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Identification and developmental expression of the *ets* gene family in the sea urchin (*Strongylocentrotus purpuratus*)

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Abstract

A systematic search in the available scaffolds of the *Strongylocentrotus purpuratus* genome has revealed that this sea urchin has 11 members of the *ets* gene family. A phylogenetic analysis of these genes showed that almost all vertebrate *ets* subfamilies, with the exception of one, so far found only in mammals, are each represented by one orthologous sea urchin gene. The temporal and spatial expression of the identified ETS factors was also analyzed during embryogenesis. Five *ets* genes (*Sp-Ets1/2*, *Sp-Tel*, *Sp-Pea*, *Sp-Ets4*, *Sp-Erf*) are also maternally expressed. Three genes (*Sp-Elk*, *Sp-Elf*, *Sp-Erf*) are ubiquitously expressed during embryogenesis, while two others (*Sp-Gabp*, *Sp-Pu.1*) are not transcribed until late larval stages. Remarkably, five of the nine sea urchin *ets* genes expressed during embryogenesis are exclusively (*Sp-Ets1/2*, *Sp-Erg*, *Sp-Ese*) or additionally (*Sp-Tel*, *Sp-Pea*) expressed in mesenchyme cells and/or their progenitors. Functional analysis of *Sp-Ets1/2* has previously demonstrated an essential role of this gene in the specification of the skeletogenic mesenchyme lineage. The dynamic, and in some cases overlapping and/or unique, developmental expression pattern of the latter five genes suggests a complex, non-redundant function for ETS factors in sea urchin mesenchyme formation and differentiation.

Keywords: *ets* gene family; Sea urchin; Mesenchyme; *Strongylocentrotus purpuratus*

Introduction

The *ets* gene family of transcription factors is noted for its wide distribution among metazoans (Degnan et al., 1993). Genes belonging to this family have been identified in a variety of animals, including sponges and ctenophores, but not in plants, fungi, yeast or any protozoan (Laudet et al., 1993). There are 27 paralogous *ets* genes in *Homo* (Hollenhorst et al., 2004), 8 in *Drosophila* (Hsu and Schulz, 2000), 14 in *Ciona* (Leveugle et al., 2004; Yagi et al., 2003) and 10 in *Caenorhabdtis* (Hart et al., 2000). Three main features characterize this family and allow for sub-classification. These are: (i) sequence homology of highly conserved domains such as the ETS-(DNA-binding-) domain, shared by all members, and the pointed (PNT) domain, which is conserved in a subset of family members and also found in various receptors, protein kinases and adaptor proteins (Graves and Petersen, 1998; Sharrocks et al., 1997); (ii) the widespread and diverse interaction with co-regulatory partner proteins, which is a consequence of domain conservation and often reflected in similar functions observed within members belonging to the same subfamily (Li et al., 2000); (iii) the ability to act as a nuclear target for signal-transduction pathways, in particular those mediated by MAP kinases, which bind to specific docking domains and target conserved phosphor-acceptor motifs found in a subset of family members (reviewed in Yordy and Muise-Helmericks, 2000).

Functional analyses of these genes have revealed essential, non-redundant roles in many different developmental processes including cell proliferation, apoptosis, differentiation, migration, transformation and hematopoiesis (for a recent review see Sharrocks, 2001).

Genes belonging to only two members of this family have so far functionally been characterized in sea urchin. These are the *Sp-Ets4* gene, identified as a regulator of the mechanism that establishes the animal–vegetal axis of the sea urchin embryo

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(Wei et al., 1999a), and three homologues of the human *ETS-1* gene, the founder member of the ETS domain family that was first discovered from the E24 avian leukemia virus as the *v-ets* (*e twenty-six*) oncogene (Karim et al., 1990). For these sea urchin genes, an important role in the process of micromere specification and skeletogenic cell differentiation has been recently uncovered in three different species (Kurokawa et al., 1999; P. Oliveri, personal communication; Rottinger et al., 2004). The central function exhibited by these sea urchin *ets* genes, together with the high degree of conservation of ETS domains displayed by *ets* family members, which is often reflected in overlapping binding specificities (Sharrocks et al., 1997), persuaded us to investigate this gene family systematically in the sea urchin. Moreover, the availability of the *Strongylocentrotus purpuratus* genome sequence (released by Human Genome Sequencing Center at Baylor College of Medicine) and the phylogenetic position of sea urchins as non-chordate deuterostomes offered the opportunity of re-evaluating the evolution of this important gene family with particular regard to the emergence of vertebrates. Here we report the isolation, domain structure, phylogenetic analyses and expression profiling during embryonic development of eleven *ets* genes identified in the *S. purpuratus* genome. Given the well documented amenability of the sea urchin embryo for experimental analysis of gene regulatory networks (Davidson et al., 2002; Oliveri and Davidson, 2004), this work may provide the essential framework for investigations into the complex molecular interactions and multiple biological roles of ETS proteins *in vivo*.

Materials and methods

Animals

Adult *S. purpuratus* were obtained from Pat Leahy (Kerchoff Marine Laboratory, California Institute of Technology, USA). Spawning was induced by vigorous shaking of animals or by intracoelomic injection of 0.5 M KCl. Embryos were cultured at 15°C in Millipore filtered Mediterranean seawater (MFSW) diluted 9:1 in deionized H_2O.

Gene search and phylogenetic analyses

S. purpuratus Ets proteins were identified with BLAST searches against the traces, contigs, scaffolds and Glean3 database at the HGSC, Baylor College of Medicine site: http://www.hgsc.bcm.tmc.edu/blast/?organism=Spurpuratus. Identified Glean3 predictions were manually annotated and validated where possible with known ESTs or by PCR amplification and sequencing. A phylogenetic tree comparing sea urchin *ets* genes and homologues from multiple species was constructed in order to name accurately the newly identified genes. Orthologous sequences were obtained by database searches using BLASTP and TBLASTX (http://www.ncbi.nlm.nih.gov/BLAST/). Protein domains were identified using SMART (Letunic et al., 2006; Schultz et al., 1998) (http://smart.embl-heidelberg.de/) and Pfam (Bateman et al., 2004) (http://www.sanger.ac.uk/Software/Pfam/) databases. Accession numbers of all the sequences are listed in Table S1. Multiple sequence alignment of ETS domains was generated by CLUSTAL X 1.83 (Thompson et al., 1994) then manually optimized with GeneDoc (http://www.psc.edu/biomed/genedoc). Phylogenetic reconstruction was carried out using the neighbor-joining method, and bootstrap values determined by 1000 replicates. The tree was also generated using maximum parsimony methods with bootstrap replicates of 1000. The final output of the phylogenetic tree was obtained using Treeview software version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Isolation of sea urchin ets cDNAs

The cDNAs corresponding to the complete coding sequences of *Sp-Elk*, *Sp-Erg* and *Sp-Elf* were obtained by screening arrayed cDNA libraries from *S. purpuratus* 20 h and 40 h embryos (Cameron et al., 2000) following an established protocol (Rast et al., 2000). For the *ets* genes, and where necessary, PCR and 3′ or 5′ RACE (Invitrogen, Carlsbad, CA) of *S. purpuratus* cDNA were performed to confirm transcript sequence of predicted genes. Isolated cDNAs were fully sequenced using an Automated Capillary Electrophoresis Sequencer *3730 DNA Analyzer* (Applied Biosystems, Foster City, CA). The list and reference numbers of all *ets* cDNAs are reported in Table S2 of Supplementary material.

Quantitative PCR (QPCR)

Total RNA was extracted from embryos at various stages (egg, 8, 10, 15, 18, 21, 24, 33, 40, 45, 48, 52, 72 h post fertilization, hpf) using Eurozol reagent (Euroclone, Celbio, Milan, Italy). Residual DNA was digested with DNase I using a DNA-free kit (Ambion, Austin, TX). First-strand cDNA was synthesized in a 50 μl reaction from 1 μg of total RNA using random hexamers and the TaqMan Reverse transcription Kit (Applied Biosystems). Specific primer sets (Table S3) for each gene were designed using the Primer3 program (Rozen and Skaletsky, 2000) (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). Primer sets were chosen to amplify products 100–200 bp in length. Blast searches were used to ensure that primers were specific for each individual *ets* gene. cDNA was diluted to a concentration of 1 embryo/μl. Reactions were performed in triplicate using the Chromo 4 real-time detector (BioRad, Hercules, CA) with SYBR Green chemistry (Applied Biosystems). Data for each gene were normalized against ubiquitin mRNA, which is known to be expressed at constant levels during the first 72 h of development (Nemer et al., 1991). Primer efficiencies (i.e., the amplification factor for each cycle) were found to exceed 1.9. Calculations from QPCR raw data used the formula $1.9^{\Delta Ct}$, where 1.9 is the multiplier for amplification per PCR cycle, and ΔCt is the cycle threshold difference with ubiquitin found for that sample. Absolute quantification of the number of transcripts was obtained by using SpZ12-1 as an internal standard. The number of SpZ12-1 transcripts in embryos of the relevant stages had been measured earlier by RNA titration (Wang et al., 1995).

Whole-mount in situ hybridization (WMISH)

Fragments of *Sp-Elk*, *Sp-Ese*, *Sp-Erf*, *Sp-Ets*, *Sp-Gabp*, *Sp-Pea* and *Sp-Tel* were amplified from cDNA templates by PCR using specific primers (Table S3, Supplementary material). PCR products were purified and cloned into pCRII-TOPO (Invitrogen) according to the manufacturer's instructions and the identity of inserts confirmed by sequencing. For *Sp-Erg*, a fragment of 450 bp was subcloned into $pBSK^+$ (Stratagene, La Jolla, CA) using *Kpn*I and *Eco*RI sites. For *Sp-Elf*, a 1856 bp long fragment was derived from the cDNA clone 1K4 (20 h library). The position of each probe with reference to the corresponding ETS protein sequence is depicted with a blue line in Fig. 1B.

Whole-mount *in situ* hybridization was performed as described by Minokawa et al. (2004). The accuracy of whole-mount *in situ* hybridization data was confirmed by control experiments using sense probes (not shown). Both antisense- and sense-digoxigenin-labeled RNA probes were obtained using a DIG-RNA labeling kit (Roche, Indianapolis, IN), following the manufacturer's instructions and using 1 μg of linearized plasmids. RNA probes were purified using Mini Quick Spin RNA Columns (Roche). Following staining, embryos were mounted in glycerol and analyzed using a Zeiss Axio Imager M1 microscope operating in DIC mode.

