). At the mid-blastula stage (10-12 hpf), *SphesC* transcripts were downregulated in the central region of the vegetal plate; i.e. in a region that contained the large micromere, as shown by a decrease in the average pixel intensity of the F-WMISH signal (Fig. 1B,B′,F).

To determine whether this temporal pattern of gene expression was shared by other sea urchin species, we carried out a similar analysis using *L. variegatus* embryos. *LvhesC* was cloned using degenerate RT-PCR and RACE (random amplification of cDNA ends). A comparison between *SphesC* and *LvhesC* revealed that the two genes were ~80% identical at the nucleotide level (see Fig. S1 in the supplementary material) and showed that they encoded proteins with ~85% amino acid identity (see Fig. S2 in the supplementary material). Phylogenetic analysis using ClustalW and

the Phylogenetic Analysis Using Parsimony (PAUP) software demonstrated that *LvhesC* and *SphesC* are orthologous genes (see Fig. S3 in the supplementary material). We examined the developmental expression of *LvhesC* by WMISH and found that the pattern was very similar to that of *SphesC* (see Fig. S4 in the supplementary material).

F-WMISH confirmed that *Lvalx1* was activated specifically in the eight daughter cells of the large micromeres, beginning at ~5 hpf (seventh cleavage), as reported previously (Ettensohn et al., 2003). Quantitative, two-color F-WMISH showed that, at this stage, the level of *LvhesC* mRNA in the large micromere progeny was equivalent to that in the remainder of the embryo (Fig. 1C,C′,F). At the hatched blastula stage (8 hpf), *LvhesC* transcripts were downregulated in the central region of the vegetal plate (Fig. 1D,D′,F). *LvhesC* expression also declined in the apical plate region, as has been described in *S. purpuratus* (Smith and Davidson, 2008).
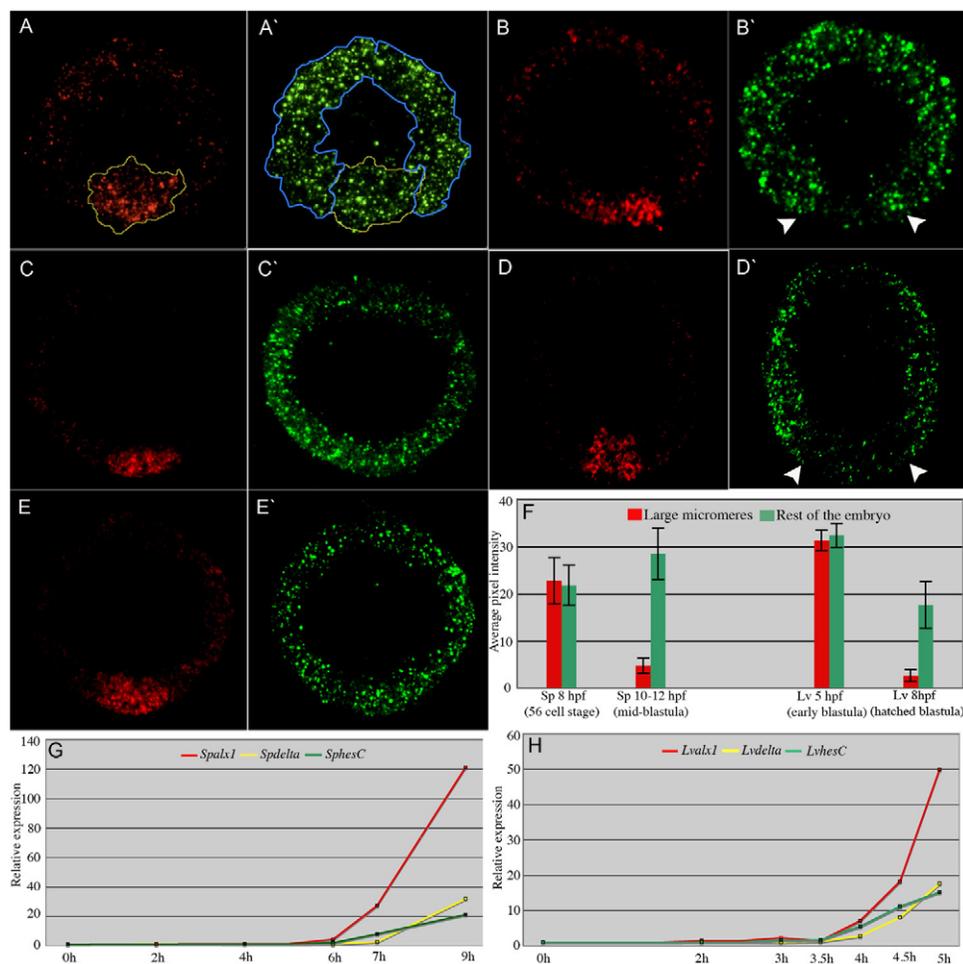
Fig. 1. Accumulation of *alx1* transcripts precedes the clearing of *hesC* mRNA from the large micromere territory. Two-color F-WMISH was performed using digoxigenin-labeled *hesC* probes (green) and fluorescein-labeled *alx1* or *delta* probes (red). Each image is a projection of 3-5 confocal sections. (**A-B′**) *Spalx1* and *SphesC* expression in *S. purpuratus*, shown at 8 hpf (56-cell stage; A,A′) and 10-12 hpf (mid-blastula stage; B,B′). (**C-D′**) *Lvalx1* and *LvhesC* expression in *L. variegatus*, shown at 5 hpf (128 cell stage; C,C′) and 8 hpf (mid-blastula stage; D,D′). (**E,E′**) *Lvdelta* and *LvhesC* expression at 5 hpf (128-cell stage). Arrowheads in B′ and D′ indicate the vegetal region of *hesC* clearing. (**F**) Quantification of *hesC* F-WMISH signal. Average pixel intensities were determined for the large micromere territory (i.e. the *alx1*-expressing region) and for the remainder of the embryo, as illustrated in A and A′ (see Materials and methods). For each species and time point analyzed, data from 6-12 different embryos were used to calculate mean average pixel intensities for the two regions (red and green bars). Black bars show standard errors. (**G,H**) qPCR analysis of *alx1*, *delta* and *hesC* expression in *S. purpuratus* (G) and *L. variegatus* (H). For each time point, the average C_t value for each gene was normalized against the average C_t value of an internal standard mRNA (*ubiquitin* for *L. variegatus* and *z12* for *S. purpuratus*). Values shown on the *y*-axis reflect relative numbers of *hesC* transcripts at the various stages, with the maternal expression level arbitrarily set to 1.

