Erratum

A Raf/MEK/ERK signaling pathway is required for development of the sea urchin embryo micromere lineage through phosphorylation of the transcription factor Ets

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We apologise to the authors and readers for this mistake.

A Raf/MEK/ERK signaling pathway is required for development of the sea urchin embryo micromere lineage through phosphorylation of the transcription factor Ets

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Summary

In the sea urchin embryo, the skeleton of the larva is built from a population of mesenchymal cells known as the primary mesenchyme cells (PMCs). These derive from the large micromeres that originate from the vegetal pole at fourth cleavage. At the blastula stage, the 32 cells of this lineage detach from the epithelium and ingress into the blastocoel by a process of epithelial-mesenchymal transition. We report that shortly before ingression, there is a transient and highly localized activation of the MAP-kinase ERK in the micromere lineage. We show that ingression of the PMCs requires the activity of ERK, MEK and Raf, and depends on the maternal Wnt/β-catenin pathway. Dissociation experiments and injection of mRNA encoding a dominant-negative form of Ras indicated that this activation is probably cell autonomous. We identified the transcription factors Ets1 and Alx1 as putative targets of the phosphorylation by ERK. Both proteins contain a single consensus site for phosphorylation by the MAP kinase ERK. In addition, the Ets1 protein sequence contains a putative ERK docking site. Overexpression of *ets1* by injection of synthetic mRNA in the egg caused a dramatic increase in the number of cells becoming mesenchymal at the blastula stage. This effect could be largely inhibited by treating embryos with the MEK inhibitor U0126. Moreover, mutations in the consensus phosphorylation motif substituting threonine 107 by an aspartic or an alanine residue resulted respectively in a constitutively active form of Ets1 that could not be inhibited by U0126 or in an inactive form of Ets1. These results show that the MAP kinase pathway, working through phosphorylation of Ets1, is required for full specification of the PMCs and their subsequent transition from epithelial to mesenchymal state.

Key words: ERK, MAP-kinase, MEK *ets*, Epithelial-mesenchymal transition, Primary mesenchyme, Ingression, Micromere, Sea urchin embryo

Introduction

In the sea urchin embryo, most of the mesoderm derives from two populations of mesenchymal cells called the primary and secondary mesenchyme cells (PMCs and SMCs, respectively). Precursors of these cell types are generated during cleavage at the vegetal pole of the embryo. At fourth cleavage, blastomeres of the vegetal half divide asymmetrically to form macromeres and micromeres. The macromeres divide equally, while micromeres again divide asymmetrically to form the large and the small micromeres. The large micromeres are the precursors of the PMCs. At the late blastula stage, the 32 cells of this lineage lose their typical epithelial character, become motile and detach from the epithelium of the vegetal pole. They ingress into the blastocoel during a process of epithelial-mesenchymal transition, migrate on the blastocoel wall and finally fuse to form a syncytium with a precise geometry, which will become the skeleton of the larva by biomineralisation (Angerer and Angerer, 2003; Brandhorst and Klein, 2002; Decker and Lennarz, 1988; Ettensohn and Sweet, 2000).

The SMCs derive from the macromeres. They delaminate from the tip of the archenteron throughout gastrulation by a process that also involves an epithelial-mesenchymal transition. They contribute to a variety of mesodermal tissues including pigment cells, blastocoelar cells, muscle fibers and to coelomic pouches (Cameron et al., 1991; Cameron et al., 1987; Gibson and Burke, 1985; Logan and McClay, 1997; Ruffins and Ettensohn, 1996; Tamboline and Burke, 1992).

In addition to their role in formation of the skeleton of the larva, micromeres have three other important functions in intercellular signaling. First, soon after their formation at fourth cleavage and during the two next divisions, they send a signal to the surrounding macromeres which is required for the specification of the endoderm (Horstadius, 1973; Ransick and Davidson, 1993). The nature of the early signal responsible for endoderm induction is not known. The second signaling function of the micromeres occurs between the seventh and ninth cleavage, when they are still within the vegetal plate epithelium. During this period, micromeres emit a signal that is crucial for induction of the SMC fate (McClay et al., 2000; Sweet et al., 1999). This signal has been identified as the Delta ligand that activates the Notch receptor (Sherwood and McClay, 1999; Sweet et al., 2002). Finally, during gastrulation, the PMCs send an inhibitory signal to the SMCs that prevents them from differentiating as skeletogenic mesenchyme. When micromeres are removed at the 16 cell stage or when the PMCs

are eliminated surgically at the mesenchyme blastula stage, skeletogenic cells nevertheless form. In the absence of PMCs, pigment cells and blastocoelar cells adopt a skeletogenic fate by a process called conversion (Ettensohn, 1992; Ettensohn and McClay, 1988).

A large body of evidence indicates that the fate of the large micromeres is specified autonomously. Micromeres isolated or transplanted to any ectopic location in the embryo always differentiate on schedule into skeletogenic cells according to their normal fate (Angerer and Angerer, 2003; Brandhorst and Klein, 2002; Davidson et al., 1998; Horstadius, 1973; Okazaki, 1975; Ransick and Davidson, 1993). The maternal determinants responsible for this autonomous differentiation are not known. However, several zygotic genes involved in the very early steps of specification of the micromeres have been described recently (Croce et al., 2001; Davidson et al., 2002a; Ettensohn et al., 2003; Fuchikami et al., 2002; Kurokawa et al., 1999) and epistatic analyses have helped to order them in a molecular pathway (Oliveri et al., 2002). One of the earliest events in this pathway is the nuclear accumulation of β-Catenin in the micromeres nuclei at the 32-cell stage (Emily-Fenouil et al., 1998; Logan et al., 1999). β-Catenin then activates the expression of a homeobox gene called *pmar1*, which is to date the earliest zygotic gene expressed specifically in this lineage. Pmar acts as a transcriptional repressor and therefore it is inferred that its role is to allow the expression of other zygotic specification genes by releasing the repressive action of an ubiquitous repressor. Among the zygotic factors acting downstream of *pmar*, the *ets1* and *alx1* transcription factors play crucial roles. Both genes are expressed very early in the micromere lineage and both genes are strictly required for formation of the PMCs (Ettensohn et al., 2003; Kurokawa et al., 1999). When the function of either gene is blocked, no PMCs form and several downstream differentiation markers are downregulated.

In this study, we show that the specification of cell fate in the micromeres and their conversion from epithelial to migratory behaviour require a functional Raf/MEK/ERK signaling pathway. Inhibition of this pathway blocks PMC ingression and arrests the program of specification of the micromeres and SMCs at an early step. We further show that Ets1 is a key target of ERK, and that ERK may be activated in a cell-autonomous manner.

Materials and methods

Animals, embryos and treatments

Adult sea urchins (*P. lividus*) were collected in the bay of Villefranche. Embryos were cultured as described elsewhere (Lepage and Gache, 1989; Lepage and Gache, 1990). Dissociation of blastomeres and treatments with LiCl were performed as described previously (Ghiglione, 1993). Treatments with $ZnCl_2$ was carried out by exposing embryos to 0.5 mM of $ZnCl_2$ in artificial sea water (ASW) from 30 minutes to 48 hours. Embryos were treated with PD98059 or U0126 (Calbiochem) diluted from stocks in DMSO in 24-well plates protected from light. 3-30 μM U0126 completely blocked ingression of PMCs. It was used at 5 or 10 μM in most experiments. PD98059 (10 μM) delayed but did not block micromere ingression.

Detection of phosphorylated ERK by immunostaining

A monoclonal antibody specific for the dually phosphorylated form of MAP kinase (ERK1 and ERK2) (Sigma M8159) was used for immunolocalisation, with a vectastain ABC kit (alkaline phosphatase) and the chromogenic substrates NBT/BCIP. In control experiments, the primary antibody was omitted.

Western blotting

Protein samples (10 μg/lane) were separated by SDS-gel electrophoresis and electrophoretically transferred to 0.2 μm nitrocellulose filters. After blocking for 2 hours in 5% dry milk in TBST and incubation overnight with the anti ERK antibody, bound antibodies were revealed by ECL immunodetection.

In situ hybridization

In situ hybridization was performed following a protocol adapted from Harland (Harland, 1991) with antisense RNA probes and staged embryos. Most of the probes used in this study were isolated in the course of an in situ hybridization screen (T. Lepage, unpublished). The gene markers for secondary mesenchyme were *AA29* and *23F*, which encode enzymes of the nucleotide metabolism. The endodermal marker gene *42C* gene encodes a methyl transferase. *ets1*, *ske-T*, *alx1*, *Delta*, *msp130*, *CyIa*, *goosecoid* and *brachyury* are the *P. lividus* homologs of these genes. All probes were synthesized with the T7 RNA polymerase after linearization by *Not*I.