Results

Identification, domain structure and phylogenetic analysis of sea urchin ets genes

The completion of the sequencing of the *S. purpuratus* genome and the release of the first draft genome assembly by

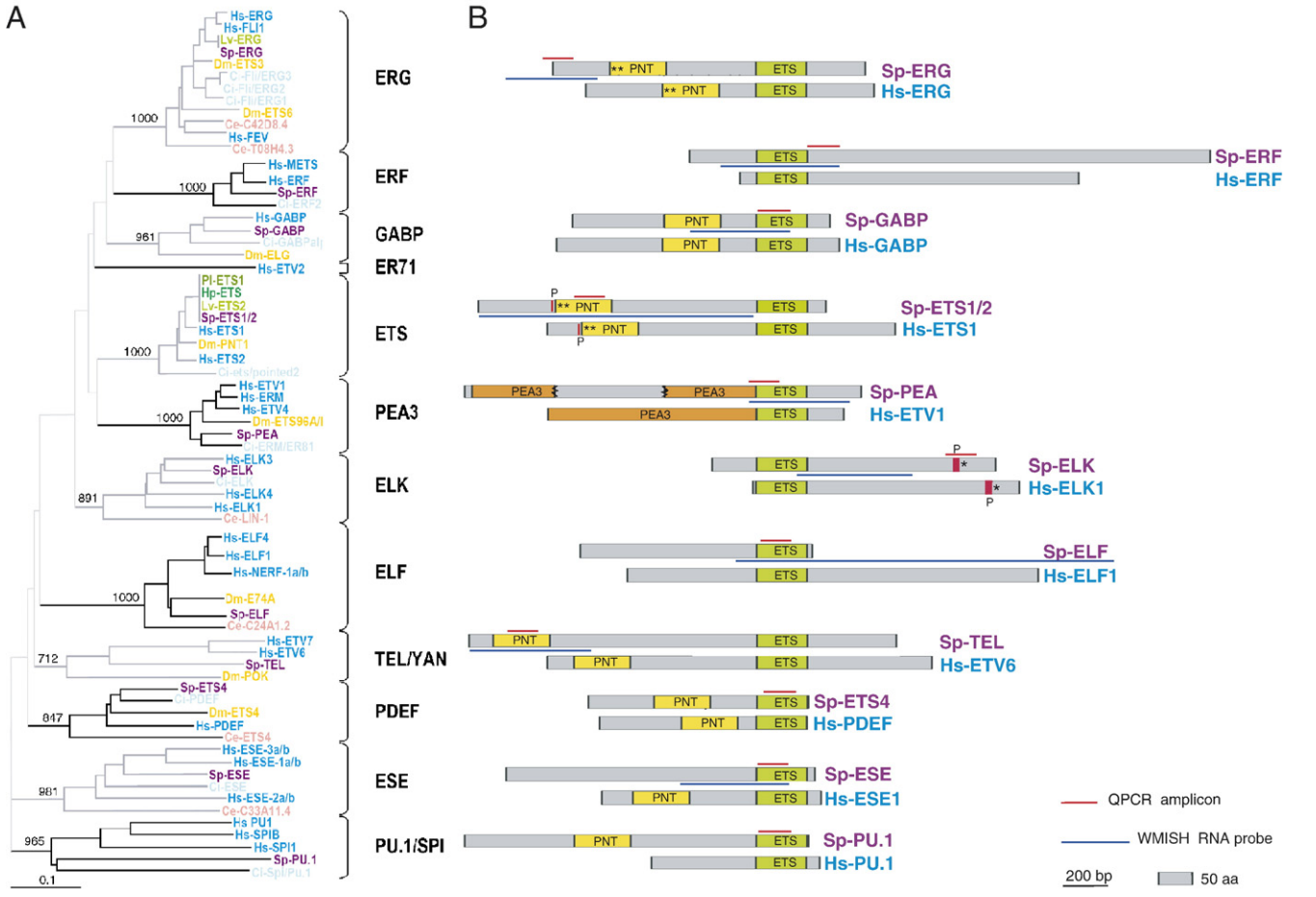


Fig. 1. Phylogenetic analysis of *S. purpuratus ets* genes. (A) Neighbor-joining phylogeny of ETS proteins comprising: 14 sea urchin (*Strongylocentrotus purpuratus* in purple text, other species in green), 27 human (*Homo sapiens*, blue text), 11 *Ciona intestinalis* (pale blue text), 8 *Drosophila melanogaster* (yellow text) and 6 *Caenorhabditis elegans* (pink text) members. The tree was created from the alignment of the amino acid sequence of the ETS, DNA binding, domain using CLUSTAL X and Treeview (see Materials and methods for details). *C. intestinalis* and *C. elegans* genes with incomplete or highly divergent ETS domains were not included in this analysis. The tree was rooted using the SPI/PU.1 group as the outgroup. Numbers give the bootstrap support for given nodes in the phylogram. For sake of simplicity, only upstream-to-group nodes are displayed, the inter-group bootstrap robustness ranging from 236 to 1000. Validation of the tree using maximum parsimony methods confirmed all group nodes. Groups are named according to Laudet et al. (1999) and Sharrocks (2001). All named proteins are appended with the species designation (one letter for the genus, one for the species; see Table S1 for complete names and sequence accession numbers). (B) Schematic structure of *S. purpuratus* ETS proteins in comparison with closest human counterparts. ETS, DNA binding domain, green; PNT, pointed domain, yellow; PEA3, PEA3 activation domain, orange; P, MAP kinase phosphorylation site, recognition motif in red. Asterisks mark the position of conserved MAP kinase docking sites (see text for details; sequence alignment not shown). Aligned along sea urchin ETS proteins are the amplicons used for quantitative PCR (QPCR) and the probes used for whole month *in situ* hybridization (WMISH) analyses, depicted as red and blue lines, respectively.

HGSC at Baylor College of Medicine in November 2005 allowed the identification of all the ETS-related genes in sea urchin. An initial BLASTN homology search through the deposited unassembled traces, followed by analyses of contigs and scaffolds later, was conducted using the ETS DNA binding domain (the "ETS domain"; Graves and Petersen, 1998) of human genes representative of all ETS subgroups (Laudet et al., 1999; Sharrocks, 2001). Sequences corresponding to 11 putative *ets* sea urchin genes were found. Phylogenetic comparison of these urchin ETS domains with those of human, *Ciona intestinalis*, *Drosophila* and *Caenorhabditis elegans* was used to identify sea urchin homologues of specific human genes (Fig. 1A). All 11 sea urchin homologues could be assigned to subgroups where the members of the subgroup are more closely related to each other than they are to other *ets* genes, regardless of species of origin. The putative urchin *ets* genes have therefore been identified and named according to the subgroup to which they belong, as follows: *Sp-Ets1/2*, *Sp-Erg*, *Sp-Ese*, *Sp-Tel*, *Sp-Pea*, *Sp-Ets4*, *Sp-Elk*, *Sp-Elf*, *Sp-Erf*, *Sp-Gabp* and *Sp-Pu1*.

Alignment of sea urchin ETS domains with their most closely related human and *Drosophila* (or *C. elegans*) homologues revealed 13 absolutely conserved amino acids, 7 of which fall within the predicted helix–turn–helix region (Mavrothalassitis et al., 1994) (Fig. 2A). Apart from these thirteen amino acids (in the region of 84 to 90 amino acids encompassing the ETS domain), each sea urchin *ets* gene revealed extended stretches of high similarity with the members of the subgroup to which it belongs, as shown by color boxes in Figs. 2A and B. In these figures, for sake of simplicity, each sea urchin gene is compared to only one of the often several human paralogs belonging to the same group. Given the very high conservation among human *ets* homologues of the same paralog