These studies confirmed that in *L. variegatus*, as in *S. purpuratus*, *alx1* was activated selectively in the large micromere progeny prior to the clearing of *hesC* transcripts from these cells.

In *L. variegatus*, expression of *delta* in the large micromere territory begins at ~5 hpf (Sweet et al., 2002). We carried out two-color F-WMISH analysis of *L. variegatus* embryos labeled with *Lvdelta* and *LvhesC* probes, and found that expression of *LvDelta* in the large micromere territory also preceded the clearing of *LvhesC* transcripts from the vegetal region (Fig. 1E,E'). Although we did not carry out equivalent, double-label F-WMISH studies of *delta* and *hesC* expression in *S. purpuratus*, it has already been reported that *Spdelta* is first transcribed at the late fifth cleavage stage (8 hours to 8 hours, 40 minutes after fertilization) (Smith and Davidson, 2008). This is the same cleavage division at which we first detect *Spalx1* expression in the large micromeres, and 2-3 hours before *SphesC* mRNA clears from the micromere territory (Fig. 1A,A',F). Therefore, these studies indicate that at least two early genes in the skeletogenic GRN, *alx1* and *delta*, are activated in the large micromeres prior to any measurable decline in *hesC* transcript levels.

In *S. purpuratus*, *hesC* transcripts are present maternally at relatively low levels (~500 transcripts/egg) and increase sharply in abundance beginning at about the 56-cell stage, indicating that zygotic transcription of the gene is taking place by this stage (Revilla-i-Domingo et al., 2007). We used qPCR to compare directly the temporal patterns of *hesC*, *alx1* and *delta* expression in both *S. purpuratus* and *L. variegatus* (Fig. 1G,H). Activation of *alx1* and *hesC* transcription occurred at approximately the same time in both species, while *delta* lagged slightly behind. Significantly, at the same developmental stages that were used for our quantitative F-WMISH analysis of *hesC* expression prior to vegetal clearing (i.e. at 8 hpf in *S. purpuratus* and 5 hpf in *L. variegatus*), *hesC* transcript levels were more than 10-fold higher than maternal levels. Therefore, these qPCR studies showed that, in both species, *hesC* transcripts that were visualized by F-WMISH at the developmental stages shown in Fig. 1 were predominantly zygotic in origin. We conclude that *hesC* is transiently transcribed in the large micromere territory and that the lineage-specific activation of the skeletogenic GRN occurs at a stage when *hesC* mRNA is uniformly distributed throughout the embryo.

Ets1 is not required for the onset of *Lvalx1* expression, but is required for its maintenance

The observation that *Lvalx1* is activated before the clearing of *LvhesC* transcripts from the large micromere territory prompted us to examine other factor(s) that might trigger the onset of *Lvalx1* expression. Ets1 has been identified as a positive regulator of *alx1* (Rottinger et al., 2004; Oliveri et al., 2008) and the presence of maternal *ets1* transcripts was demonstrated previously in the sea urchin (Kurokawa et al., 2000; Oliveri et al., 2008). To test whether maternal Ets1 protein plays a role in activating *alx1*, we carefully compared the spatial and temporal patterns of expression of the mRNAs and proteins encoded by *Lvets1* and *Lvalx1*. WMISH studies confirmed that *Lvets1* mRNA was expressed ubiquitously in *L. variegatus* embryos until ~6 hpf (mid-blastula stage; Fig. 2A). At this developmental stage, very strong expression of *Lvalx1* mRNA was apparent in the large micromere territory in sibling embryos (Fig. 2C). At the hatched blastula stage (8 hpf), *Lvets1* mRNA, like *Lvalx1* mRNA, was restricted to the large micromere lineage (Fig. 2B,D).