RNA injection

The coding sequence of *ets1* and *alx1* were amplified by PCR using the Pfu DNA polymerase and inserted at the *Bam*HI-*Xho*I sites (*ets*) or *Bam*HI and *Eco*RI sites (*alx*) of *pCS2+* (Turner and Weintraub, 1994) to generate *pCS2-ets1* and *pCS2 alx1*. RNA encoding wild-type Ets1, Ets1 T107D and Ets1 T107A, and Ets1 VP16 were injected at 200μg/ml. RNA encoding Alx1 was injected at 160 μg/ml.

pCS2-DN-TCF encodes a dominant negative TCF and was made by deleting the β-catenin binding domain of the sea urchin TCF (C. Gache, unpublished). The dominant negative *Ras* construct used was the human *Ha-Ras* cDNA which carries the Asn17 mutation cloned in pSP64T and the constitutively active Ras was the $p21^{v\text{-}Ha\text{-}ras}$ (Feig and Cooper, 1988; Whitman and Melton, 1992). DnRas RNA was injected at concentrations up to 2mg/ml while $p21^{v\text{-}Ha\text{-}ras}$ RNA was injected at 50 to 150 μg/ml. The dominant-negative Raf construct is a kinase defective mutant that carries an alanine in place of serine at position 621 (Fabian et al., 1993); DNRaf mRNA was injected at 800 μg/ml. The zebrafish MAP kinase phosphatase construct contains the MKP3-coding sequence clones in pCS2 (Kudoh et al., 2001); MKP3 mRNA was used at 450 μg/ml.

Site-directed mutagenesis and construction of expression plasmids

To make *pCS2 ets T107D* and *pCS2 ets T107A*, the ACG codon encoding threonine in position 107 of *pCS2 ets* was mutated to GAT or GCG by splicing PCR using the following oligonucleotides: Ets T107D Fw, 5′-CCCCCGCCAGATCCAGGCACCAACGCT-3′ and Ets T107D Rev, 5′-AGCGTTGGTGCCTGGATCTGGCGGGGG-3′; Ets T107A Fw, 5′-CCCCCGCCAGCGCCAGGCACC and Ets T107A Rev, 5′-GGTGCCTGGCGCTGGCGGGGG-3′. For *pCS2 VP16 Ets* construction, the region encoding amino acids 413-490 from VP16 (accession number HEHSV165) was PCR amplified using (VP16 sequences in capitals) VP16-Fw-*Bam*HI (5′-CGCGGATCCACC-ATGGCCCCCCCGACCGATGTCAGC-3′) and VP16-Ets-Rev (5′-GCAGTGCATAGATGCCATCCCACCGTACTCGTCAAT-3′) and spliced by PCR to the full-length Ets sequence amplified using Ets Fw (5′-ATGGCATCTATGCACTGCTCC-3′) and Ets TGA Xho (5′-AGGCTCGAGTCAGTCGTCATCGCGTGCACC-3′).

Results

A highly localized and transient activation of the MAP kinase pathway precedes ingression of the PMCs

We analyzed the spatial activation of ERK during sea urchin

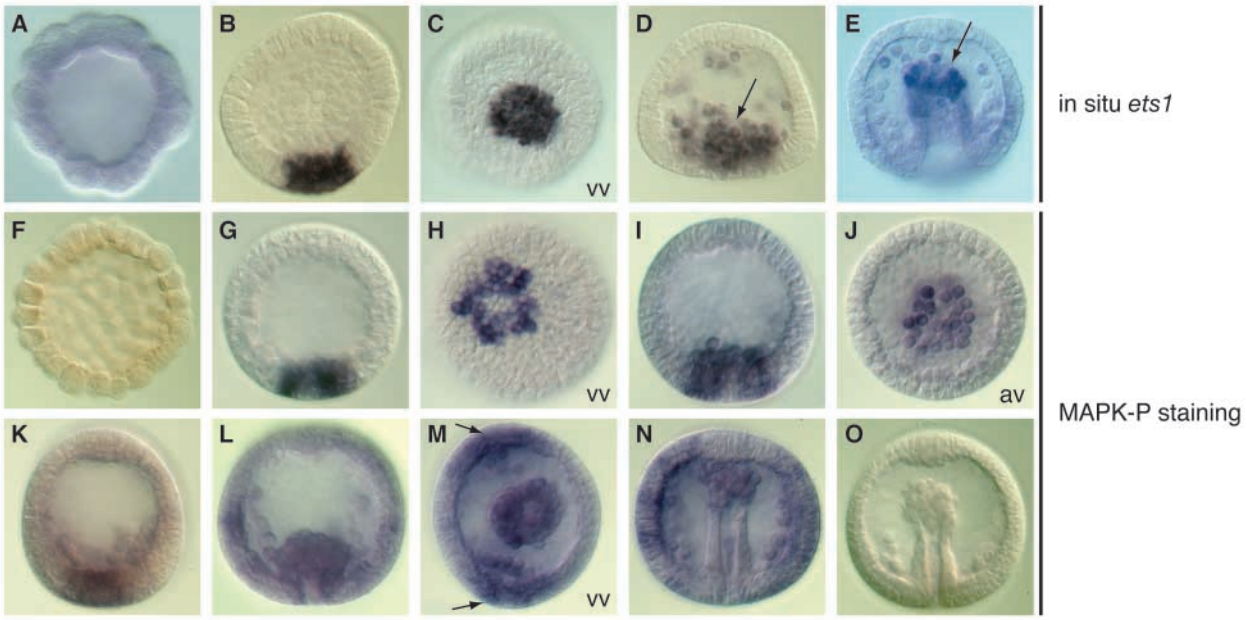

Fig. 1. ERK is activated transiently in the PMC precursors a few hours after zygotic expression of *ets1*. (A-E) In situ hybridization with an *ets1* probe. (B-D) Starting at the early blastula stage up to the mesenchyme blastula stage, *ets1* expression is restricted to the PMCs (arrow in D). (E) At the end of gastrulation, *ets1* transcripts are also enriched in the SMCs (arrow). (F-N) Whole-mount immunolocalization of activated ERK using an anti-MAPK-P antibody. (A) Sixty-cell stage, (F) early blastula, (B,C) prehatching blastula, (G,H) hatching blastula, (I,J) early mesenchyme blastula, (D,K) mesenchyme blastula, (L,M) early gastrula (arrows in M indicate staining in the ectoderm adjacent to the PMC clusters) and (E,N,O) late gastrula. (O) Control embryo stained with the secondary antibody alone. All the embryos are viewed from the side with the animal pole at the top except when mentioned: av, animal view; vv, vegetal view.

development. Embryos were fixed at various stages and stained using a monoclonal antibody that recognizes a phosphorylated epitope in an activated form of ERK (Fig. 1) (Yung et al., 1997). Immunostaining at the cleavage and early blastula stages did not reveal any activation of ERK (Fig. 1F). Staining was first detected at the hatching blastula stage in a small ring of about 30 cells around the vegetal pole (Fig. 1G-H), surrounding a group of about eight cells with a much reduced level of activated ERK. The pattern of the stained cells resembled that of the primary mesenchyme cells precursors surrounding the small micromeres. Double immunostaining with an antibody recognizing the ectodermal marker hatching enzyme (Lepage et al., 1992) confirmed that the stained cells surround the vegetal pole (data not shown). Strong staining in the micromere lineage was observed for about 2 hours until the early mesenchyme blastula stage, when the micromeres start to delaminate into the blastocoel (Fig. 1J). Staining in the PMCs continued to be detected during ingression (Fig. 1I,J), then decreased after the end of delamination (Fig. 1K). At this stage, weak staining was also detected in the presumptive SMC territory at the vegetal pole (Fig. 1K). During gastrulation, ERK activation was detected predominantly in the invaginating archenteron, in the PMC clusters and in the adjacent ectoderm (Fig. 1M). At the late gastrula stage, the regions showing the highest level of staining were the tip of the archenteron and the ectoderm adjacent to the PMCs. Activation of ERK overlapped temporally with expression of *ets1* during formation of the PMCs and SMCs (Kurokawa et al., 1999) (Fig. 1A-E). These observations show that ERK is activated broadly and in a highly dynamic pattern during sea urchin development. Notably, there is strong transient activation in the PMCs starting just prior to their ingression.

Inhibition of the ERK signaling pathway prevents PMC ingression

To test the role of ERK, we used U0126, a potent and specific inhibitor of the ERK activating kinase MEK (Davies et al., 2000; DeSilva et al., 1998). Development of the embryos treated from the two-cell stage was normal up to the blastula stage, cleavage and hatching occurred on schedule. Strikingly, U0126 treatment then prevented the delamination of the PMCs, although a flattened vegetal plate formed normally (Fig. 2A,E). Treated embryos thus remained as an empty ball of epithelial cells until the onset of gastrulation (compare Fig. 2A with Fig. 2E). Initiation of gastrulation was slightly delayed in the treated embryos but in most of the embryos the flattened vegetal plate, still containing the micromere progeny, eventually started to buckle and an archenteron formed and elongated normally (Fig. 2B,F). These observations indicate that ERK phosphorylation is required for PMC ingression but not for gastrulation

U0126 experiments also indicated that ERK activity participates in SMC formation and/or behaviour. A significant but reduced number of mesenchymal cells was observed around the tip of the archenteron in treated embryos at the time when SMCs budded from this site in controls (Fig. 2C,G). In both treated and untreated embryos, these cells emitted filipodia, detached and migrated into the blastocoel very much like normal SMCs. We conclude that unlike the PMCs, some SMCs cells are able to undergo epithelial mesenchymal