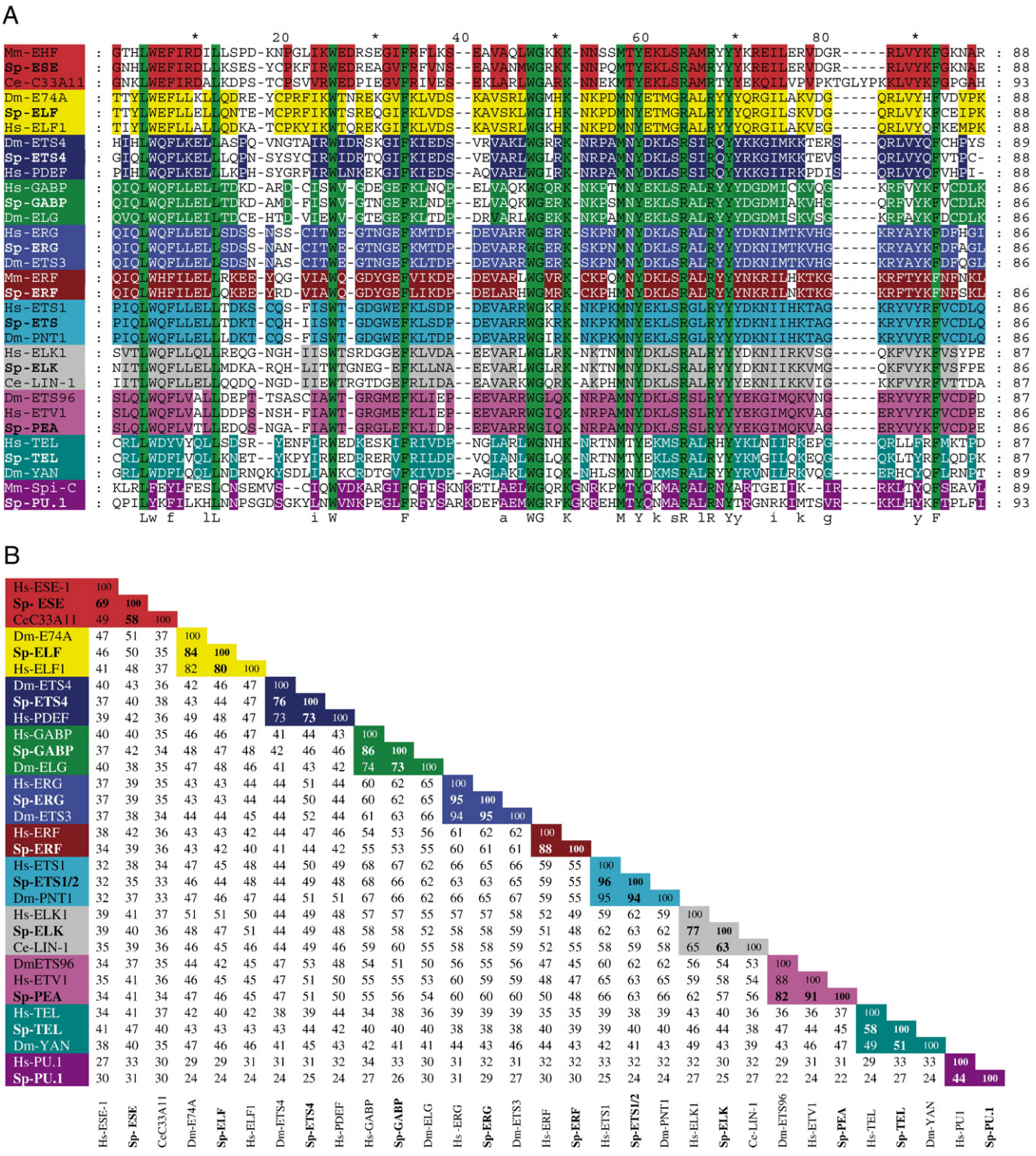


Fig. 2. Multiple sequence alignment of the ETS DNA-binding domain. (A) The amino acid sequence of the ETS domain of the 11 *S. purpuratus* ETS proteins and their closest human and *Drosophila* (or *C. elegans*) relatives were aligned using CLUSTAL X and colored in GeneDoc. Bright green, completely conserved residues. Differently colored boxes highlight similarities within subgroups. Bottom row, consensus sequence. Right column, total number of amino acids included in the ETS domain. (B) Pair wise percent of identities of the ETS proteins aligned in panel A. The statistical report was generated using GeneDoc and manually colored according to panel A.

group, the arbitrary choice of any paralog – here taken as a vertebrate counterpart – does not affect sequence comparison analysis. The most striking conservation is observed within the ETS (96% identity between Sp-ETS1/2 and Hs-ETS1, and 94% between Sp-ETS1/2 and Dm-PNT1) and ERG subgroups (95% identity between Sp-ERG and both Hs-ERG and Dm-ETS3). Significant homology (average 80% identity) is also displayed in the ELF, PDEF, GABP, ERF, ELK and PEA subgroups. Lower identities were observed within the subgroups ESE (69% between Sp-ESE and Hs-ESE-1, and 58% between Sp-ESE and

CeC33A11), and TEL/YAN (58% between Sp-TEL and Hs-TEL, and 51% between Sp-TEL and Dm-YAN). Even the least similar pair, Sp-PU.1 and Hs-PU.1, has 44% identity and 53% similarity. In most cases (nine out eleven), the highest similar pair within each subgroup is observed between sea urchin and human proteins, consistent with the phylogenetic position of echinoderms in the deuterostomian clade.

In total, six cDNA sequences that contain an ETS domain and that could be assigned to three different *ets* genes, have been previously identified in four sea urchin species. These are: *SpEts4* (Wei et al., 1999b), which falls in the PDEF subgroup; *LvErg* (Qi et al., 1992) and *SpErg* (Zhu et al., 2001), which belongs to the ERG subgroup; *HsEts* (Kurokawa et al., 1999), *LvEts2* (Chen et al., 1988), *SpEts1* and *PlEts1* (Rottinger et al., 2004), which all fall in the ETS subgroup (Fig. 1A). The last four genes are all orthologues of the group identified by human *ETS1* and *ETS2*, as well as *Drosophila pnt1* and *pnt2*. We therefore propose to re-name the only *S. purpuratus* gene belonging to the ETS subgroup as *Sp-Ets1/2*. For two of the three previously identified *S. purpuratus ets* genes, namely *SpEts1* (hereby called *Sp-Ets1/2*) and *SpEts4*, complete cDNA sequence was available. To determine the transcript sequence and structure of all predicted sea urchin *ets* genes, a combination of cDNA library screening (for *Sp-Erg*, *Sp-Elf* and *Sp-Elk*), 5′ and 3′ RACE sequencing, PCR cloning and analysis of existing EST databases was used (see Table S2 for details).

The domain structure of each *S. purpuratus* ETS protein in comparison with its closest human homolog is depicted in Fig. 1B. In addition to the ETS domain already mentioned, a significant fraction of known *ets* genes share a second highly conserved domain, the POINTED (PNT) domain (Klambt, 1993). The presence and position of the PNT domain are conserved in five of the six sea urchin human gene homologues that possess the PNT domain. The only exception is observed in the more divergent ESE and PU.1/SPI subgroups: while Hs-ESE1 possesses a PNT domain and Hs-PU.1 does not, the reverse situation is found in sea urchin, where Sp-ESE does not have a PNT domain, while Sp-PU.1 displays one PNT domain (see Fig. 1B). Interestingly, the latter gene is considerably longer that the human counterpart. When overall domain organization is taken into account, in particular (i) the position of the ETS domain with respect to the N- and C-terminal region, (ii) the relative positions of the ETS and PNT domains, and (iii) the total length of the proteins, it appears that the Sp-ERG/Hs-ERG, Sp-ERF/Hs-ERF, Sp-GABP/Hs-GABP, Sp-ETS1/2/Hs-ETs1, Sp-PEA/Hs-ETV1, Sp-ELK/Hs-ELK1, Sp-TEL/Hs-ETV6 and Sp-ETF4/Hs-PDEF pairs are most similar. An exception is the Sp-ELF/Hs-ELF1 pair where the sea urchin protein appears truncated at the C-terminus, although it presents a considerably longer 3′ untraslated region (see Table S2). On the other hand, several additional elements of conservation are found within each group. The PEA3 domain, a 300 amino acid domain found just upstream to the ETS domain in all proteins belonging to the PEA3 subgroup (Pfam entry ETS_PEA3_N; de Launoit et al., 1997), is present, although interrupted, in Sp-PEA. Moreover, several motifs, known targets of MAP kinase phosphorylation, appear also conserved in three sea urchin ETS proteins. Depicted in Fig. 1B are: the MAP kinase ERK docking sites present in proteins of the ERG and ETS subgroups (Seidel and Graves, 2002), which appear conserved in the PNT domain of Sp-ETS1/2 and Sp-ERG (double asterisk); the IHFWStLSP MAP kinase recognition motif found in all members of the ELK subgroup in mammals (Yang et al., 1998a,b) as well as the FQFP motif, shown to promote docking by the ERK MAPK in *C. elegans* Lin-1 (Jacobs et al., 1998) and in mammalian ELK/SAP proteins (Jacobs et al., 1999), which are both present in Sp-ELK (the red vertical bar and an asterisk, respectively; sequence alignment not shown).

Expression of sea urchin ets genes during embryonic development

S. purpuratus completes embryogenesis, from egg to larva, in 72 h and QPCR was used to determine which *ets* genes were active during this period. Expression levels of the 11 sea urchin *ets* genes were measured quantitatively at 10–13 time points, from egg (time zero) to larval stage (72 hpf). The results of these measurements, reported in individual graphs, one for each gene, are shown in Fig. 3. Five genes (*Sp-Ets1/2*, *Sp-Ets4*, *Sp-Tel*, *Sp-Erf*, *Sp-Pea*) are significantly represented in maternal mRNA. With the exception of the exceptionally high number of transcripts per egg exhibited by *Sp-Ets1/2* (>40,000), average maternal mRNA levels were about 2300, ranging from 800 to 4000, transcripts per egg. Expression levels of *Sp-Ets1/2* are noticeably high (>20,000 copies per embryo) during cleavage up to 10 hpf, then decrease steeply to about 7000 transcripts per embryo at early blastula stage (15 hpf), and maintain quite high levels throughout gastrulation (average 4000 transcripts per embryo). In striking contrast, two genes (*Sp-Gabp* and *Sp-Pu.1*) are not expressed during embryogenesis, but are transcribed during late larval stages and/or in adult tissues (not shown). The range of expression levels among the remaining nine *ets* genes over developmental time is rather broad, varying from the few hundreds to as many as 8300 mRNA molecules per embryo. Three genes, *Sp-Ets4*, *Sp-Tel* and *Sp-Elk*, show the highest levels of expression from cleavage to early blastula stage (8–15 hpf). Four other genes, *Sp-Erg*, *Sp-Ese*, *Sp-Pea*, *Sp-Elf*, reach maximal expression levels during gastrulation, from 24 to 48 hpf. *Sp-Erf* shows a biphasic profile with two peaks, one at cleavage (8 hpf) and the other during gastrulation (33 hpf).