We used a polyclonal antiserum that recognizes LvEts1 (Ettensohn et al., 2007) to examine the distribution of this protein at different developmental stages. Immunostaining studies showed that
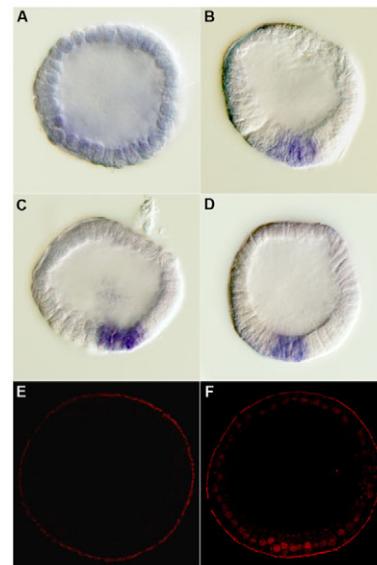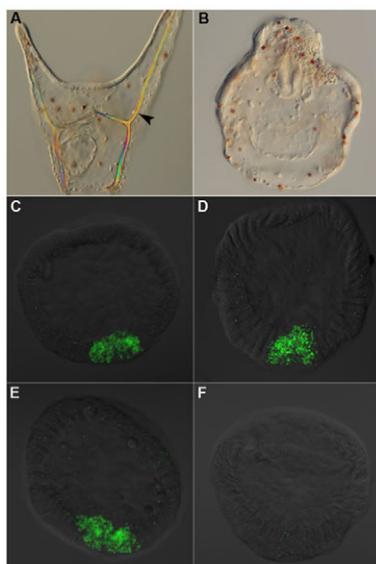

Fig. 2. *Lvalx1* mRNA expression precedes the nuclear localization of LvEts1 protein. (**A-D**) WMISH analysis of *Lvets1* (A,B) and *Lvalx1* (C,D) expression. Maternal *Lvets1* mRNA is present in all cells of the embryo until 6 hpf (mid-blastula stage; A), but by 8 hpf (hatched blastula stage), maternal transcripts decline in most cells and the zygotic expression of *Lvets1* is restricted to the large micromere territory (B). In sibling embryos, strong expression of *Lvalx1* is seen in the large micromere territory at 6 hpf (C) and at 8 hpf (D). (**E,F**) Immunolocalization of LvEts1 protein. Nuclear LvEts1 protein is not detectable by immunostaining at 6 hpf (E), a stage at which *Lvalx1* transcripts are already strongly expressed. At 8 hpf, LvEts1 protein is concentrated in the nuclei of all blastomeres and is present at the highest levels in presumptive PMCs (F).

LvEts1 was not detectable in nuclei at 6 hpf (Fig. 2E). At 8 hpf, however, the protein was concentrated in the nucleus of every blastomere and was visibly enriched in the nuclei of the large micromere progeny (Fig. 2F). We do not know whether the expression of LvEts1 protein outside the large micromere territory at this stage is a consequence of the translation of ubiquitous maternal transcripts or reflects transient widespread zygotic transcription of *Lvets1* during early development. If the former is the case, then the reason for the significant lag in the nuclear accumulation of the protein following fertilization is unclear. In any event, the absence of detectable nuclear LvEts1 protein at 6 hpf, when *Lvalx1* transcripts are expressed at very high levels, suggests that LvEts1 does not play a role in the onset of *Lvalx1* transcription.

To test more directly the role of LvEts1 in the onset of *Lvalx1* expression, we overexpressed a dominant-negative form of Ets1 that lacked the N-terminal activation domain (dnLvEts1) (Kurokawa et al., 1999). We considered it probable that this dominant-negative form would interfere with the function of both maternal and zygotic pools of endogenous LvEts1 protein. Consistent with previous findings in a different species (Kurokawa et al., 1999), embryos injected with mRNA encoding dnLvEts1 failed to form PMCs and completely lacked skeletal elements, even after prolonged periods in culture (Fig. 3A,B). F-WMISH analysis showed, however, that there was no change in *Lvalx1* expression at 6 hpf in dnLvEts1-expressing embryos as compared with sibling control embryos that had been injected with 20% glycerol (Fig. 3C,E). Soon thereafter,

the expression of *Lvalx1* declined markedly in embryos expressing dnLvEts1, and by 8 hpf, *Lvalx1* transcripts were no longer detectable by F-WMISH (Fig. 3D,F). These findings indicate that LvEts1 is required for the maintenance, but not for the activation, of *Lvalx1* expression.

Misexpression of wild-type Ets1 results in the formation of supernumerary mesenchymal cells (Kurokawa et al., 1999; Rottinger et al., 2004). The precise fate of these cells is unclear; it has been reported that they express some skeletogenic genes but not others (Kurokawa et al., 1999). Based on these findings, it seemed possible that misexpression of Ets1 might be sufficient to activate *alx1* in non-micromere-derived cells. We tested this possibility by injecting mRNA (8 mg/ml) encoding wild-type LvEts1 into fertilized eggs. We chose this concentration because it reliably induced the formation of large numbers of supernumerary mesenchymal cells in ~75-80% of injected embryos. *Lvalx1* expression was analyzed by WMISH at the hatched blastula stage and during the phase of supernumerary mesenchymal cell ingression. These studies showed that there was no expansion of the expression domain of *Lvalx1* at either developmental stage (Fig. 4C,D). By contrast, >50% of the injected embryos showed a moderate reduction in the number of cells expressing *Lvalx1* as compared with sibling uninjected embryos (*n*=35 at each developmental stage). We also examined skeletogenic specification in these embryos by immunostaining with monoclonal antibody 6a9, an antibody that recognizes MSP130 family proteins. Again, we observed no increase in the numbers of skeletogenic cells (data not
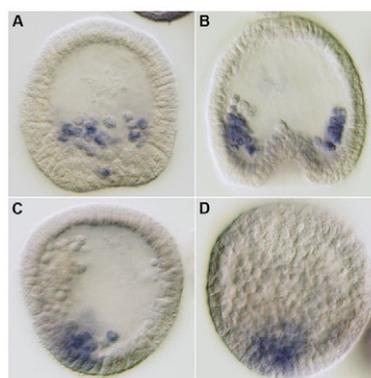
shown). These findings indicate that the striking conversion of cells to a mesenchymal phenotype induced by the misexpression of Ets1 is not accompanied by the ectopic activation of *alx1*. Taken together, our studies suggest that LvEts1 is neither necessary nor sufficient for the initial activation of *Lvalx1*, although the protein plays an important role in maintaining *Lvalx1* expression in the large micromere territory.