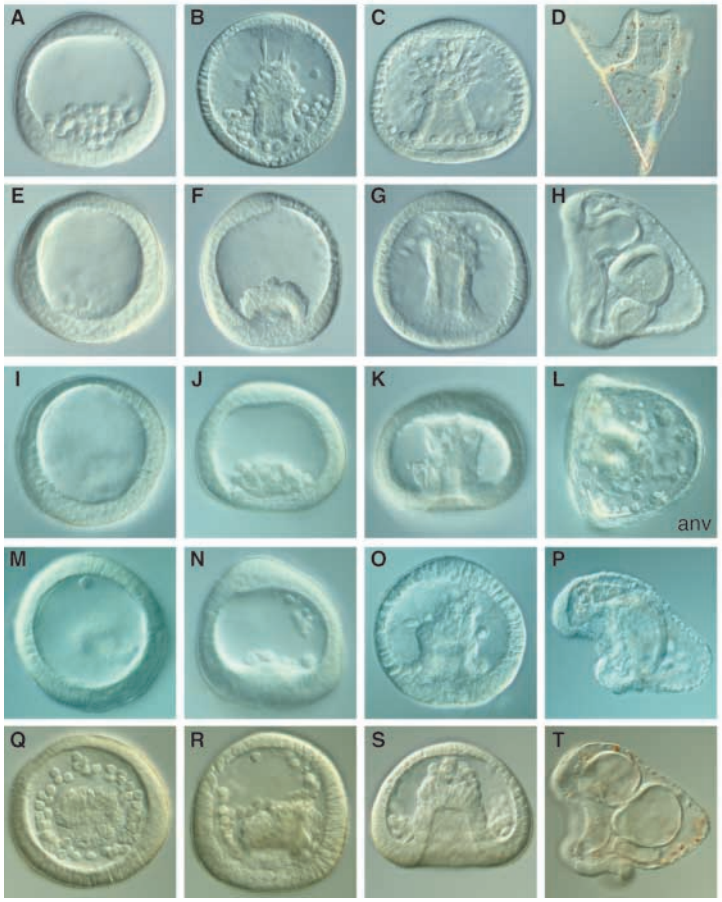

Fig. 2. U0126 treatment and injections of mRNA encoding a MAP Kinase Phosphatase (*MKP3*) or a dominant-negative Raf (*dnRAF*) block PMC ingression and differentiation. (A-D) Control embryos. (E-H) Embryos treated with 10 μM U0126, (I-L) embryos injected with *MKP3* mRNA, (M-P) *dnRaf* injections, (Q-T) *dnRas* injections. Note the absence of PMCs in the embryos treated with UO126 (E,F), or injected with MKP3 RNA (I,J) or dnRaf RNA (M,N). (A,E,I,M,Q) Mesenchyme blastula, (B,F,J,N,R) early gastrula, (C,G,K,O,S) late gastrula and (D,H,L,P,T) pluteus stage. anv, anal view. In control embryos, the PMCs ingress into the blastocoel (A) and form a ring around the archenteron and two bilateral clusters during gastrulation (B).

transition in the presence of the MEK inhibitor. Even when inhibitor treatment began at the late mesenchyme blastula-stage or early gastrula stage, or when fresh inhibitor was added during gastrulation, SMCs formed at the tip of the archenteron, ruling out the possibility that the drug became less effective during long treatments.

When observed at the pluteus stage (48/60 hours), embryos that had been treated with the inhibitor from the two-cell stage onwards, had a significantly reduced number of pigment cells and mesenchymal cells (Fig. 2D,H). The archenteron was regionalized normally into hindgut, midgut and foregut, and a stomodeum opening was present on the oral ectoderm. Although spiculogenesis was completely absent, the embryos had acquired an oral-aboral polarity, as shown by the presence of a mouth, a ciliary band and the differentiation of squamous aboral ectoderm. More than 98% of the treated embryos survived and showed these defects. In the remaining 2% of the embryos, treatment with the inhibitor caused exogastrulation (not shown).

To confirm the specificity of the defects caused by the inhibitor, we overexpressed a MAP kinase phosphatase (MKP3) by RNA injection into the egg as an independent mean of inhibiting ERK function. As with U0126 treatment, microcroinjection of MKP3 mRNA in to the egg completely prevented formation of the PMCs but did not inhibit gastrulation and formation of SMCs (Fig. 2I-L). Similarly, injection of mRNA encoding a dominant-negative form of the human Raf kinase which acts upstream of MEK, suppressed formation of the PMCs (Fig. 2M-P). These results show that a conserved Raf/MEK/ERK signaling pathway plays an essential role in PMC formation.

The small GTP-binding protein Ras is an important mediator of the MAPK pathway activated by receptor tyrosine kinases and has been implicated in regulating cell scattering and motility induced by growth factors such as SF/HGF (Graziani et al., 1993; Hartmann et al., 1994) and EGF (Boyer et al., 1997). We found that overexpression of a dominant-negative form of Ras (dnRas) by RNA injection into sea urchin eggs did not block ingression of the micromeres but inhibited their differentiation into spicules (Fig. 2Q-T). This result suggests that the transient activation of ERK in the micromere lineage and ingression of the PMCs is not dependent on Ras-mediated receptor signaling, while differentiation of PMCs into spicules is likely to require Ras-dependent steps. However, the dnRas construct we used was of human origin and, therefore, the possibility remains that it does not completely block Ras activity in the sea urchin embryo.

The MAP kinase signaling pathway is required for PMC ingression, then for skeletogenesis

In the sea urchin embryo, ablation of the micromeres at the 16-cell stage can be compensated later by the SMCs, some of which transfate and differentiate into skeletogenic mesenchyme (Ettensohn, 1992; Ettensohn and McClay, 1988). This phenomenon of conversion did not occur in embryos cultured in the continuous presence of the MEK inhibitor, as they lacked spicules. ERK activity may thus be required for the transfating of the SMCs or for spiculogenesis. Alternatively, the PMCs may not require ERK to send the signal that suppress transfating. To address this issue, we treated groups of embryos for various periods of time starting at the two-cell stage. Embryos were scored at the mesenchyme blastula and gastrula stage for the presence of micromeres, at the late gastrula stage for the presence of cells emigrating from the archenteron and at 48-72 hours for the presence of pigment cells and spicules (Fig. 3). All treatments starting before the hatching blastula stage blocked ingression of the PMCs and produced embryos lacking skeleton (Fig. 3A,B). However, when the inhibitor was added

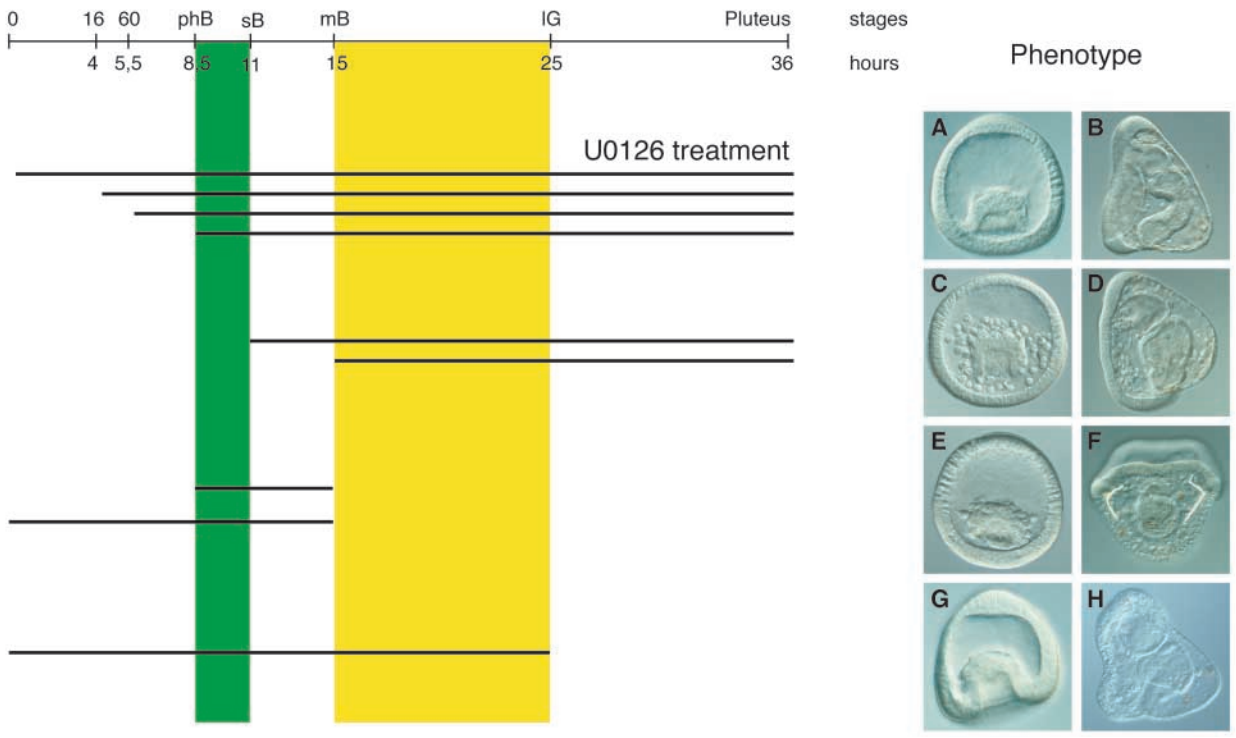

Fig. 3. Activation of MAP kinase is essential for PMC ingression and skeletogenesis. Embryos were incubated at the indicated times with 10 μM of the MEK inhibitor and scored at the early gastrula stage for the presence of micromeres, and at the pluteus stage (48-72 hours) for the presence of spicules. Pictures of representative embryos obtained after the treatments are shown on the right. When U0126 treatment was started before hatching and was continued up to the pluteus stage, PMC ingression and spicule formation was blocked (A,B). If the inhibitor was added after beginning of hatching, PMC ingressed into the blastocoel but did not differentiate further (C,D). When the inhibitor was added at the two-cell stage or prehatching blastula stage PMCs formation was prevented (E), but if the inhibitor was removed soon after the beginning of invagination of the archenteron, spicules nevertheless formed (F). Spicules did not form when the inhibitor was removed after the late gastrula stage (G,H). ERK activity is required first between the prehatching blastula stage and the swimming blastula stage for ingression of the PMCs (green), and then during gastrulation for differentiation of the PMCs into spicules (yellow).