The expression patterns of all sea urchin *ets* genes during embryogenesis, with only the exception of *Sp-Ets4* which has been previously characterized (Wei et al., 1999a,b), has been assessed during development by whole-mount *in situ* hybridization. Five stages were analyzed for each gene: cleavage stage, from 4th to 6th (5–8 hpf), when the large micromeres, founder blastomeres of the primary mesenchyme cells (PMC), segregate by unequal cleavage at the vegetal pole; early blastula stage (13–15 hpf), when prospective PMCs (inner ring) and prospective secondary mesenchyme cells (SMC, outer ring) lie as concentric circles at the vegetal pole of the one cell thick spherical embryo; mesenchyme blastula (22–24 hpf), when PMCs are completing their ingression into the blastocoel from the thickened vegetal epithelium; early gastrula (28–36 hpf),

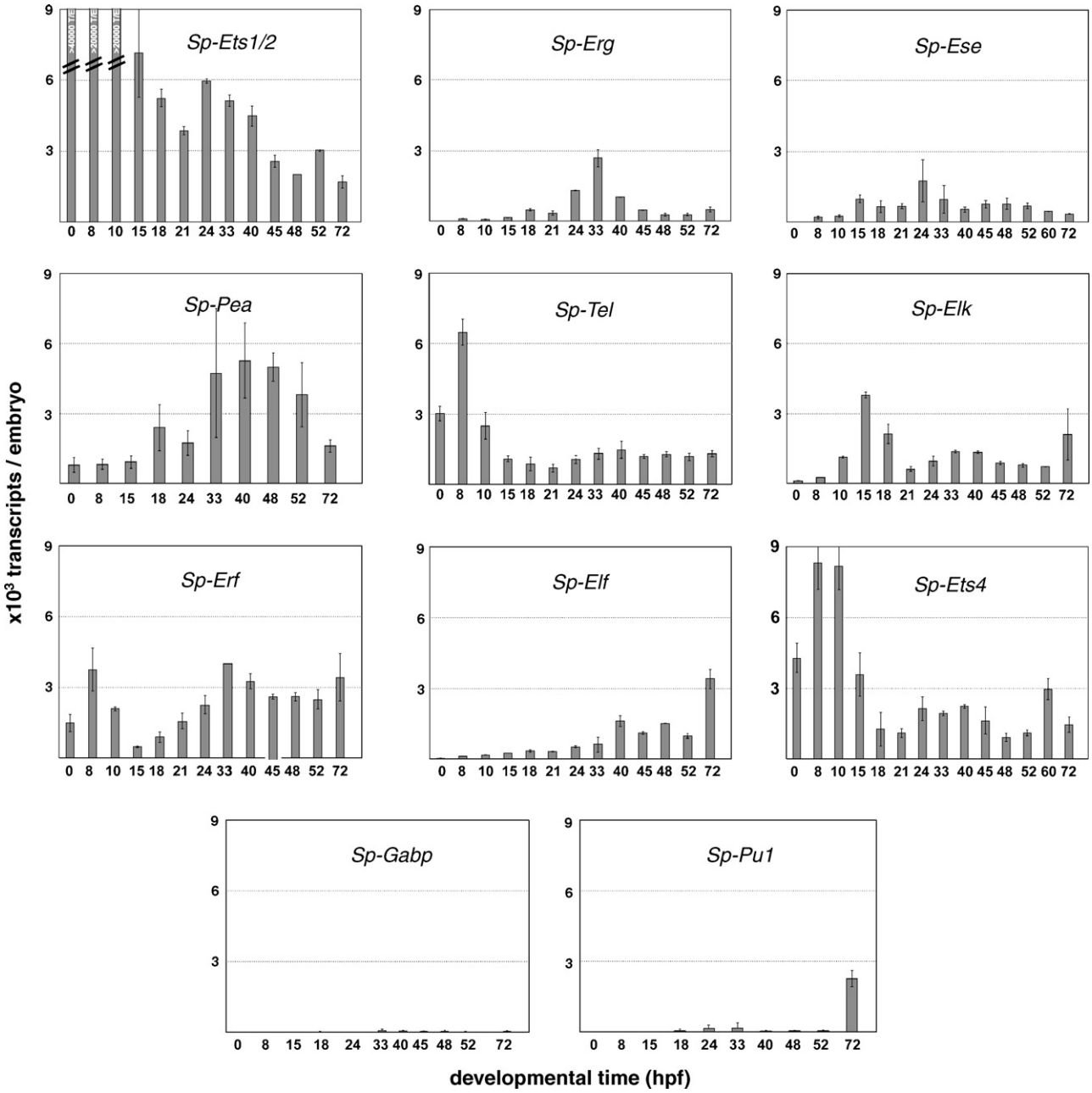


Fig. 3. Quantitative profile of expression of *S. purpuratus ets* genes during embryonic development (0–72 h post fertilization, hpf). The number of transcripts per embryo was calculated from QPCR data using SpZ12 (Wang et al., 1995) as internal standard (see Materials and methods for details). All quantitative measurements were done in triplicate on at least two different batches of embryo cDNA. Average calculations over the various measurements ± standard deviations per individual time points are reported for each gene as grey columns with error bars.

when PMCs migrate on the inner surface of the gastrula wall to arrange in a ring around the blastopore and SMCs delaminate off the tip of the invaginating archenteron; late gastrula (45–48 hpf), when PMCs begin to secrete the larval skeleton at their two ventral–lateral clusters and SMCs keep on migrating from the tip of the fully elongated archenteron to reach their definitive location into the ectoderm (pigment cells), in various regions of the blastocoel (blastocoelar cells), and along the foregut (muscle cells). The spatial expression patterns obtained for eight sea urchin *ets* genes are reported in Fig. 4 and described in the following sections, grouped according to pattern.

Sp-Ets1/2, Sp-Erg and Sp-Ese

Sp-Ets1/2, among *ets* genes, and probably among all transcription factors, is the gene most represented in the sea urchin maternal mRNA stockpile. This initial set of transcripts, probably reinforced by zygotic transcription (starting from 8 hpf; data not shown), is uniformly distributed in all blastomeres during early cleavage (Fig. 4, A1). From 10 h of development, when a sharp decrease in *Sp-Ets1/2* expression levels was observed (Fig. 3), a clearance of transcripts was noted starting from the animal pole (not shown). At 13 hpf, *Sp-*

Ets1/2 pattern of expression resolves as a ring of cells at the vegetal pole of the embryo that includes all micromere descendants, the prospective PMCs (Fig. 4, A2). *Sp-Ets1/2* is transcribed at high levels in PMCs while they ingress into the blastocoel (Fig. 4, A3). Expression in the skeletogenic lineage is retained throughout gastrulation (Fig. 4, A4, A5) and during the process of differentiation. When invagination of the archenteron begins, *Sp-Ets1/2* is also activated in the prospective secondary mesenchyme cells (SMC) at the vegetal pole of the embryo (Fig. 4, A4). At late gastrula stage, *Sp-Ets1/2* mRNA is also detectable in several SMCs delaminating from the tip of the gut and migrating towards the oral ectoderm (Fig. 4, A5).

Sp-Erg exhibits a pattern of expression very similar to *Sp-Ets1/2*, the main difference being that its expression levels are much lower than *Sp-Ets1/2* and maternal transcripts are undetectable (Fig. 3). Moreover, *Sp-Erg* is not expressed during cleavage (Figs. 3 and 4, B1). Similarly to *Sp-Ets1/2*, at 15 hpf, all future PMCs are stained by the *Sp-Erg* probe (Fig. 4, B2) and this expression is retained in the PMCs while they ingress into the blastocoel. Unlike *Sp-Ets1/2*, which is confined to the skeletogenic lineage until late mesenchyme blastula, *Sp-Erg* expression in the SMC mesoderm begins before PMC ingression (not shown). At mesenchyme blastula, *Sp-Erg* mRNA is present in both ingressed PMCs and prospective SMCs, arranged at this stage as a circle at the vegetal pole (Fig. 4, B3). Around 33 h of development, coinciding with the maximal levels of *Sp-Erg* expression (Fig. 3), *Sp-Erg* transcripts disappear from the PMCs while transcription is retained by the SMCs, now delaminating from the tip of the archenteron (Fig. 4, B4). The number of cells expressing *Sp-Erg* gradually decreases as gastrulation proceeds. At late gastrula stage, *Sp-Erg* is present only in a few SMCs migrating towards the blastocoel wall, where the mouth will open (Fig. 4, B5).

Sp-Ese instead presents a more limited domain of expression. It is undetectable during cleavage (Figs. 3 and 4, C1) and its levels of expression slightly increase at early blastula stage when the gene is ubiquitously expressed (Fig. 4, C2). *Sp-Ese* transcripts become localized to a few prospective SMCs at the time of PMC ingression. At mesenchyme blastula, a period that coincides with maximum levels of *Sp-Ese* mRNA in the embryo (Fig. 3), strong asymmetric expression is observed at the vegetal pole (Fig. 4, C3). Double *in situ* RNA hybridization together with a specific aboral ectoderm mRNA probe, *Spec1* (Lynn et al., 1983), indicates that the prospective SMCs expressing *Sp-Ese* at this stage, are facing the future oral ectoderm (data not shown). According to fate map studies (Ruffins and Ettensohn, 1996), these cells most likely are the precursors of blastocoelar SMCs. Few of the delaminating SMCs express *Sp-Ese* as the gut starts to invaginate (Fig. 4, C4). The number of SMCs that express *Sp-Ese* progressively diminishes during gastrulation. At late gastrula, as few as two to four cells at the tip of the archenteron are positive for *Sp-Ese* staining (Fig. 4, C5).

Sp-Pea and Sp-Tel

Sp-Pea transcripts are uniformly distributed in the embryo during early embryogenesis until the onset of gastrulation. Expression levels measured by QPCR (see Fig. 3) are such that, except for cleavage stage (Fig. 4, D1), less than 10 transcripts per embryo can be detected. Consistent with this low copy number per cell, staining is almost undetectable in these embryos (Figs. 4, D2, D3). As gastrulation begins, levels of *Sp-Pea* expression increase considerably (Fig. 3) and staining can be observed at the tip of the invaginating archenteron (Fig. 4, D4). Expression in this domain is retained until late gastrula, when staining is also observed in the oral ectoderm, more intense at the apical plate (Fig. 4, D5).

Sp-Tel displays maximal levels of expression during cleavage (8 hpf, Fig. 3), when it is ubiquitously expressed (Fig. 4, E1). *Sp-Tel* transcripts become localized during early blastula, when they are confined to micromere descendants (Fig. 4, E2). PMCs retain *Sp-Tel* expression while ingressing into the blastocoel (Fig. 4, E3), but lose it immediately after, at beginning of gastrulation, when transcripts are now localized at the tip of the invaginating archenteron (Fig. 4, E4). Expression at the tip of the archenteron continues throughout gastrulation, but is less intense at late gastrula, when expression is also detected in the endoderm around the blastopore and in the oral ectoderm (Fig. 4, E5).

Sp-Elk, Sp-Erf and Sp-Elf

Sp-Elk, *Sp-Erf*, and *Sp-Elf* expression patterns are grouped together because unlike the previously described genes, they share a very low degree of localized expression in the embryo. Extreme cases are represented by both *Sp-Erf* and *Sp-Elf* that are ubiquitously expressed throughout embryogenesis (Fig. 4, panels in rows G and H). Moreover, the levels of expression of *Sp-Elf* (Fig. 3) are undetectable until late gastrulae, when transcripts appear uniformly distributed, although probably more concentrated in the gut (Fig. 4, G5). Ubiquitous *Sp-Erf* expression is quite strong during cleavage (Fig. 4, G1), but slowly decreases until early blastula. Expression levels increase considerably again during gastrulation (Fig. 3), although the ubiquitous distribution of *Sp-Erf* transcripts renders the level of this gene to less than 10 copies per cell. In agreement with the time course data, staining in these embryos is very faint, just above background levels (compare in Fig. 4 panels G2–G5 and panels in row 4).