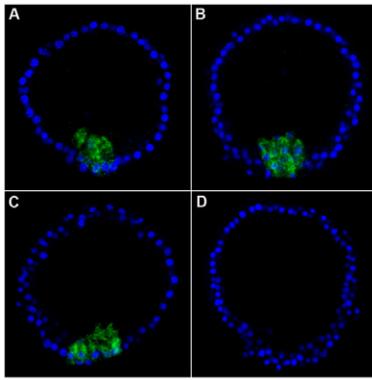## MAPK signaling is required for the maintenance, but not for the activation, of *Lvalx1* expression

Rottinger et al. (Rottinger et al., 2004) showed that *alx1* expression was inhibited when embryos were treated with U0126, a MEK inhibitor. They provided evidence that one important role of MAPK signaling is to promote the phosphorylation of Ets1. Previous work showed that U0126 has the same general effects on embryonic development (i.e. a complete inhibition of PMC formation and skeletogenesis) in *L. variegatus* as it does in other species, and is effective at similar concentrations (Ettensohn et al., 2007). We analyzed the effect of U0126 on *alx1* expression in *L. variegatus*, focusing specifically on the initial phase of expression. Embryos treated continuously with 6 µM U0126 from the 2-cell stage exhibited strong *Lvalx1* expression at 7 hpf (late blastula stage) (Fig. 5C). Such embryos were indistinguishable from control embryos treated with DMSO alone (Fig. 5A). By contrast, by 9 hpf (hatched blastula stage), no *Lvalx1* expression was detectable in U0126-treated embryos (Fig. 5B,D). The effects of U0126 on *Lvalx1* expression were therefore very similar to those of dnLvEts1 and reinforced the view that the activation and the maintenance of *Lvalx1* expression are controlled by different mechanisms.

The expression of *Lvalx1* and *Lvdelta*, but not that of *Lvpmar1*, is dependent upon unequal cell division

To determine whether the unequal cleavage divisions that occur at the vegetal pole influence the activation of *alx1* expression, we equalized both the fourth and fifth cleavage divisions using low

Fig. 3. LvEts1 is required for the maintenance, but not for the activation, of *Lvalx1* expression. (**A**) Control pluteus larva at 72 hpf. Arrowhead indicates skeletal rods. (**B**) Sibling embryo expressing *dnLvets1*, also at 72 hpf. *dnLvets1* mRNA-injected embryos fail to form skeletal elements even after prolonged periods in culture. (**C-F**) *Lvalx1* expression in embryos overexpressing *dnLvets1*. (C,D) Control embryos injected with 20% glycerol alone and examined at 6 hpf (mid-blastula stage; C) and 8 hpf (hatched blastula stage; D). (E,F) Embryos injected with *dnLvets1* mRNA and examined at 6 hpf (E) and 8 hpf (F). Each panel is a merged image of a *z*-projection of several confocal slices and a single DIC image taken at the midpoint of the stack. *dnLvets1* mRNA-injected embryos exhibit normal levels of *Lvalx1* expression at 6 hpf (compare C with E), but by 8 hpf, *Lvalx1* expression is no longer detectable (compare D with F).
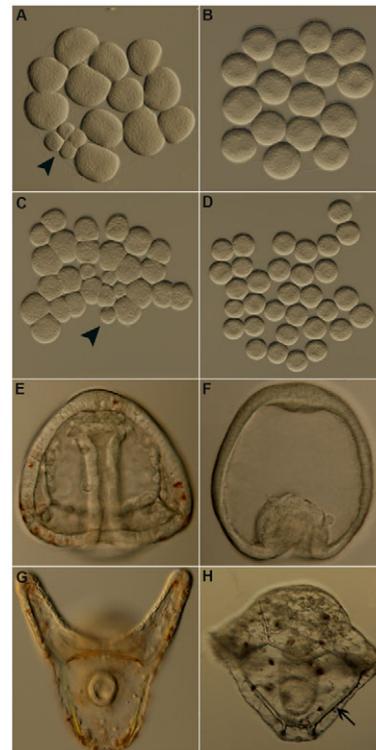

Fig. 4. Misexpression of *Lvets1* mRNA converts many cells of the embryo to a mesenchymal fate but *Lvalx1* expression remains restricted to the large micromere lineage. *Lvalx1* expression was examined in *Lvets1* mRNA-injected embryos when sibling control embryos were at the mesenchyme blastula (MB) or early gastrula (EG) stages. (**A**,**B**) Control embryos showing normal expression of *Lvalx1* at the MB stage (A) and the EG stage (B). (**C**,**D**) *Lvets1* mRNA-injected embryos showing that *Lvalx1* expression is restricted to the PMCs at the MB stage (C) and later in development, when additional mesenchymal cells form (D).