at the hatching blastula stage, ingression was delayed by about 2 hours but eventually occurred (Fig. 3C). In these embryos, the mesenchyme cells remained as individual cells and did not coalesce into a syncytium or produce spicules. Embryos treated with the MEK inhibitor from the late mesenchyme blastula stage or gastrula stage onwards also lacked spicules (Fig. 3D). When the embryos were treated with U0126 from the two-cell stage but washed out at the mesenchyme blastula stage, spiculogenesis was largely rescued in most embryos (140/160) although the shape of these spicules was often abnormal (Fig. 3E,F). We do not know if the skeletogenic cells formed in these embryos by delayed delamination of the PMCs or by conversion of the SMCs. When the inhibitor was washed out at the late gastrula stage, most embryos (151/155) lacked spicules (Fig. 3G,H). Taken together, these observations show that there are at least two crucial requirements for ERK activity: first between the prehatching blastula stage and the swimming blastula stage for the epithelial mesenchymal transition of the PMCs (green labeling in Fig. 3); and then during gastrulation for differentiation of the PMCs into spicules (yellow labeling in Fig. 3).

The ERK signaling pathway is required to maintain the program of specification of the micromeres

To determine if MEK/ERK signaling participates in specifying PMC fate, we compared the expression of gene markers in untreated and in inhibitor-treated embryos (Fig. 4; Table 1). *ets1* and *alx1* (Kurokawa et al., 1999; Ettensohn et al., 2003) act early in the hierarchy of genes regulating PMC formation and upstream of the expression of the T-box gene *ske-T/Tbrain* (Davidson et al., 2002b; Ettensohn et al., 2003), while *msp130*, which encodes a cell surface protein, is expressed much later during PMC differentiation.

Expression of *ets1* was largely normal in embryos treated with the MEK inhibitor (Fig. 4D) and was maintained in the center of the vegetal plate of mesenchyme blastulae (Fig. 4E). During gastrulation, strong expression was detected in part of the archenteron (Fig. 4F), indicating that micromere descendants continued to express *ets1* without leaving the epithelium. The other PMC marker genes behaved very differently. Expression of *alx* was severely reduced at the blastula stage by inhibition of MEK/ERK signaling and almost absent from mesenchyme blastula and late gastrula stage embryos (Fig. 4J-L) indicating that ERK activity is required for maintenance of *alx1* expression. Expression of the downstream gene *skeT* was also severely reduced (Fig. 4P-R), whereas expression of *msp130* was abolished (Fig. 4V-X). These results show that the MAP kinase pathway is crucially required for the normal expression of some of the key transcription factors that participate in micromere specification.

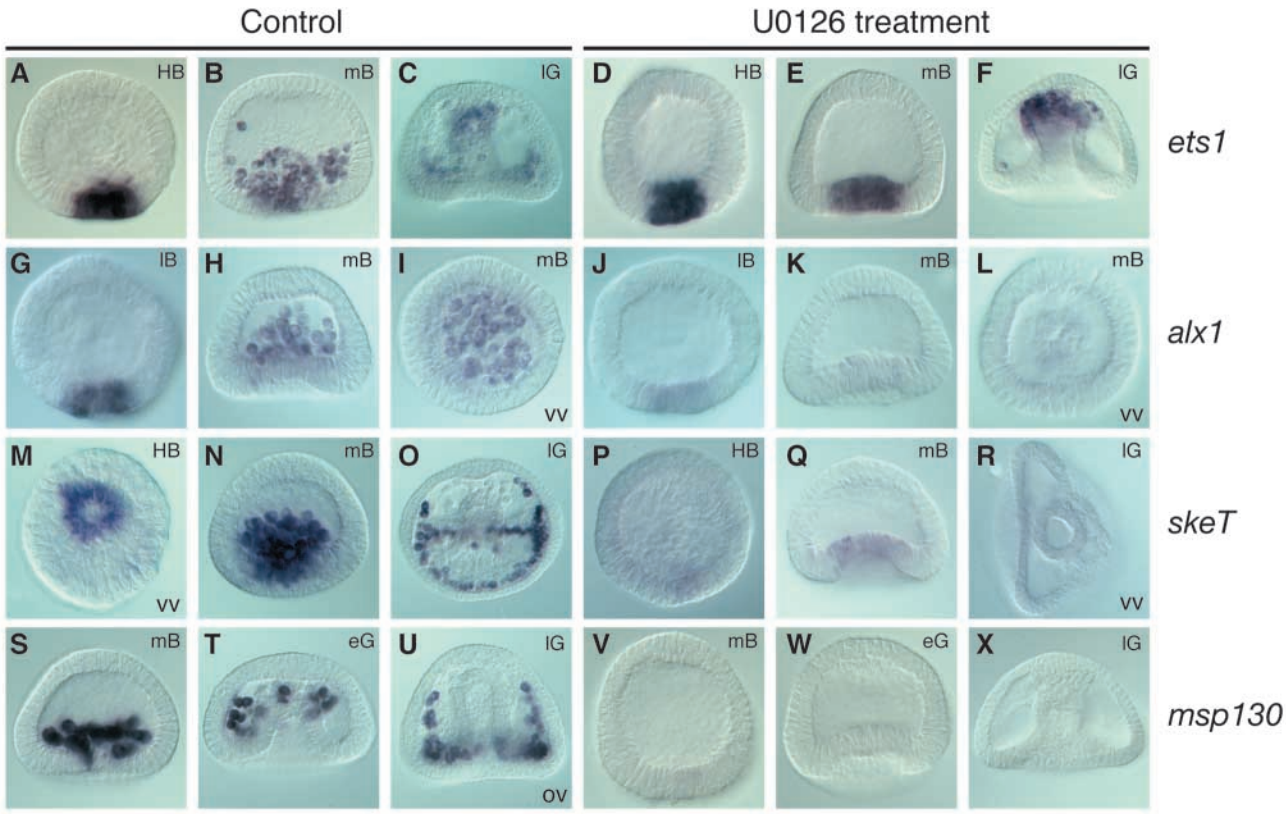

Fig. 4. Effects of inhibiting MAP Kinase function on the gene expression program of the PMCs. Embryos were placed into MFSW containing DMSO (control) or 10 μM U0126 starting at the two-cell stage. After fixation at the desired stage, in situ hybridization was then performed with the following probes *ets1* (A-F), *alx1* (G-L), *skeT* (M-R) and *msp130* (S-X). Control embryos on the left, U0126-treated embryos on the right of the figure. These experiments were repeated several times with different batches of embryos. Representative embryos are shown. U0126 treatment reduces the level of *alx1*, *skeT* and *msp130* transcripts. Expression of *ets1* remains unaffected by the treatment. HB, hatching blastula; lB, late blastula; mB, mesenchyme blastula; eG, early gastrula; lG, late gastrula; vv, vegetal view; ov, oral view.

MEK/ERK signaling is required for full induction of the SMC fates

SMC fates (notably pigment cells and blastocoelar cells) are induced from macromeres decendants by adjacent micromeres expressing Delta during the late blastula stage, between the eighth and ninth cleavage (McClay et al., 2000; Sherwood and McClay, 1997; Sherwood and McClay, 1999; Sherwood and McClay, 2001; Sweet et al., 2002; Sweet et al., 1999). SMC induction therefore coincides temporally with the activation of ERK in the micromeres.

Table 1. Expression of various gene markers after U0126 treatment

Probe	Stage	++	+	+/–	–
ets	mB	240/240			
alx	lB		31/157	126/157	
alx	mB/lG			17/160	143/160
skeT	HB			34/276	242/276
msp130	mB			9/274	265/274
Delta	HB	7/303	296/303		
Delta	mB/lG	379/379			
AA29	mB	96/224	77/224	51/224	
23F	mB	4/272	268/272		
CyIa	mB				279/279
CyIa	lG	126/126			
gsc	mB	218/218			
brachyury	mB	332/332			
42C	mB	260/260			

HB, hatching blastula; lB, late blastula; lG, late gastrula; mB, mesenchyme blastula; ++, normal expression; +, reduced but detectable expression; +/– weak or absent expression; –, no expression.