On the other hand, a certain degree of localized expression is exhibited by *Sp-Elk*. Ubiquitously distributed in the early blastula (15 hpf, Fig. 4, F2), when it reaches its maximal level of expression (Fig. 3), *Sp-Elk* also appears localized faintly in the vegetal plate of mesenchyme blastulae (Fig. 4, F3). In addition, there appears to be significant expression at late gastrula stage in the oral ectoderm and in the endoderm, but more intense at the oral side and at the tip of the archenteron (Fig. 4, F5).

Discussion

Phylogenetic analysis of sea urchin ets genes

The study of metazoan multigene families is a valuable approach to understanding genome evolution. The *ets* gene

family is well known for its wide distribution among metazoans and considerable expansion by gene duplications in vertebrate genomes (Graves and Petersen, 1998; Leveugle et al., 2004). Sea urchins are in a key evolutionary position for these studies since they diverged from other deuterostome lineages prior to the origin of chordates. Therefore they provide an invaluable outgroup for assessment of what might be ancient in chordates, what is chordate-specific, what is protostome-specific. Here we report the identification and developmental expression of 11 *ets* genes from the newly sequenced *S. purpuratus* genome. Eight of these genes had not been previously studied in the sea urchin. The phylogenetic analysis of the sea urchin *ets* genes (Figs. 1 and 2) showed that almost all vertebrate *ets* subfamilies, with the exception of only one so far found exclusively in mammals, are represented by a single sea urchin gene ortholog. In most cases, these subfamilies are constituted by a set of two or three paralogs in the human genome, which has a total of 27 genes.

Gene duplications have been extensively studied in some *ets* subfamilies. In particular, focus has fallen on duplications within the ERG and ETS groups, traditionally considered as sister groups also because of the striking chromosomal location of *ets1* and *ets2* (of the ETS group), and *erg* and *fli* (of the ERG group), in mammals. Indeed, *ets1* and *fli* are close neighbors on one chromosome and *ets2* and *erg* are located with a very similar arrangement but on a different chromosome. Our phylogenetic analysis is not consistent with the proposed monophyly of ETS and ERG groups. Moreover, the fact that *ets* and *erg* orthologues in *Drosophila* are found on different chromosomes (Hsu and Schulz, 2000) does not support the idea that a common ancestor of these genes was duplicated to give rise to *ets* and *erg* genes which were maintained on the same locus. Different evolutionary models have been proposed to account for the peculiar arrangement of these genes in mammals (reviewed by Laudet et al., 1999). Debates center on the concern of when, in relative evolutionary time, the common ancestor of these genes duplicated to give rise to extant *ets* family members, if and when the two genes were transferred to the same locus (as proposed by Laudet et al., 1999) and finally when this locus further duplicated to generate the arrangement presently found in mammals. It has been proposed that duplication within the ERG group predates the deuterostome to protostome divergence (Hart et al., 2000). The existence of only one sea urchin ERG member and one ETS member highlighted in this study, together with the presence of only one ETS member in *Drosophila* and *Ciona* genomes, suggests that duplication within these groups occurred after vertebrates emerged and that multiplicity of ERG members found in non-vertebrate genomes (such as *C42D8.4* and *T08H4.3* in *C. elegans*, *ets3* and *ets6* in *Drosophila*, and three, all very closely related, *Fli/Erg* genes in *Ciona*) is due to a high frequency of lineage-specific duplications in these genomes. Assessment of the chromosomal positions of *Sp-Ets1/2* and *Sp-Erg* should help answer the question as to whether the founder genes of ETS and ERG subfamilies (which were probably generated by an ancient duplication that predated deuterostome/protostome divergence) were transferred into the same genetic locus before or after emergence of chordates.

Genes belonging to the PU.1/SPI subgroup, which are required for the development of both innate and adaptive immunity (for a recent review see Gangenahalli et al., 2005), have been so far thought to be a chordate-specific feature. Interestingly, a PU.1/SpiB/SpiC gene, *Sp-PU.1*, was identified in the sea urchin (this study and J. Rast, unpublished results). In contrast, no protostome orthologues have been found. Notably, the sea urchin gene retains a pointed domain unlike chordate genes of this subfamily (including the *C. intestinalis* ortholog *CiSpi/Pu.1*; data not shown). Finally, we also identified a sea urchin ortholog of the ERF subfamily, a group that to date also does not include protostome members.

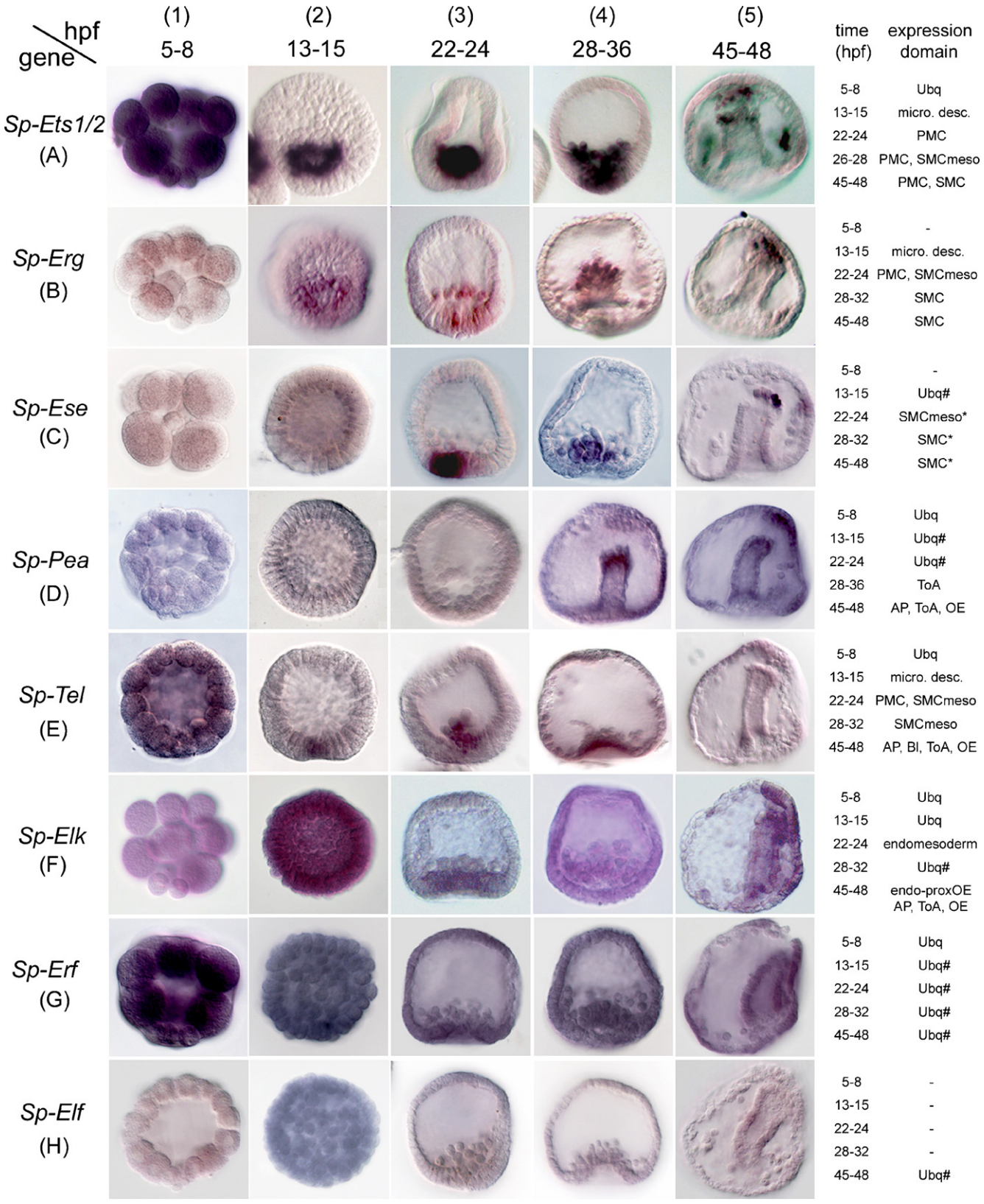
In conclusion, our analysis suggests that prior to emergence of chordates the *ets* family already comprised at least 11 genes, two of which are probably unique to the deuterostome lineage, and that acted as founder members of the extant vertebrate *ets* family (which consists of 27 paralogs belonging to 12 subfamilies).

Comparison of developmental expression patterns of sea urchin ets genes with mammalian and protostome ets genes

The gene expression analysis performed in this study (Figs. 3 and 4) revealed that temporal and spatial activation of most sea urchin *ets* genes employed in embryogenesis is sharply regulated. This feature appears more remarkable when the