Fig. 5. The MAPK pathway is required for the maintenance of *Lvalx1* expression. Embryos were treated with 6 μm U0126 and *Lvalx1* expression was assayed at 7 hpf (hatched blastula stage) and 9 hpf (pre-ingression blastula stage). Each panel is a merged image of *z*-projections of confocal stacks (green, *Lvalx1*; blue, Hoechst). (**A**,**B**) DMSO-treated control embryos showing normal *Lvalx1* expression (green) at 7 hpf (A) and 9 hpf (B). (**C**,**D**) U0126-treated embryos showing normal expression of *Lvalx1* at 7 hpf (C) but a striking loss of *Lvalx1* expression by 9 hpf (D).

Fig. 6. SDS treatment equalizes the fourth and fifth cleavage divisions and blocks PMC formation in *L. variegatus*. (**A-H**) Embryos were visualized using DIC optics; cleavage-stage embryos shown in A-D were flattened with a coverslip. Left panels show control embryos at the 16-cell (A), 28-cell (C), late gastrula (E) and pluteus stages (G). Arrowheads indicate micromeres. Right panels show sibling SDS-treated embryos at the same developmental stages. In most SDS-treated embryos, all cells are approximately equal in size after the fourth and fifth cleavage divisions (B,D). These embryos lack PMCs (F) and form reduced skeletons (H, arrow).

concentrations of SDS. Approximately 72% of SDS-treated embryos (*L. variegatus*) underwent an equal fourth cleavage division and ~67% of the treated embryos underwent equal divisions at both the fourth and fifth cleavages (Fig. 6A-D). The remaining embryos formed variable numbers of micromeres. As reported by Langelan and Whiteley (Langelan and Whiteley, 1985), SDS-treated embryos lacked PMCs, gastrulated in a delayed fashion and formed spicules after a considerable delay (Fig. 6E-H). Skeleton formation in these embryos was probably a consequence of the transfating of non-micromere-derived cells. F-WMISH analysis of SDS-treated embryos revealed a striking reduction in *Lvalx1* expression (Fig. 7D,E) compared with sibling controls (Fig. 7A,B) at 8 hpf (hatched blastula stage) and 10 hpf (pre-ingression blastula stage). Only 30-35% of the SDS-treated embryos exhibited any detectable *Lvalx1* expression at these stages. At 12 hpf, when sibling control embryos were at the mesenchyme blastula stage (Fig. 7C), *Lvalx1* expression was detectable in ~50% of the SDS-treated embryos, but usually in a much smaller number of cells than in control embryos (Fig. 7F). The residual expression of *Lvalx1* at early developmental stages in SDS-treated embryos is probably a consequence of the incomplete effect of the detergent on the pattern of cleavage.

We next asked whether the effect of the cleavage pattern on *Lvalx1* activation might be mediated by *pmar1*. Two hundred control embryos and 200 sibling SDS-treated embryos were collected at 6 hpf (mid-blastula stage) and the expression of *Lvpmar1*, *Lvalx1* and *Lvdelta* was assessed by RT-PCR using equivalent serial dilutions of the cDNA samples. SDS had no effect on *Lvpmar1* expression, but the expression of *Lvalx1* and *Lvdelta* was clearly reduced (Fig. 7G). We also examined *Lvpmar1* expression at earlier developmental stages (3-4 hpf) and found, in three independent trials, that equal cleavage had no effect on transcript levels (data not shown). The effect of SDS on *Lvpmar1*, *Lvalx1* and *Lvdelta* expression was confirmed by qPCR (Fig. 7H). These experiments showed that, although *pmar1* is ordinarily expressed specifically in the micromeres immediately after they form, unequal cleavage is not required for the

transcriptional activation of this gene. The level of *Lvpmar1* expression on a per-embryo basis was not affected by SDS-treatment, but the level of expression per cell might have been altered owing to changes in cell size. Unlike *Lvpmar1*, *Lvalx1* and *Lvdelta* were dependent on unequal cell division for their activation; this might have been a consequence of changes in the level of *pmar1* expression per cell, or could have occurred by other mechanisms.