To test the impact of MEK/ERK signaling on SMC specification, we analyzed the expression of the *P. lividus* Delta gene. In agreement with previous reports in other sea urchin species (Oliveri et al., 2002; Sweet et al., 2002), we found that Delta is first expressed in the micromeres, starting at about the 128-cell stage. As Delta expression declines in the PMCs following their ingression, a second wave of Delta expression is initiated in the macromeres-derived mesendodermal cells. We found that interfering with ERK signaling significantly reduced expression of Delta in the micromeres at the late blastula stage (Fig. 5D and Table 1). By contrast, at the mesenchyme blastula stage, relatively normal levels of Delta expression were maintained in the presumptive SMCs following treatment with the inhibitor (Fig. 5E,F).

We then analyzed the expression after U0126 treatment of three genes markers expressed in the presumptive mesenchyme cells. The first of these genes is the cytoskeletal actin gene *CyIa*, which shows a broad and very dynamic expression in all germ layers (Thiebaud et al., 1990). At the mesenchyme blastula stage, *CyIa* is expressed strongly in a crescent-like domain within the SMC territory probably corresponding to

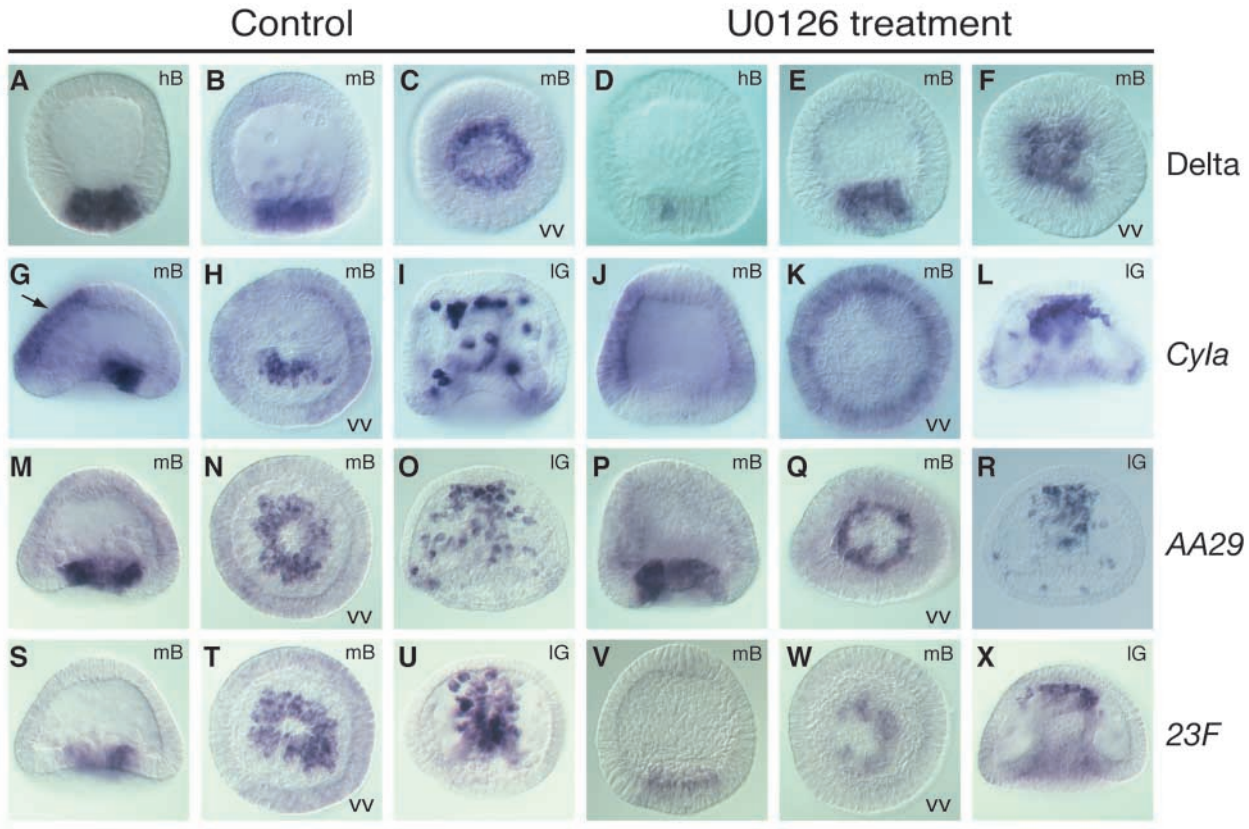

Fig. 5. Effects of inhibiting MAP kinase signaling on specification of the SMCs. Embryos were treated as above and whole-mount in situ hybridization was then performed for Delta (A-F), *CyIa* (G-L), *AA29* (M-R) and *23F* (S-X). Control embryos on the left, U0126-treated embryos on the right of the figure. U0126 treatment decreases the level of expression of Delta at the early blastula (B5) but not at later stages. The mesodermal expression of *CyIa* is suppressed at the mesenchyme blastula stage, but unaffected at later stages. The oral ectoderm expression (arrow in G) is unaffected. The expression of *AA29* and *23F* is reduced but not suppressed by the treatment with U0126. Stages are the same as in Fig. 4.

presumptive pigment cells (Fig. 5G,H). Later during gastrulation, mesenchymal cells strongly expressing *CyIa* bud off the oral side of the archenteron and migrate into the blastocoel (Fig. 5I). These are likely to be blastocoelar cells, which form after the pigment cells in this region. The two other differentiation markers used, *AA29* and *23F*, are expressed in the presumptive blastocoelar cells: zygotic expression of both genes is initiated symmetrically in the presumptive SMC territory at the early mesenchyme blastula stage (Fig. 5M,N,S,T). It persists during gastrulation in newly formed mesenchymal cells (Fig. 5O,U) and in a population of cells dispersed throughout the blastocoel at the pluteus stage.

U0126 treatment was found to abolish the expression of *CyIa* in the putative pigment cell precursors, whereas the ectodermal expression was unaffected. By contrast, the late expression of *CyIa* in presumptive blastocoelar cells at the gastrula stage seemed largely unaffected. Both *AA29* and *23F* were expressed in cells that migrated from the archenteron tip in U0126-treated embryos, but only about half the normal number were present at the blastula and gastrula stages (Fig. 5P,Q,V,W and Table 1). Moreover, *AA29* and *23F*-expressing mesenchymal cells did migrate from the tip of the archenteron during gastrulation. Taken together, these observations suggest that the pigment cell and blastocoelar cell fates are affected differently when the MEK/ERK pathway is blocked. These observations are consistent with the lack of pigment cells in the U0126 embryos because among SMC derivatives, pigment cells have the highest requirement for Delta, whereas specification of blastocoelar cells can occur via Notch/Delta signaling among macromeres descendants (Sweet et al., 2002).

Finally, we confirmed that ERK signaling is not involved in the early signaling that is required for endoderm induction, which occurs at a time when MAPK activation was not significantly detected. Early expression of *42C*, an endodermal marker expressed in presumptive hindgut and midgut from the mesenchyme blastula stage onwards was unaffected by treatment with U0126 (Fig. 6A-F; Table1). Moreover, gut formation and patterning appeared normal when MEK/ERK signaling was inhibited from fertilization onwards. We also confirmed that U0126 treatment did not affect the expression of *goosecoid* and *brachyury*, two early markers of oral /aboral polarity (Angerer et al., 2001; Croce et al., 2001; Gross and McClay, 2001), (Fig. 6G-R).

In summary, these results demonstrate that the activity of MEK is required for the maintenance of a high level of expression of Delta in the micromeres and for the full expression of SMC differentiation genes, but not for endoderm formation or the establishment of oral aboral polarity.