Fig. 4. Spatial pattern of expression of *S. purpuratus ets* genes. Reported are whole-mount *in situ* hybridizations (WMISH) of the *ets* genes that showed detectable expression, as measured by QPCR, in the interval from 5 to 48 hpf (see Fig. 3). WMISH probes used are depicted in Fig. 1B. Excluded from this analysis is *Sp-Ets4*, whose complete pattern of expression has been published previously (Wei et al., 1999a). Panels are arranged as a table, one gene per row, one developmental stage per column. Left column, gene names (A–H). Top row, developmental time intervals (hours post fertilization, hpf; 1–5). With a few exceptions, in each column embryos of similar age are displayed in analogous orientations, as follows. Column 1, cleavage stages (5–8 hpf). 16 cells, 4th cleavage embryos are shown for all genes except *Sp-Pea*, *Sp-Tel* and *Sp-Erf*, for which later (from 5th to 7th) cleavage stages are presented. All embryos are viewed along the A/V axis (animal top, vegetal down) except for *Sp-Ese*, which is viewed from the vegetal pole. Column 2, early blastulae (13–15 hpf). Animal–vegetal sections are displayed for all embryos except for *Sp-Ets1/2* and *Sp-Erg*, where embryos were slightly rotated to show the vegetal pole. Column 3, mesenchyme blastulae (22–24 hpf). All embryos are seen in animal–vegetal view, vegetal pole down. Column 4, early to mid gastrulae (28–36 hpf). In most cases embryos at about 32 hpf, when the blastopore has opened and archenteron begins invagination, are displayed. Earlier (28 hpf) and later (36 hpf) stages are shown for *Sp-Ets1/2* and *Sp-Pea*, respectively. All embryos are viewed as in column 3. Column 5, late gastrulae (45–48 hpf). All embryos show a lateral view, with the oral side facing right, except the *Sp-Ets1/2* stained embryo, which is viewed from the oral side. On the right column, tables summarize the territories of expression for each gene. AP, apical plate; Bl, blastopore; endo-prox. OE, endoderm-proximal to oral ectoderm; micro. desc., micromere descendants; SMC, secondary mesenchyme cells; SMCmeso, SMC pregastrular mesoderm; ToA, tip of archenteron; OE, oral ectoderm; Ubq, ubiquitous. Asterisks (*) indicate that only a subset of cells in the given territory is expressing the gene. Pounds (#) mark situations where the gene is ubiquitously expressed at less than ten copies per cell (calculated according to QPCR experiments; see Fig. 3). Dashes (–) indicate cases of undetectable levels of expression. The corresponding embryo pictures also serve as background level and sense control (not shown) comparison.

developmental expression patterns of these genes are analyzed in comparison with their closest mammalian and protostome orthologues (shown in Table 1). When compared to mouse *ets* genes, which are in most cases broadly expressed in all three embryonic layers, sea urchin homologues often present more localized domains of expression. Striking examples are illustrated by *Sp-Ets1/2*, *Sp-Erg* and *Sp-Ese* which, in the sea urchin embryo, are restricted to a few cells all belonging to



mesenchymal lineages by the time of their onward specification (Fig. 4). The orthologues of these genes are widely expressed in mesodermal tissues including extraembryonic mesoderm, cartilage, bone, as well as neural crest cells and central nervous system of the mouse embryo (Kola et al., 1993; Vlaeminck-Guillem et al., 2000). Biological roles, when assessed in the mouse embryo, are as diverse as control of trophoblast stem cell formation, for *Elf5* (*ESE2* in humans; Donnison et al., 2005), differentiation of intestinal epithelium, for *Elf3* (*ESE1* in humans; Ng et al., 2002) and regulation of T- and B-cell differentiation/survival, for *Ets-1* (Bories et al., 1995; Muthusamy et al., 1995). As expected from their broad expression, knock-out of these genes often led to embryonic lethal mice, as in the case of *Ets2* (Yamamoto et al., 1998) and *Fli1* (Spyropoulos et al., 2000).

Although very different in the extent and multiplicity of expression domains, *ets* genes share some common paradigms among metazoans. For example, *Sp-Ets1/2* expression observed in sea urchin mesenchymal cells (Fig. 1, row A) is reminiscent of the expression of *Ets1* in mesenchymal cells of several developing organs in the mouse (Kola et al., 1993), as well as the expression of *pointed* in migrating midline glial cells in *Drosophila* (Klambt, 1993). These cells, although of different embryonic origin, all share a common feature, as they are actively migrating within the developing embryo. Conserved patterns of expression in the embryo suggest conserved developmental functions. All three genes have been implicated in epithelial–mesenchymal transition and cell migration (Bartel et al., 2000; Hsu and Schulz, 2000; Rottinger et al., 2004). Interestingly these genes also share other conserved domains outside the ETS domain such as a pointed domain which contains a MAP kinase docking site (see Fig. 1B). Conserved protein domains also suggest conserved interactions. Indeed, the well documented regulation of *Ets1*, *Ets2* and *pointed* by MAP kinase phosphorylation (reviewed in Yordy and Muise-Helmericks, 2000), appear conserved in sea urchin orthologues (Fernandez-Serra et al., 2004; Rottinger et al., 2004).

The wide expression domains displayed by *ets* genes in mammals has several times raised the question as to how these genes acquire the specificity of function demonstrated by genetic studies in which mutation of individual members causes distinct phenotypes (Graves and Petersen, 1998; Sharrocks, 2001). This dilemma is even more evident if the high conservation among ETS (DNA-binding) domains, which is often reflected in overlapping binding specificities, is taken into account. The extent of this problem has been recently estimated in a study where the mRNA levels of the 27 paralogous human *ets* genes were systematically measured in 23 tissues and cell lines (Hollenhorst et al., 2004). Two-thirds of these genes were found expressed significantly in most cell types. On the other hand, *in*

Table 1
Major domains of expression and/or function of *ets* genes in sea urchin, mouse, fly and worm embryos [a]

Sea urchin gene	Domain of expression in the sea urchin embryo (hpf) [b]	Mouse gene	Major domains of embryonic expression and/or gene KO phenotype in mouse (ref.)	Fly or worm gene	Main function in fly or worm (ref.)
Sp-Elf	Ubiquitous (40 h)	*MmElf-1* *MmMef*	Thymocytes, epithelial cells, skin (1) Reduced NK cells (2)	*DmE74-A*	Metamorphosis (19)
Sp-Elk	Ubiquitous (15 h)	*MmElk-3* *MmNet*	Impaired neuronal gene activation (3) Vascular Defects (4)	*Celin-1*	Vulva development (20)
Sp-Erf	Ubiquitous (8 h)	*MmErf*	(–)	–	–
Sp-Erg	PMC and SMC (33 h)	*MmErg* *MmFli-1*	Mesodermal tissues, neural crests (5) Hemorrhage, decreased lymphocytes (6)	*DmEts-3* *DmEts-6*	CNS development (21)
Sp-Ese	SMC meso (24 h)	*MmElf-3* *MmElf-5*	Intestinal differentiation defects (7) Extraembryonic mesoderm (8)	*CeC33A11*	Unknown
Sp-Ets1/2	Micro. desc. (15 h)	*MmEts-1* *MmEts-2*	Mesodermal cells, cartilage and bone, CNS (9) Ubiquitous; embryonic lethal (10)	*DmPnt-1*	Eye development, neurogenesis, tracheal cell migration (22)
Sp-Ets4	Non-veg. ectoderm (15 h)	*MmPdef*	(–)	*DmEts-4*	Germ cell development (21)
Sp-Gabp	Undetectable	*MmGabpα*	Developing myotome; embryonic lethal (11)	*DmElg*	Oogenesis (23)
Sp-Pea	Tip of archenteron (40 h)	*MmPea-3* *MmEr81*	CNS, mesodermal tissues (12); sterile males (13) Lack some neuronal connections (14)	*DmEts-96*	Unknown
Sp-Pu.1	Undetectable	*Mm Spi-B* *MmPu.1*	Spleen, thymus (15) B-cells defects (16) Embryonic lethal, hematopoietic abnormalities (17)	–	–
Sp-Tel	Ubiquitous (8 h)	*MmTel-1*	Neural tissues, lungs, kidneys, liver; embryonic lethal (18)	*DmYan*	Usually paired with Pn (24)

(–), not known in mouse embryos.
–, not found in *Drosophila* nor *C. elegans*.
Gene names use the following species designations: *Ce*, *Caenorhabditis elegans*; *Dm*, *Drosophila melanogaster*; *Mm*, *Mus musculus*; *Sp*, *Strongylocentrotus purpuratus*.
Ref., references: 1, (Bassuk et al., 1998); 2, (Lacorazza et al., 2002); 3, (Cesari et al., 2004); 4, (Ayadi et al., 2001); 5, (Vlaeminck-Guillem et al., 2000); 6, (Spyropoulos et al., 2000); 7, (Ng et al., 2002); 8, (Donnison et al., 2005); 9, (Kola et al., 1993); 10, (Yamamoto et al., 1998); 11, (Ristevski et al., 2004); 12, (Chotteau-Lelievre et al., 1997); 13, (Laing et al., 2000); 14, (Arber et al., 2000); 15, (Lefebvre et al., 2005); 16, (Su et al., 1997); 17, (McKercher et al., 1996); 18, (Wang et al., 1997, 1998); 19, (Janknecht et al., 1989); 20, (Miley et al., 2004); 21, (Chen et al., 1992); 22, (Klambt, 1993); 23, (Pribyl et al., 1991); 24, (Lai and Rubin, 1992).

[a] Each sea urchin gene is confronted with (i) those mouse paralogs for which either the gene knock-out phenotype and/or the expression pattern in the mouse embryo are known and (ii) a protostome paralog from *D. melanogaster* or *C. elegans.*

[b] For each sea urchin gene, the expression domain at about the time of its maximal level of zygotic expression before late gastrula stage (48 hpf) is reported. Data are from this study, except for *Sp-Ets4* (Wei et al., 1999a,b). Non-veg., non vegetal. Other abbreviations are as in Fig. 4.

vivo DNA binding by chromatin immunoprecipitation demonstrated that there is promoter specificity in spite of extensive co-expression (Hollenhorst et al., 2004). Selectivity of promoter activation in different tissues has been also demonstrated in transgenic sea urchin embryos (Consales and Arnone, 2002). The restricted pattern of expression of *ets* genes in the sea urchin embryo, which correlates with the reduced size (about 1000 cells for the gastrulating embryo) and simpler mode of development as compared to the mouse embryo, offers the unique opportunity to study, *in vivo*, the molecular mechanisms which confer specificity of function to this important class of genes.