Pmar1 protein is stable in both the large and small micromeres

Pmar1 mRNA is detectable in the micromeres immediately after they are born and continues to be expressed in both the large and small micromeres until the blastula stage (Oliveri et al., 2002). By contrast, *alx1* and *delta* are activated specifically in the large micromeres and are restricted to this lineage throughout later development (Sweet et al., 2002; Ettensohn et al., 2003). Misexpression studies show that Pmar1 is sufficient to activate the GRN widely throughout the embryo; why then does Pmar1 not ordinarily activate *alx1* and *delta* in the small micromeres? One possibility is that, despite the presence of *pmar1* mRNA in the small micromeres, post-transcriptional mechanisms prevent the accumulation of functional Pmar1 protein in these cells.
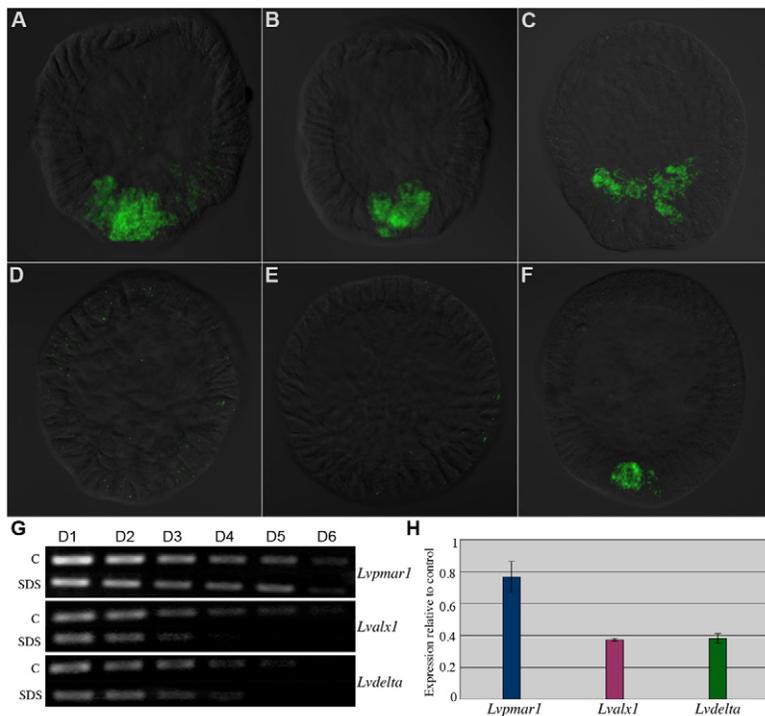
Fig. 7. The activation of *Lvalx1* and *Lvdelta*, but not that of *Lvpmar1*, is dependent on unequal cleavage division. (**A-F**) F-WMISH analysis of *Lvalx1* expression in SDS-treated embryos. Each photomicrograph is a merged image of a *z*-projection of a confocal stack and a single DIC section at the midpoint of the stack. (A-C) Control embryos showing normal *Lvalx1* expression at 8 hpf (hatched blastula; A), 10 hpf (pre-ingression blastula; B) and 12 hpf (mesenchyme blastula; C). (D-F) Sibling SDS-treated embryos at 8 hpf (D), 10 hpf (E) and 12 hpf (F). *Lvalx1* expression is not detectable at 8 hpf or 10 hpf. At 12 hpf, ~50% of the SDS-treated embryos lack any *Lvalx1*-positive cells, while the remaining embryos have greatly reduced numbers of *Lvalx1*-positive cells (an example of such an embryo is shown in F). (**G,H**) Analysis of gene expression in equally cleaving embryos. Total RNA was isolated from 200 SDS-treated embryos and 200 control embryos at 6 hpf. The expression of *Lvpmar1*, *Lvdelta* and *Lvalx1* was analyzed by RT-PCR using serial dilutions (D1-D6) of the two cDNA samples (G), and by qPCR (H). SDS treatment had no effect on *Lvpmar1* expression, but levels of *Lvalx1* and *Lvdelta* mRNA were significantly reduced. The bars in H show levels of expression in SDS-treated embryos relative to sibling controls. Standard errors based on two independent trials are also indicated.

First, to further support the idea that *pmar1* provides a positive regulatory input into *alx1*, we used F-WMISH to show that overexpression of *Lvpmar1* was sufficient to activate *Lvalx1* throughout the embryo (Fig. 8A,B). This finding supported earlier studies demonstrating an increase in *alx1* mRNA levels as measured by qPCR (Ettensohn et al., 2003), and experiments showing that misexpression of *pmar1* induces the ectopic activation of *delta* (Oliveri et al., 2002). Selective protein degradation regulates the expression of other transcription factors in the sea urchin embryo (Weitzel et al., 2004; Angerer et al., 2005). Therefore, to test whether Pmar1 might be selectively degraded in the small micromeres, we microinjected mRNA encoding a GFP-tagged form of LvPmar1 (coding region only) into fertilized eggs and monitored the expression of fluorescent protein in living embryos by confocal microscopy. LvPmar1-GFP was expressed in all cells of the embryo during cleavage, including both the large and small micromere lineages (Fig. 8C,D), and continued to be stably expressed in both territories until at least the blastula stage, after the onset of *Lvalx1* expression. This pattern of protein expression contrasts with that of other GFP-tagged transcriptional regulators; for example, β-catenin-GFP is rapidly degraded in animal blastomeres during early cleavage (Weitzel et al., 2004). These findings argue against the hypothesis that LvPmar1 is degraded selectively in the small micromeres and indicate that other mechanisms prevent the activation of the GRN in these cells.

DISCUSSION

Much progress has been made in dissecting the GRN that underlies the development of the skeletogenic primary mesenchyme (Oliveri et al., 2008; Ettensohn, 2009). The identification of Pmar1 as an important early activator of this pathway, combined with evidence that this protein functions as a transcriptional repressor, led to the hypothesis that Pmar1 activates the GRN by blocking the expression of a second repressor. HesC has been identified as this second

repressor based on the following criteria: (1) *hesC* transcript levels are downregulated following forced misexpression of Pmar1; (2) *hesC* transcripts disappear from the micromere territory during early development; and (3) MO-mediated inhibition of HesC translation results in an increase in mesenchymal cells and in the ectopic expression of *delta* (Revilla-i-Domingo et al., 2007). Based on these findings, *hesC* has been considered the linchpin of a double-repression gate, and the evolutionary invention of the *pmar1*/*hesC* gate has been put forward as a pivotal event during the evolution of skeletogenesis in sea urchins (Davidson and Levine, 2008; Gao and Davidson, 2008).