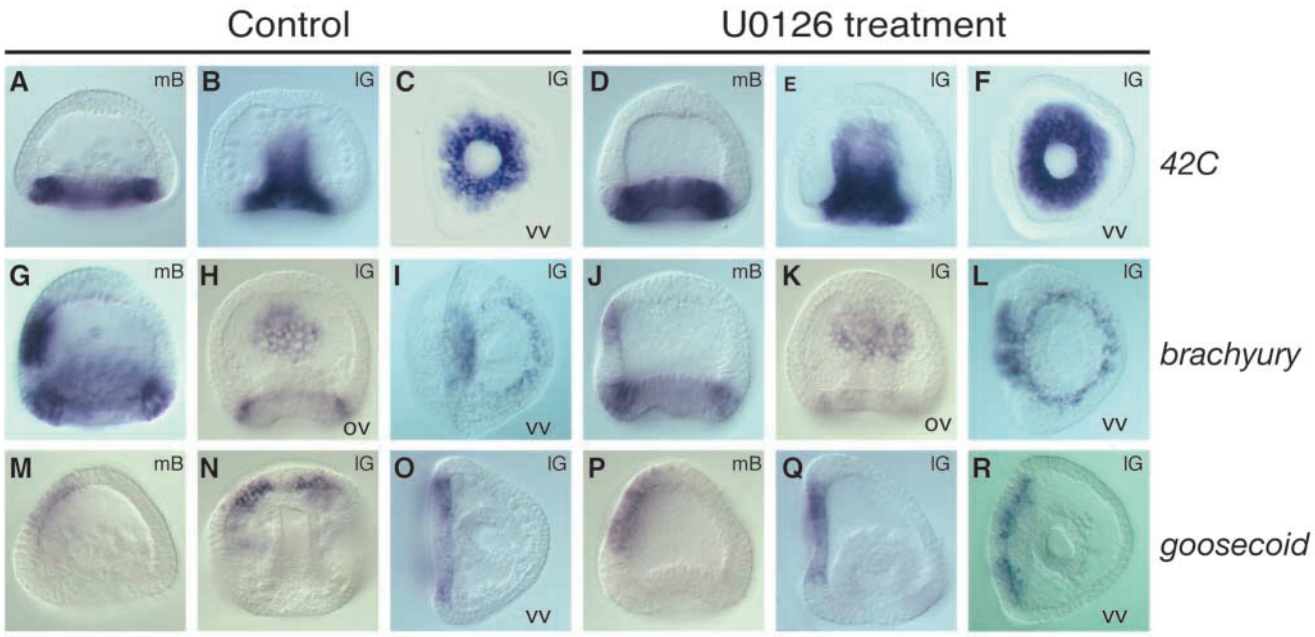

Fig. 6. Inhibition of MAP Kinase signaling does not disrupt specification of the endoderm territory and formation of the oral/aboral axis. Expression of *42C* (A-F), *brachyury* (G-L) and *goosecoid* (M-R) genes after inhibition of the ERK function. Control embryos on the left, U0126-treated embryos on the right. U0126 treatment does not affect the expression of endoderm (*42C*) and oral ectoderm (*goosecoid* and *brachyury*) marker genes. mB, mesenchyme blastula; lG, late gastrula; vv, vegetal view; ov, oral view.

The sea urchin Ets transcription factor as a direct target of MEK/ERK signaling

As shown in Fig. 4, *ets1* is expressed at a relatively normal level in the presence of U0126 while several other genes acting further downstream in the network are strongly downregulated. This observation suggests that one target of MAP kinase could be Ets1 itself, or a transcription factor acting early in parallel with Ets1 such as the homeobox gene product Alx1 (Ettensohn et al., 2003). Sequence analysis showed that the sea urchin Ets1 protein contains a PPTP motif, and the Alx1 protein contains a PSTP conforming to the PXS/TP consensus MAP-kinase phosphorylation site (Marshall, 1994) (Fig. 7A,C). In addition, we found that the sea urchin Ets1 protein sequence contains a stretch of amino acids KTDF**L**SRAPP**F**MGD very similar to the KEXF**L**X**L**XPX**F**XGD ERK2 docking site, the most critical Phe residue being conserved (bold indicates the most conserved residues) (Seidel and Graves, 2002) (Fig. 7B). To test the importance of phosphorylation of the MAP kinase consensus site for activation of the sea urchin Ets1 in vivo, we substituted the threonine phospho-acceptor residue of Ets1 by an aspartic (*etsT107D*) or alanine (*etsT107A*) residue. We also constructed a chimeric mRNA encoding the whole Ets1 protein sequence fused to the potent transactivation domain of the viral VP16 protein (*ets-VP16*). Both the introduction of a constitutive negative charge and the fusion to a strong transactivating domain were designed to bypass the requirement for phosphorylation. Conversely, the conversion of the phosphoacceptor residue into alanine should produce an inactive or weak dominant-negative form (Brunner et al., 1994).

As described in another sea urchin species, injection of *ets1* mRNA in *P. lividus* eggs caused a dramatic phenotype. This was characterized by a wave of massive cell ingression and cell migration that spread from the vegetal pole towards the animal hemisphere (Kurokawa et al., 1999) (Fig. 7E). A similar phenotype results from overexpression of the homeobox gene *pmar* (Oliveri et al., 2002), which acts upstream of *ets1*. When embryos overexpressing *ets1* were treated with U0126 starting at the 16-cell stage, the massive epithelial-mesenchymal transition was largely inhibited (Fig. 7I). Fifteen hours after fertilization, when *ets1* injected control embryos showed a large excess of mesenchymal cells at the vegetal pole, *ets1* injected U0126-treated embryos still had an empty blastocoel. Mesenchymal cells eventually formed in a disorganized manner, then development arrested and the embryos died. Formation of an excess of mesenchymal cells at the vegetal pole was also observed in embryos that had received the *ets T107D* mRNA (Fig. 7F) or the *Ets-VP16* mRNA (Fig. 7H), but treatment with U0126 did not block these effects (Fig. 7J and data not shown). Overexpression of *etsT107A* mRNA did not promote formation of mesenchymal cells, nor did it block ingression of PMCs, suggesting that substitution of the threonine phosphoacceptor site into alanine resulted in an inactive protein (Fig. 7G). These results suggest that phosphorylation within the MAP kinase consensus site is a key step in the regulation of the activity of the sea urchin *Ets1*.

Equivalent experiments with *alx1*, which also contains a consensus MAP kinase phosphorylation site and is required for ingression and differentiation of the micromeres (Ettensohn et al., 2003), were not possible because overexpression of *alx1* inhibited rather than promoted ingression of the micromeres. This suggests that dosage of *alx1* transcript is critical for its function.

Finally, we tested whether overexpression of a constitutively active form of Ras (CA-Ras) could promote formation of primary mesenchyme cells. PMCs formed normally in

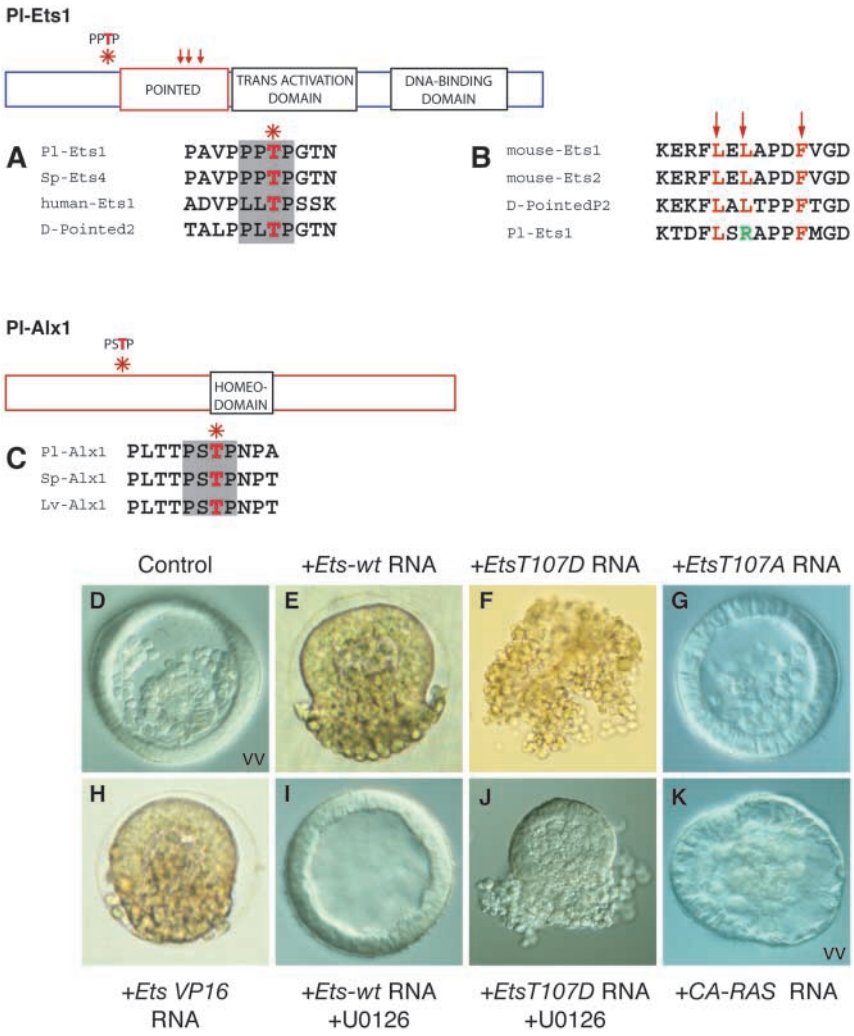

Fig. 7. Structural features of the sea urchin Ets1 and Alx1 proteins. (A,B) The *P. lividus* Ets1 protein contains a conserved MAPK phosphorylation consensus site and a putative ERK docking site in the Pointed domain. Partial sequence alignments between two sea urchin Ets1 protein sequences and the human Ets1, Ets2 and the *Drosophila* Pointed proteins showing the conservation of the PXTP motif (A) and ERK docking site (B). (C) The N-terminal region of the *P. lividus* Alx1 protein contains a putative MAPK phosphorylation consensus site (PSTP). Comparison of three different sea urchin Alx1 sequences showing conservation of this site. (D-K) Effects of mutations in the putative MAPK phosphorylation site of Ets1 on formation of PMCs and effects of activated Ras mRNA injections. (A) Control mesenchyme blastula stage. Overexpression of wild-type *ets1* (E), *ets1 T107D* (F) or *ets1 VP16* (H) converts a large number of cells into mesenchymal cells. Treatment with U0126 can block the effects of overexpressing *ets1* (I) but not *ets1 T107D* (J). Mutating the putative MAPK phosphorylation site of the sea urchin Ets1 (*etsT107A*) abolishes its ability to promote epithelial-mesenchymal transition (G). (K) Overexpression of *CA-Ras* does not cause the same phenotype as overexpression of *ets1* but causes a global epithelial remodeling. (vv) vegetal view.

embryos injected with mRNA encoding an activated form of Ras. However, during early gastrulation we found that most cells of the injected embryos rounded up and detached from the hyaline layer. In large sectors of the injected embryos, the ectoderm became very thin (Fig. 7K). Cells continuously detached from the epithelium while the remaining cells stretched to cover the embryo. This broad disorganisation of the epithelium appeared very different from the localized epithelial-mesenchymal transition caused by overexpression of *ets1*, *ets1-VP16*, *ets1-T107D* (Fig. 7) or *pmar1* (Oliveri et al., 2002), and it occurred at a later stage. This suggests that the remodeling of the ectoderm caused by artificial activation of the pathway at the level of Ras is a process different from the epithelial-mensenchymal transition induced by overexpression of *ets1* or *pmar*.