ets genes in the sea urchin mesoderm

An interesting feature of the *ets* genes that has emerged in this study is the expression bias of this family to a specific domain in the sea urchin embryo. Five of the nine sea urchin *ets* genes transcribed during embryonic development are predominantly expressed in the mesoderm. Sea urchin embryonic mesoderm consists entirely of two types of mesenchyme cell. Primary mesenchyme cells (PMCs) are the first mesoderm cells that ingress to the blastocoel before any gut invagination has begun, while secondary mesenchyme cells (SMCs) enter the blastocoel during gastrulation. Whole-mount hybridization analysis performed in this study (Fig. 4) showed that three of the sea urchin *ets* genes (*Sp-Ets1/2, Sp-Erg, Sp-Ese*) are exclusively and two more (*Sp-Tel*, *Sp-Pea*) are additionally expressed in these mesenchyme cells and/or their progenitors. These data are summarized in Table 2 where domains of *ets* gene expression are reported as a function of developmental time with particular regard to the mesenchyme cell specification schedule. PMCs, the sole descendants of the large micromeres, segregate as founder cells of the skeletogenic lineage by an unequal division at 5th cleavage (6 hpf in *S. purpuratus*). These four founder cells undergo three more rounds of cell division to appear as a two cells thick ring at the vegetal pole at early blastula stage (13–15 h). They begin to ingress into the blastocoel a few hours later (19–23 hpf). SMCs derive from the most vegetal tier of micromeres that lies above the large micromeres at the 60-cell stage (7 hpf). SMC founder cells segregate from the future endoderm following a micromere-induced Delta/Notch signal at about 10–12 hpf, when they are arranged as a ring one cell thick surrounding micromere descendants (the prospective PMCs). These prospective SMCs are then found as a compact circle at the vegetal pole when ingression of PMCs is completed (23–24 hpf). SMCs begin to enter the blastocoel soon after and continue to delaminate from the tip of the growing archenteron throughout gastrulation (28–48 hpf). The expression domains (reported in color in Table 2, where the PMC and SMC lineages are depicted in dark and light grey, respectively) show co-localization of several ETS factors at critical times of mesenchyme development. Particularly significant with respect

Table 2
Domains of *ets* gene expression in the sea urchin mesoderm

MESODERMAL DOMAIN / developmental time (hpf)	13–15	16–19	20–24	25–27	28–33	34–39	40–48
Micromeres descendants (prospective PMCs)	(600) (10) (90)						
Primary mesenchyme cells (PMC)			(190) (30) (30)				
SMC pregastrular mesoderm (prospective SMCs)			(30) (150)				
Secondary mesenchyme cells (SMC) and/or tip of archenteron							

Sp-Ets1/2 (n)
Sp-Erg (n)
Sp-Ese* (n)
Sp-Pea
Sp-Tel (n)

V **PMC ingression** V **gastrulation**

* indicates expression in a restricted region within the domain.
A, animal; Ab, aboral; O, oral; V, vegetal.
(n) = approximate number of transcripts per cell calculated according to QPCR (Fig.3) and WMISH (Fig.4) data.

to the mesenchyme cell specification schedule is the co-expression of *Sp-Ets1-2*, *Sp-Erg* and *Sp-Tel* in the micromere descendants at early blastula stage (13–15 hpf) and the co-expression of *Sp-Erg* and *Sp-Ese* first (16–19 hpf), *Sp-Pea*, *Sp-Tel* and *Sp-Ets1-2* later (20–24 hpf), in SMC mesoderm prior to onset of gastrulation.

Functional analysis of sea urchin orthologues belonging to the ETS subfamily has previously demonstrated their essential role in the specification and differentiation of the skeletogenic mesenchyme lineage. Evidence for this was obtained by injection of a dominant negative form of *Hp-Ets* (Kurokawa et al., 1999), overexpression of *Pl-Ets1* by mRNA injection (Rottinger et al., 2004) and knockdown of *Sp-Ets1/2* by morpholino antisense oligonucleotide (MASO) injection (P. Oliveri, unpublished results). On the other hand, preliminary functional knock-out data obtained by MASO injection (F. Rizzo, M. Fernandez-Serra and M.I. Arnone, unpublished results) suggest that despite very similar binding affinities and co-expression, these mesenchyme-specific *ets* genes show non-redundant functions in the sea urchin embryo. A detailed analysis of the function of these genes during sea urchin mesenchyme specification and differentiation will significantly contribute to the understanding of the role of ETS factors in mesoderm formation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.012.

References

Arber, S., Ladle, D.R., Lin, J.H., Frank, E., Jessell, T.M., 2000. ETS gene *Er81* controls the formation of functional connections between group Ia sensory afferents and motor neurons. Cell 101, 485–498.

Ayadi, A., Zheng, H., Sobieszczuk, P., Buchwalter, G., Moerman, P., Alitalo, K., Wasylyk, B., 2001. Net-targeted mutant mice develop a vascular phenotype and up-regulate *egr-1*. EMBO J. 20, 5139–5152.

Bartel, F.O., Higuchi, T., Spyropoulos, D.D., 2000. Mouse models in the study of the Ets family of transcription factors. Oncogene 19, 6443–6454.

Bassuk, A.G., Barton, K.P., Anandappa, R.T., Lu, M.M., Leiden, J.M., 1998. Expression pattern of the Ets-related transcription factor Elf-1. Mol. Med. 4, 392–401.

Bateman, A., Coin, L., Durbin, R., Finn, R.D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E.L., Studholme, D.J., Yeats, C., Eddy, S.R., 2004. The Pfam protein families database. Nucleic Acids Res. 32, D138–D141.

Bories, J.C., Willerford, D.M., Grevin, D., Davidson, L., Camus, A., Martin, P., Stehelin, D., Alt, F.W., 1995. Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. Nature 377, 635–638.

Cameron, R.A., Mahairas, G., Rast, J.P., Martinez, P., Biondi, T.R., Swartzell, S., Wallace, J.C., Poustka, A.J., Livingston, B.T., Wray, G.A., Ettensohn, C.A., Lehrach, H., Britten, R.J., Davidson, E.H., Hood, L., 2000. A sea urchin genome project: sequence scan, virtual map, and additional resources. Proc. Natl. Acad. Sci. U. S. A. 97, 9514–9518.

Cesari, F., Brecht, S., Vintersten, K., Vuong, L.G., Hofmann, M., Klingel, K., Schnorr, J.J., Arsenian, S., Schild, H., Herdegen, T., Wiebel, F.F., Nordheim, A., 2004. Mice deficient for the ets transcription factor *elk-1* show normal immune responses and mildly impaired neuronal gene activation. Mol. Cell. Biol. 24, 294–305.

Chen, Z.Q., Kan, N.C., Pribyl, L., Lautenberger, J.A., Moudrianakis, E., Papas, T.S., 1988. Molecular cloning of the ets proto-oncogene of the sea urchin and analysis of its developmental expression. Dev. Biol. 125, 432–440.

Chen, T., Bunting, M., Karim, F.D., Thummel, C.S., 1992. Isolation and characterization of five *Drosophila* genes that encode an ets-related DNA binding domain. Dev. Biol. 151, 176–191.

Chotteau-Lelievre, A., Desbiens, X., Pelczar, H., Defossez, P.A., de Launoit, Y., 1997. Differential expression patterns of the PEA3 group transcription factors through murine embryonic development. Oncogene 15, 937–952.

Consales, C., Arnone, M.I., 2002. Functional characterization of Ets-binding sites in the sea urchin embryo: three base pair conversions redirect expression from mesoderm to ectoderm and endoderm. Gene 287, 75–81.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown, C.T., Livi, C.B., Lee, P.Y., Revilla, R., Rust, A.G., Pan, Z., Schilstra, M.J., Clarke, P.J., Arnone, M.I., Rowen, L., Cameron, R.A., McClay, D.R., Hood, L., Bolouri, H., 2002. A genomic regulatory network for development. Science 295, 1669–1678.

Degnan, B.M., Degnan, S.M., Naganuma, T., Morse, D.E., 1993. The *ets* multigene family is conserved throughout the Metazoa. Nucleic Acids Res. 21, 3479–3484.

de Launoit, Y., Baert, J.L., Chotteau, A., Monte, D., Defossez, P.A., Coutte, L., Pelczar, H., Leenders, F., 1997. Structure–function relationships of the PEA3 group of Ets-related transcription factors. Biochem. Mol. Med. 61, 127–135.

Donnison, M., Beaton, A., Davey, H.W., Broadhurst, R., L'Huillier, P., Pfeffer, P.L., 2005. Loss of the extraembryonic ectoderm in Elf5 mutants leads to defects in embryonic patterning. Development 132, 2299–2308.

Fernandez-Serra, M., Consales, C., Livigni, A., Arnone, M.I., 2004. Role of the ERK-mediated signaling pathway in mesenchyme formation and differentiation in the sea urchin embryo. Dev. Biol. 268, 384–402.

Gangenahalli, G.U., Gupta, P., Saluja, D., Verma, Y.K., Kishore, V., Chandra, R., Sharma, R.K., Ravindranath, T., 2005. Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. Stem Cells Dev. 14, 140–152.

Graves, B.J., Petersen, J.M., 1998. Specificity within the ets family of transcription factors. Adv. Cancer Res. 75, 1–55.

Hart, A.H., Reventar, R., Bernstein, A., 2000. Genetic analysis of ETS genes in *C. elegans*. Oncogene 19, 6400–6408.

Hollenhorst, P.C., Jones, D.A., Graves, B.J., 2004. Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. Nucleic Acids Res. 32, 5693–5702.

Hsu, T., Schulz, R.A., 2000. Sequence and functional properties of Ets genes in the model organism *Drosophila*. Oncogene 19, 6409–6416.

Jacobs, D., Beitel, G.J., Clark, S.G., Horvitz, H.R., Kornfeld, K., 1998. Gain-of-

function mutations in the *Caenorhabditis elegans lin-1* ETS gene identify a C-terminal regulatory domain phosphorylated by ERK MAP kinase. Genetics 149, 1809–1822.

Jacobs, D., Glossip, D., Xing, H., Muslin, A.J., Kornfeld, K., 1999. Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. Genes Dev. 13, 163–175.

Janknecht, R., Taube, W., Ludecke, H.J., Pongs, O., 1989. Characterization of a putative transcription factor gene expressed in the 20-OH-ecdysone inducible puff 74EF in *Drosophila melanogaster*. Nucleic Acids Res. 17, 4455–4464.

Karim, F.D., Urness, L.D., Thummel, C.S., Klemsz, M.J., McKercher, S.R., Celada, A., Van Beveren, C., Maki, R.A., Gunther, C.V., Nye, J.A., et al., 1990. The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. Genes Dev. 4, 1451–1453.

Klambt, C., 1993. The *Drosophila* gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. Development 117, 163–176.

Kola, I., Brookes, S., Green, A.R., Garber, R., Tymms, M., Papas, T.S., Seth, A., 1993. The Ets1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation. Proc. Natl. Acad. Sci. U. S. A. 90, 7588–7592.