Our WMISH studies show that the activation of *alx1* and *delta* selectively in the large micromere territory occurs prior to the clearing of *hesC* mRNA from these cells. We considered the possibility that maternal *hesC* transcripts might be non-translatable, or could encode a non-functional form of the HesC protein, and that WMISH signal from maternal transcripts might prevent us from detecting a local repression of zygotic *hesC* transcription. qPCR studies, however, showed that levels of maternal hesC transcripts were low (see also Revilla-i-Domingo et al., 2007) and that at the developmental stages that were used for quantitative F-WMISH analysis, most *hesC* transcripts were zygotic in origin. Therefore, our combined qPCR and WMISH data indicate that *hesC* is transiently transcribed in the large micromere territory and that *alx1* and *delta* are expressed selectively by these cells at a stage when zygotic *hesC* transcripts are ubiquitous. Our qPCR analysis also showed that the activation of *alx1* occurred at approximately the same time as the activation of *hesC* (Fig. 1G,H). It is therefore difficult to envision how regional differences in *hesC* transcription, which would require some time to produce differences in HesC protein levels, could influence the early spatial pattern of *alx1* expression. These considerations, however, do not preclude an essential role for *hesC* repression in the later maintenance phase of *alx1* expression (see below).
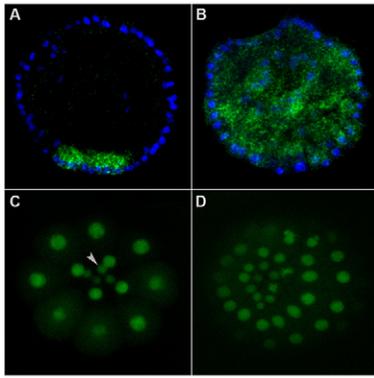
Fig. 8. Ectopically expressed LvPmar1 is stable in all cells and activates *Lvalx1* expression. (**A**,**B**) *Lvpmar1* mRNA was injected into fertilized eggs (B) and *Lvalx1* expression was assessed by F-WMISH at 6 hpf (mid-blastula stage). Merged images of confocal stacks are shown (green, *Lvalx1*; blue, Hoechst). Control embryos show expression of *Lvalx1* in the large micromere territory at 6 hpf (A), whereas misexpression of LvPmar1 induces *Lvalx1* expression in all cells (B). (**C**,**D**) LvPmar1 protein is stable in both the large and small micromeres. Fertilized eggs were injected with mRNA encoding the coding region of LvPmar1 fused to GFP and living embryos were examined by confocal microscopy. Projections of the vegetal hemisphere of a 32-cell stage embryo (D) and an ~128-cell stage embryo (E) are shown, viewed from the vegetal pole. LvPmar1-GFP is stable in all vegetal blastomeres, including the small micromeres (arrowhead in C).

Considerable evidence indicates that *pmar1* is a pivotal regulator of the micromere-PMC GRN. Misexpression of *pmar1* is sufficient to activate the skeletogenic GRN in non-micromere-derived cells (Oliveri et al., 2002; Oliveri et al., 2003; Nishimura et al., 2004; Yamazaki et al., 2005; Yamazaki et al., 2009) (this study). It has been more difficult to test directly the function of *pmar1* in the large micromere territory (where the gene is ordinarily expressed) owing to the difficulty in blocking the expression of multiple tandem *pmar1* genes using MOs. A VP16-Pmar1 fusion protein, which probably acts in a dominant-negative fashion, blocks PMC formation and reduces the levels of several skeletogenic mRNAs, at least at late developmental stages (Yamazaki et al., 2005). This loss-of-function analysis supports the view that *pmar1* is required for the deployment of the skeletogenic GRN in the large micromere territory. Pmar1 (a known repressor) presumably mediates the activation of *alx1*, *delta* and other early genes in the network by blocking the expression of a second repressor, as discussed by Oliveri et al. (Oliveri et al., 2002). Our findings therefore point to one of two possibilities: (1) the existence of an as-yet-undiscovered repressor downstream of *pmar1* but distinct from *hesC*, or (2) a *pmar1*-independent mechanism of GRN activation, which might be reinforced later in development by the *pmar1-hesC* derepression system.

The conclusion that the skeletogenic GRN is activated by mechanisms that are independent of *hesC* repression seems at odds with evidence that inhibition of *hesC* function is sufficient to activate the GRN ectopically (Revilla-i-Domingo et al., 2007; Smith and Davidson, 2008). It should be noted that in a recent structure-function analysis of Pmar1, one mutant construct (N-HD-A-C) that lacked a portion of the C-terminal region of the protein was reported to downregulate *hesC* mRNA levels throughout the embryo without expanding the expression of *alx1*, *tbr* or *ets1* (Yamazaki et al., 2009).

This observation suggests that downregulation of *hesC* might not be sufficient to activate the skeletogenic GRN in non-micromere lineages. If, as the earlier data suggest, inhibition of *hesC* function is sufficient to activate the network, then one interpretation is that *alx1*, *delta* and other early genes can be activated by more than one regulatory mechanism. According to this view, the repression of *hesC* is sufficient to activate these genes in non-micromere lineages, but during normal development (i.e. in the large micromere lineage), a separate regulatory pathway is used very early in development that activates the network, essentially bypassing *hesC* repression, which is relegated to a maintenance function. A prediction of this view is that genes such as *alx1* and *delta* will have multiple activation modules, any one of which can activate transcription once engaged. The cis-regulatory architecture of *alx1* has not been described, but *delta* has been analyzed extensively in this regard (Revilla-i-Domingo et al., 2004; Revilla-i-Domingo et al., 2007; Smith and Davidson, 2008). Consistent with the above model, two separate regulatory modules have been identified that are sufficient to drive expression of *delta* in the micromere territory; both modules are responsive to *pmar1* but only one contains putative HesC binding sites.