Activation of MEK/ERK in the PMCs requires the maternal TCF/β-Catenin pathway

We tested whether activation of ERK in the micromeres was dependent on the maternal β-Catenin pathway, which is responsible for patterning along the AV axis. Injection of mRNA encoding ΔN-TCF/Lef, a dominant-negative form of TCF/Lef which blocks the Wnt/β-Catenin pathway, caused a typical animalized phenotype (Fig. 8H) and prevented activation of ERK at the hatching blastula stage as revealed by immunostaining. (Fig. 8E). Similarly, ERK activation in the micromeres could not be detected in embryos treated with $ZnCl_2$ (Fig. 8B), a classical animalizing reagent. By contrast, neither treatment of embryos with lithium, which activates maternal Wnt/β-Catenin pathway by inhibiting GSK3, nor overexpression of Wnt8 (data not shown) caused an increase in the number of cells positive for activated ERK (Fig. 8C,F). These results suggest that activation of ERK requires a functional Wnt/β-Catenin pathway but that activation of this pathway is not sufficient to activate ERK or trigger ingression of the micromeres.

Activation of the MEK/ERK signaling pathway may be cell autonomous

In a number of developmental systems, activation of ERK and epithelial-mesenchymal transitions have been shown to be triggered by signals emitted by neighboring cells (Savagner, 2001). Surprisingly, we found that ingression of the PMCs was not inhibited by overexpression of a dominant-negative form of Ras, which mediates signals from membrane tyrosine kinases to ERK (Fig. 2). To address whether intercellular contact is required for the activation of ERK in the micromeres, we followed ERK phosphorylation in dissociated cells. Embryos were dissociated into single blastomeres by gentle agitation in Ca^{2+}-free sea water from just after fertilization. As a positive control for dissociation, we measured the expression of the ectodermal marker *goosecoïd*, which is strictly dependent on cell interactions (Angerer et al., 2001) (data not shown) (Fig. 8K-M). Western

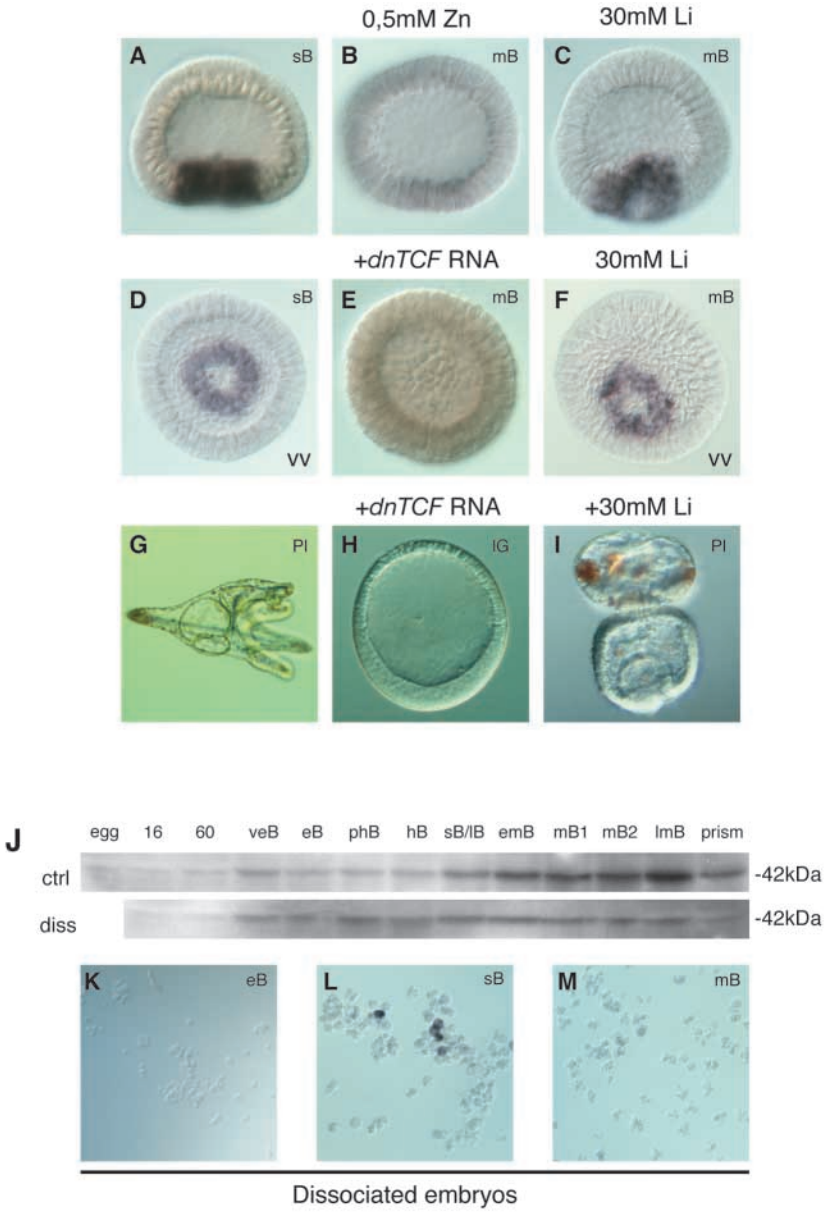

Fig. 8. Activation of the MAP Kinase is downstream of the Wnt/β-Catenin pathway but does not requires intercellular communication. Immunolocalizations using an anti MAPK-P antibody on control embryos (A,D), zinc-treated (B), dnTCF RNA-injected (animalized) (E) or lithium treated (vegetalized) (C,F) embryos. Phenotypes of a control pluteus (G), dnTCF RNA injected animalized embryo (H) and a lithium vegetalized embryo (I). (J) Time course of MAPK activation analysed by western blot analysis and effect of cell dissociation. 16, sixteen-cell stage; 60, 60-cell stage; veB, very early blastula; eB, early blastula; phB, prehatching blastula; hB, hatching blastula; sB, swimming blastula; mB, mesenchyme blastula; lG, late gastrula; Pl, pluteus; vv, vegetal view. (K-M) Activation of ERK in dissociated blastomeres analysed by immunostaining. Note that the cellular aggregates visible on the pictures were not present during the culture but formed during fixation and the immunostaining protocol.

blot analysis showed that activation of MAP kinase in dissociated blastomeres was initiated prior to hatching at about the same time as in intact embryos (Fig. 8J). During the later stages, however, a much lower level of activated MAPK-P was detected than in intact embryos. Correspondingly, about 5% of the dissociated blastomeres at the late blastula stage stained positively for activated ERK (Fig. 8L), but no specific signal could be detected at the early blastula or mesenchyme blastula stages (Fig. 8K). This percentage is close to the expected ratio of micromeres versus total number of cells (32:~500 cells) at this stage (ninth cleavage) (Lepage et al., 1992). We conclude that activation of ERK occurs on time in dissociated blastomeres and probably does not require intercellular contacts. The possibility remains that autocrine signaling by a Ras-independent pathway could be responsible for activation of ERK in the micromeres.

Discussion

We have investigated the role of the MAP kinase signaling pathway in the sea urchin embryo. We found a triple requirement for Raf, MEK and ERK activity for ingression of the micromeres, which is the first important morphogenetic event of sea urchin gastrulation (Fig. 9). In the absence of ERK signaling, the program of gene expression responsible for specification of the skeletogenic mesoderm is blocked at an early stage and the specification of a subset of SMCs fates is affected. Moreover, by overexpressing wild-type and mutated forms of Ets1 in the presence or absence of ERK function, we found that ERK controls epithelial mesenchymal transition of the micromeres mostly through the phosphorylation of Ets1. Our study also shows that in the early sea urchin embryo, activation of ERK in the micromeres is downstream of Wnt/ β-Catenin. Finally, we have shown that ERK activation can occur in dissociated cells and that Ras-mediated signaling is not required for activation of ERK or for micromere ingression, suggesting that ERK activation is a cell autonomous process.

A Raf/MEK/ERK signaling pathway required for development of the sea urchin embryo micromere lineage.