Kurokawa, D., Kitajima, T., Mitsunaga-Nakatsubo, K., Amemiya, S., Shimada, H., Akasaka, K., 1999. HpEts, an ets-related transcription factor implicated in primary mesenchyme cell differentiation in the sea urchin embryo. Mech. Dev. 80, 41–52.

Lacorazza, H.D., Miyazaki, Y., Di Cristofano, A., Deblasio, A., Hedvat, C., Zhang, J., Cordon-Cardo, C., Mao, S., Pandolfi, P.P., Nimer, S.D., 2002. The ETS protein MEF plays a critical role in perforin gene expression and the development of natural killer and NK-T cells. Immunity 17, 437–449.

Lai, Z.C., Rubin, G.M., 1992. Negative control of photoreceptor development in *Drosophila* by the product of the yan gene, an ETS domain protein. Cell 70, 609–620.

Laing, M.A., Coonrod, S., Hinton, B.T., Downie, J.W., Tozer, R., Rudnicki, M.A., Hassell, J.A., 2000. Male sexual dysfunction in mice bearing targeted mutant alleles of the PEA3 *ets* gene. Mol. Cell. Biol. 20, 9337–9345.

Laudet, V., Niel, C., Duterque-Coquillaud, M., Leprince, D., Stehelin, D., 1993. Evolution of the ets gene family. Biochem. Biophys. Res. Commun. 190, 8–14.

Laudet, V., Hanni, C., Stehelin, D., Duterque-Coquillaud, M., 1999. Molecular phylogeny of the ETS gene family. Oncogene 18, 1351–1359.

Lefebvre, J.M., Haks, M.C., Carleton, M.O., Rhodes, M., Sinnathamby, G., Simon, M.C., Eisenlohr, L.C., Garrett-Sinha, L.A., Wiest, D.L., 2005. Enforced expression of Spi-B reverses T lineage commitment and blocks beta-selection. J. Immunol. 174, 6184–6194.

Letunic, I., Copley, R.R., Pils, B., Pinkert, S., Schultz, J., Bork, P., 2006. SMART 5: domains in the context of genomes and networks. Nucleic Acids Res. 34, D257–D260.

Leveugle, M., Prat, K., Popovici, C., Birnbaum, D., Coulier, F., 2004. Phylogenetic analysis of *Ciona intestinalis* gene superfamilies supports the hypothesis of successive gene expansions. J. Mol. Evol. 58, 168–181.

Li, R., Pei, H., Watson, D.K., 2000. Regulation of Ets function by protein–protein interactions. Oncogene 19, 6514–6523.

Lynn, D.A., Angerer, L.M., Bruskin, A.M., Klein, W.H., Angerer, R.C., 1983. Localization of a family of MRNAS in a single cell type and its precursors in sea urchin embryos. Proc. Natl. Acad. Sci. U. S. A. 80, 2656–2660.

Mavrothalassitis, G., Fisher, R.J., Smyth, F., Watson, D.K., Papas, T.S., 1994. Structural inferences of the ETS1 DNA-binding domain determined by mutational analysis. Oncogene 9, 425–435.

McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H., Klemsz, M., Feeney, A.J., Wu, G.E., Paige, C.J., Maki, R.A., 1996. Targeted disruption of the *PU.1* gene results in multiple hematopoietic abnormalities. EMBO J. 15, 5647–5658.

Miley, G.R., Fantz, D., Glossip, D., Lu, X., Saito, R.M., Palmer, R.E., Inoue, T., Van Den Heuvel, S., Sternberg, P.W., Kornfeld, K., 2004. Identification of residues of the *Caenorhabditis elegans* LIN-1 ETS domain that are necessary for DNA binding and regulation of vulval cell fates. Genetics 167, 1697–1709.

Minokawa, T., Rast, J.P., Arenas-Mena, C., Franco, C.B., Davidson, E.H., 2004. Expression patterns of four different regulatory genes that function during sea urchin development. Gene Expr. Patterns 4, 449–456.

Muthusamy, N., Barton, K., Leiden, J.M., 1995. Defective activation and survival of T cells lacking the Ets-1 transcription factor. Nature 377, 639–642.

Nemer, M., Rondinelli, E., Infante, D., Infante, A.A., 1991. Polyubiquitin RNA characteristics and conditional induction in sea urchin embryos. Dev. Biol. 145, 255–265.

Ng, A.Y., Waring, P., Ristevski, S., Wang, C., Wilson, T., Pritchard, M., Hertzog, P., Kola, I., 2002. Inactivation of the transcription factor Elf3 in mice results in dysmorphogenesis and altered differentiation of intestinal epithelium. Gastroenterology 122, 1455–1466.

Oliveri, P., Davidson, E.H., 2004. Gene regulatory network controlling embryonic specification in the sea urchin. Curr. Opin. Genet. Dev. 14, 351–360.

Pribyl, L.J., Watson, D.K., Schulz, R.A., Papas, T.S., 1991. *D-elg*, a member of the *Drosophila ets* gene family: sequence, expression and evolutionary comparison. Oncogene 6, 1175–1183.

Qi, S., Chen, Z.Q., Papas, T.S., Lautenberger, J.A., 1992. The sea urchin erg homolog defines a highly conserved erg-specific domain. DNA Seq. 3, 127–129.

Rast, J.P., Amore, G., Calestani, C., Livi, C.B., Ransick, A., Davidson, E.H., 2000. Recovery of developmentally defined gene sets from high-density cDNA macroarrays. Dev. Biol. 228, 270–286.

Ristevski, S., O'Leary, D.A., Thornell, A.P., Owen, M.J., Kola, I., Hertzog, P.J., 2004. The ETS transcription factor GABPalpha is essential for early embryogenesis. Mol. Cell. Biol. 24, 5844–5849.

Rottinger, E., Besnardeau, L., Lepage, T., 2004. A Raf/MEK/ERK signaling pathway is required for development of the sea urchin embryo micromere lineage through phosphorylation of the transcription factor Ets. Development 131, 1075–1087.

Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132, 365–386.

Ruffins, S.W., Ettensohn, C.A., 1996. A fate map of the vegetal plate of the sea urchin (*Lytechinus variegatus*) mesenchyme blastula. Development 122, 253–263.

Schultz, J., Milpetz, F., Bork, P., Ponting, C.P., 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. U. S. A. 95, 5857–5864.

Seidel, J.J., Graves, B.J., 2002. An ERK2 docking site in the Pointed domain distinguishes a subset of ETS transcription factors. Genes Dev. 16, 127–137.

Sharrocks, A.D., 2001. The ETS-domain transcription factor family. Nat. Rev., Mol. Cell Biol. 2, 827–837.

Sharrocks, A.D., Brown, A.L., Ling, Y., Yates, P.R., 1997. The ETS-domain transcription factor family. Int. J. Biochem. Cell Biol. 29, 1371–1387.

Spyropoulos, D.D., Pharr, P.N., Lavenburg, K.R., Jackers, P., Papas, T.S., Ogawa, M., Watson, D.K., 2000. Hemorrhage, impaired hematopoiesis, and lethality in mouse embryos carrying a targeted disruption of the Fli1 transcription factor. Mol. Cell. Biol. 20, 5643–5652.

Su, G.H., Chen, H.M., Muthusamy, N., Garrett-Sinha, L.A., Baunoch, D., Tenen, D.G., Simon, M.C., 1997. Defective B cell receptor-mediated responses in mice lacking the Ets protein, Spi-B. EMBO J. 16, 7118–7129.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Vlaeminck-Guillem, V., Carrere, S., Dewitte, F., Stehelin, D., Desbiens, X., Duterque-Coquillaud, M., 2000. The Ets family member Erg gene is expressed in mesodermal tissues and neural crests at fundamental steps during mouse embryogenesis. Mech. Dev. 91, 331–335.

Wang, D.G., Kirchhamer, C.V., Britten, R.J., Davidson, E.H., 1995. SpZ12-1, a negative regulator required for spatial control of the territory-specific CyIIIa gene in the sea urchin embryo. Development 121, 1111–1122.

Wang, L.C., Kuo, F., Fujiwara, Y., Gilliland, D.G., Golub, T.R., Orkin, S.H., 1997. Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. EMBO J. 16, 4374–4383.

Wang, L.C., Swat, W., Fujiwara, Y., Davidson, L., Visvader, J., Kuo, F., Alt, F.W., Gilliland, D.G., Golub, T.R., Orkin, S.H., 1998. The TEL/ETV6

gene is required specifically for hematopoiesis in the bone marrow. Genes Dev. 12, 2392–2402.

Wei, Z., Angerer, L.M., Angerer, R.C., 1999a. Spatially regulated SpEts4 transcription factor activity along the sea urchin embryo animal–vegetal axis. Development 126, 1729–1737.

Wei, Z., Angerer, R.C., Angerer, L.M., 1999b. Identification of a new sea urchin ets protein, SpEts4, by yeast one-hybrid screening with the hatching enzyme promoter. Mol. Cell. Biol. 19, 1271–1278.

Yagi, K., Satou, Y., Mazet, F., Shimeld, S.M., Degnan, B., Rokhsar, D., Levine, M., Kohara, Y., Satoh, N., 2003. A genomewide survey of developmentally relevant genes in *Ciona intestinalis*: III. Genes for Fox, ETS, nuclear receptors and NFkappaB. Dev. Genes Evol. 213, 235–244.

Yamamoto, H., Flannery, M.L., Kupriyanov, S., Pearce, J., McKercher, S.R., Henkel, G.W., Maki, R.A., Werb, Z., Oshima, R.G., 1998. Defective trophoblast function in mice with a targeted mutation of Ets2. Genes Dev. 12, 1315–1326.

Yang, S.H., Whitmarsh, A.J., Davis, R.J., Sharrocks, A.D., 1998a. Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. EMBO J. 17, 1740–1749.

Yang, S.H., Yates, P.R., Whitmarsh, A.J., Davis, R.J., Sharrocks, A.D., 1998b. The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. Mol. Cell. Biol. 18, 710–720.

Yordy, J.S., Muise-Helmericks, R.C., 2000. Signal transduction and the Ets family of transcription factors. Oncogene 19, 6503–6513.

Zhu, X., Mahairas, G., Illies, M., Cameron, R.A., Davidson, E.H., Ettensohn, C.A., 2001. A large-scale analysis of mRNAs expressed by primary mesenchyme cells of the sea urchin embryo. Development 128, 2615–2627.