Our findings reinforce the view that the activation and the maintenance of *alx1* expression are controlled by very different mechanisms. Positive inputs into *alx1* from MAP kinase signaling and from *ets1* (inputs that might be related to one another) regulate the maintenance, but not the onset, of *alx1* expression. One essential early input into *alx1* that we have identified is a cellular, rather than molecular, one; namely, the unequal cleavage of vegetal blastomeres. It was shown previously that PMC specification is influenced by the cleavage pattern (Langelan and Whiteley, 1985), but this work predated the elucidation of the skeletogenic GRN, and ours is the first effort to analyze the molecular step(s) at which unequal cell division impinges on the network. Surprisingly, although *pmar1* is ordinarily activated in the micromeres immediately as they form, unequal cleavage is not required for the transcriptional activation of *pmar1*. Instead, a molecular step between *pmar1* expression and the activation of *alx1* and *delta* is linked to unequal cleavage. Whatever the regulatory connection, it is likely to be a novel feature of echinoid development. The embryos of a related group of echinoderms, the ophiuroids (brittle stars), exhibit equal cleavage, but nevertheless form PMCs and an embryonic skeleton [see Tominaga et al. (Tominaga et al., 2004) and references therein]. Evolution has evidently experimented freely with the upstream regulation of the skeletogenic GRN (Gao and Davidson, 2008; Ettensohn, 2009).

A key unanswered question is why the skeletogenic GRN is activated only in the large micromere territory, when *pmar1* mRNA is present in both the large and small micromeres (Oliveri et al., 2002). A variety of mechanisms can be envisioned that involve the asymmetric segregation of polarized maternal determinants, differences in nucleus-to-cytoplasm ratio and other mechanisms. Interestingly, during normal development, *hesC* expression persists in the small micromere territory after it is extinguished elsewhere in the vegetal plate (see Fig. S4 in the supplementary material) (Smith and Davidson, 2008). This suggests that Pmar1 protein is not present (or is inactive) in the small micromeres. Other transcriptional regulators undergo polarized, proteolytic degradation along the animal-vegetal axis of the sea urchin embryo (Weitzel et el., 2004; Angerer et al., 2005). Our analysis of the expression of Pmar1-GFP argues against the hypothesis that this protein is selectively degraded in the small micromeres, but other modes of post-transcriptional

regulation might be involved. Even if Pmar1 protein is present in the nuclei of both large and small micromeres, separate regulatory mechanisms might operate in the small micromeres that override the double-repression system and prevent the deployment of the skeletogenic GRN. The small micromeres express several germline markers (Juliano et al., 2006; Voronina et al., 2008) and in other animals the prospective germline is globally transcriptionally repressed during early development (Nakamura and Seydoux, 2008).

The regulative deployment of the skeletogenic GRN by NSM cells requires the ectopic activation of *alx1* via novel, *pmar1*-independent regulatory inputs (Ettensohn et al., 2007; Ettensohn, 2009). Several of the molecular conditions that might be envisioned to be required for the expression of *alx1* (i.e. activation of MAPK, expression of Ets1 and downregulation of *hesC*) appear to ordinarily be present in NSM cells, yet *alx1* is not expressed. Although it has not been tested directly, it seems unlikely that unequal cell division, which is essential for the activation of *alx1* in the micromere lineage and is a consequence of maternal cortical polarity, plays a role in the ectopic activation of *alx1* in transfating cells. Further analysis of the molecular mechanisms that activate the skeletogenic GRN, and the identification of additional inputs that are unique to the endogenous or regulative pathways, will shed light on this example of developmental plasticity.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.048652/-/DC1

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Table S1. PCR primers used

Primer name	Primer sequence (5′-3′)
Spalx1 F	CACCCGTAGAGGGCGCTATA
Spalx1 R	TGCTGGAGTCTTGCGATTCG
Spdelta F	ACGGAGCTACATGCCTGAAC
Spdelta R	TCACAATGGACCGAATCAGA
SphesC F	AGAGGCGAGCTCGCATCAAC
SphesC R	CTGGGGTCTGGACGTCTTTC
Spz12 F	AGTCGTCCAGCCATGTCTTT
Spz12 R	AAGCACACCTCGCACCTATC
Lvalx1 F	TGGAGAAGGTTTTCCAAAGG
Lvalx1 R	CGTAACCAGATCCTGGTCCT
Lvdelta F	TTGCTCTGCATTGGATTAGC
Lvdelta R	GATATCCTTGGCTTGGTGGT
LvhesC F	AAGTACCACGCTGGCTTCAC
LvhesC R	GTGCTGCAACGATCAGCTAG
Lvpmar1 F	CTGCTCACCAACCAGTCATT
Lvpmar1 R	TGTCGTCTGGTTGACCTCAT
Lvubiquitin F	CACAGGCAAGACTATCACTC
Lvubiquitin R	GATAGAGTGCGGCCGTCCTC