In this study, we have analyzed the role of ERK during sea urchin development and described its spatial and temporal activation. Immunostaining of whole embryos showed that the diphosphorylated form of ERK is highly enriched in the 32 precursors of the PMCs in the blastula and for a short period starting around hatching. This very restricted temporal and spatial pattern of ERK activation strongly suggested that ERK had a special function in this lineage. Indeed, a striking phenotype resulted from inhibition of ERK function. In embryos treated with the MEK inhibitor U0126, no PMCs formed, and a much reduced number of SMCs migrated out

from the archenteron during gastrulation. The same effects were observed after overexpression of a MAP kinase phosphatase or a dominant-negative form of Raf. In another sea urchin species, PD98059, another less potent inhibitor of MEK, delays PMC ingression (Katow and Aizu, 2002). In *P. lividus*, this inhibitor did not block PMC ingression and differentiation.

Differentiation of the endoderm and ectoderm remained largely unaffected by the inhibition of MEK signaling with U0126. The gut separated normally into three compartments and a mouth formed. Similarly, even in the absence of spicules, the ectoderm acquired a well-developed oral-aboral polarity, as indicated by the overall morphology of the larva and the normal expression of several markers. Therefore, in the sea urchin embryo, the ERK signaling pathway appears to be required mainly before gastrulation, for the formation of the mesoderm.

In vertebrates, the ERK signaling pathway has been implicated in mesoderm induction downstream of the FGF signaling cascade (Curran and Grainger, 2000; Schohl and Fagotto, 2002; Uzgare et al., 1998), in patterning the embryo along the dorsoventral axis (Furthauer et al., 1997) and in the induction of the neural tissue by FGF factors (Streit et al., 2000). As activation of this signaling pathway during gastrulation in deuterostomes is often associated with signaling from the FGF receptor, it is tempting to speculate that ligands of the FGF family are responsible for activation of ERK in the mesoderm in the sea urchin embryo. Our preliminary data using FGF pathway inhibitors indicate that this is not the case, consistent with the observation that injection of dnRas RNA does not block ingression.

Activation of ERK is required for maintaining specification of the micromeres and for formation of a subset of SMCs

We found that the expression of several important genes of the gene network regulating micromere specification was severely affected by the inhibition of MEK signaling. Expression of the homeobox gene *alx1*, which is required for ingression of the PMC and skeletogenesis, was reduced at the blastula stage and almost absent at later stages. More strikingly, the expression of *skeT*, which is restricted to the large micromere lineage from the blastula stage, and of several differentiation markers such as *msp130*, was abolished. In the absence of ERK signaling, the micromere gene expression program, which was probably well under way at the blastula stage, was arrested, the expression of several key genes was downregulated and ingression was inhibited. We conclude that, although ERK activation occurs after the initial expression of early regulators such as *pmar*, *alx1* and *ets1*, ERK function is critically required for maintenance of this specified state.

Finally, we found that blocking the MAP kinase signaling pathway diminished the early expression of Delta in the large micromere lineage, but did not significantly perturb the late Delta expression in the macromeres descendants. This effect provides an explanation for the fact that pigment cells are more affected than other SMC derivatives because pigment cells have a strict requirement for Delta signaling from the micromeres, while blastocoelar cells can form by Notch/Delta signaling among macromeres descendants (Sweet et al., 2002).

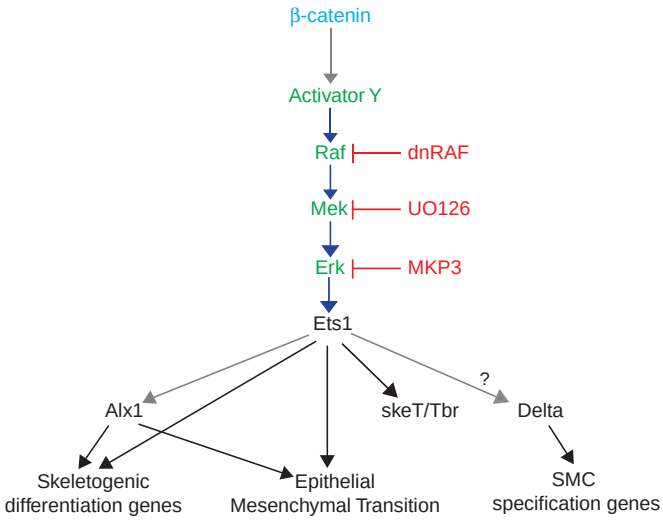

Fig. 9. Model for the role of the Raf/MEK/ERK signaling pathway in the gene network regulating mesendoderm formation. At the blastula stage, activation of the Raf/MEK/ERK signaling pathway by an unknown factor Y, allows phosphorylation of Ets1, which is crucially required for the maintenance of *alx1* and Delta expression in the micromeres, and for the initiation of *skeT/Tbr* and other downstream genes in this lineage.

The sea urchin Ets 1 is a key target of the ERK signaling pathway

A number of different transcription factor families, including the Ets family, have been implicated in epithelial mesenchymal transitions and cell migrations (Savagner, 2001). In vertebrates, *ets1* and *ets2* are expressed in several tissues undergoing morphogenetic changes. For example in *Xenopus*, *ets1* is expressed in the involuting marginal zone and in premigratory and migratory neural crest cells. In the sea urchin embryo, maternal *ets1* mRNA is ubiquitous whereas zygotic *ets1* expression becomes restricted to the precursors of the PMCs at the early blastula stage and extends to the precursors of the SMCs at the end of gastrulation. It has been shown previously that overexpression of *ets1* induces mesenchymalization of most cells of the blastula stage embryo whereas overexpression of a truncated version that inhibits Ets1 function blocks PMC ingression (Kurokawa et al., 1999). This led to the suggestion that Ets1 is a key regulator of epithelial-mesenchymal transition in the normal embryo. Our results in *P. lividus* support this idea. They further demonstrate that post-transcriptional modification of this transcription factor is a crucial step for its function. We found that the sea urchin Ets1 protein, like the vertebrate Ets1 and Ets2 and their probable *Drosophila* ortholog Pointed-P2, which contains a conserved MAP kinase phosphorylation site PXS/T upstream of the conserved N-terminal pointed domain (PNT). Phosphorylation of this site by Ras>Raf>MEK>MAP-kinase signaling is thought to stimulate the transcriptional activation function of this class of Ets family members by an unknown mechanism. We found that embryos overexpressing an activated form of Ets (EtsT107D) are largely insensitive to the MEK inhibitor. Conversely, mutation of the phosphoacceptor Thr107 to Ala abrogated the phenotypic conversion from epithelial to mesenchymal induced by *ets1* mRNA overexpression. Taken

together these results show that phosphorylation of Ets1 on Thr107 is a key event for its activation.

In addition to the presence of a MAP kinase consensus site, the sea urchin Ets1 contains a sequence very similar to the ERK2 docking site of Ets1 and Ets2. Mutation of this docking site in the vertebrate Ets1 prevents the transcriptional activation of a promoter containing a Ras Responsive Element (RRE) that contains Ets1-binding sites. As stressed by Seidel and Graves (Seidel and Graves, 2002), the presence of ERK docking sites is not widespread among Ets family members. Only 1 out of 10 of *Drosophila* Ets family members and two out of 25 human Ets protein sequences contain an ERK docking site. The combined presence of an ERK docking site and an ERK consensus phosphorylation site distinguishes a subset of Ets proteins activated by MAP kinase signaling (Oikawa and Yamada, 2003). The sea urchin Ets1, like the vertebrates Ets1/2 and *Drosophila* Pointed, appears to contain both motifs.

Activation of ERK is downstream of Wnt/β-Catenin and may be cell autonomous

The Wnt/β-Catenin pathway has been identified as the major pathway regulating patterning along the animal vegetal axis of the sea urchin embryo. Accumulation of β-Catenin in the nuclei of micromeres from the time of their segregation up to the blastula stage is required for specification of the PMCs. (Emily-Fenouil et al., 1998; Huang et al., 2000; Logan et al., 1999; Vonica et al., 2000). We found that ERK activation depends on a functional TCF/Lef factor and does not occur in animalized embryos. By contrast, artificial activation of the Wnt pathway by treatment with lithium was not sufficient to activate ERK in other cells, indicating that additional factors present only in the region of the vegetal pole are required the spatial regulation of ERK.

In most models where it has been studied, the epithelial-mesenchymal transition is a non-cell-autonomous process triggered by extracellular signals binding to their cognate receptors. Four lines of evidence suggest, however, that in the sea urchin embryo ERK activation occurs in absence of extracellular signal. First, we found that phosphorylation of ERK was transiently detectable in dissociated blastomeres and the time course of phosphorylation analyzed by western blot was not significantly different from that in intact embryos. Second, micromere formation was not blocked by overexpression of a dominant-negative version of Ras that mediates the activity of most receptor tyrosine kinases. Third, activation of ERK depends on the TCF/β-Catenin pathway, which is cell-autonomously activated in the sea urchin embryo. Finally, micromeres are capable of differentiating into skeletogenic cells when cultured in a simple medium, grafted to ectopic positions or reassociated with various tiers of blastomeres (Horstadius, 1973; Okazaki, 1975).

In most other classes of echinoderms and in some sea urchin species, embryogenesis procedes without micromeres. Embryos do not undergo an epithelial mesenchymal transition at the blastula stage and therefore resemble the *P. lividus* embryos treated with the MEK inhibitor. It will be interesting to examine how genes like *ets1* and *alx1* are expressed, and if ERK is activated at the vegetal pole in these embryos.

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