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# Zebrafish *paraxial protocadherin* is a downstream target of *spadetail* involved in morphogenesis of gastrula mesoderm

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## SUMMARY

**Zebrafish *paraxial protocadherin* (*papc*) encodes a transmembrane cell adhesion molecule (PAPC) expressed in trunk mesoderm undergoing morphogenesis. Microinjection studies with a dominant-negative secreted construct suggest that *papc* is required for proper dorsal convergence movements during gastrulation. Genetic studies show that *papc* is a close downstream target of *spadetail*, gene encoding a transcription factor required for mesodermal morphogenetic movements. Further, we show that the *floating head* homeobox gene is required in axial mesoderm to repress the expression of both *spadetail* and *papc*, promoting notochord and blocking differentiation of paraxial mesoderm. The PAPC structural cell-surface protein may provide a link between regulatory transcription factors and the actual cell biological behaviors that execute morphogenesis during gastrulation.**

Key words: Protocadherin, *spadetail*, *floating head*, Gastrulation, Convergence movements, Somitogenesis, Zebrafish

## INTRODUCTION

During vertebrate gastrulation, movements of cohesive sheets of cells lead to the formation of a body plan composed of three germ layers. Although many of the regulatory transcription and growth factors involved are being identified (Lemaire and Kodjabachian, 1996; Harland and Gerhart, 1997), little is known about the structural molecules that mediate cell interactions and cell movements during gastrulation. In zebrafish *spadetail* (*spt*) mutants, cells of the lateral mesoderm fail to converge properly toward the dorsal midline, leading to decreased numbers of somitic cells in the trunk and to an accumulation of the corresponding cells in the tail region (Kimmel et al., 1989; Ho and Kane, 1990). At late blastula stages, *spt*$^{-}$ mesodermal precursor cells on the dorsal side are less tightly packed than in wild-type embryos (Warga, 1996), an observation that suggests that altered cell adhesion may contribute to the cell movement defects.

The *spt* mutation affects predominantly the paraxial (somitic) mesoderm, whereas mutations in other genes, such as *no tail* (*ntl*, a T-box transcription factor homologous to mouse *Brachyury*) and *floating head* (*flh*, a homeobox gene homologous to the organizer-specific Xenopus gene *Xnot*), affect the axial (notochordal) mesoderm (Halpern et al., 1993, 1995; Schulte-Merker et al., 1994a,b; Talbot et al., 1995; Melby et al., 1996; Odenthal et al., 1996). An interesting gene interaction between *spt* and *flh* has been observed in double mutant studies (Amacher and Kimmel, 1998). *flh* single mutants lack a notochord, and instead axial mesoderm adopts a paraxial (muscle) fate (Talbot et al., 1995; Halpern et al., 1995; Melby et al., 1996). However, in *spt*;*flh* double mutants, this transfating event does not occur and, surprisingly, the formation of an anterior notochord, including expression of *ntl*, is restored. These results suggested that in normal development *flh* antagonizes *spt* function in axial mesoderm (Amacher and Kimmel, 1998).

In amphibians the mesodermal mantle involutes as a coherent sheet of cells, which is subsequently subdivided by the appearance of the notochord in the dorsal midline (Vogt, 1929; Keller et al., 1992). A molecule that could provide cohesion to the trunk mesodermal layer is *paraxial protocadherin* (*papc*), a Xenopus gene recently isolated during a screen for molecules expressed in Spemann's organizer (Bouwmeester et al., 1996; S. H. Kim et al., unpublished observations). Protocadherins belong to a large family of transmembrane cell surface proteins from which the cadherins have evolved (Suzuki, 1996). The classical cadherins are strong homophilic cell adhesion molecules present in most solid tissues (Takeichi, 1995; Gumbiner, 1996). In comparison, protocadherins tend to have weaker cell adhesion activity in cell culture (Suzuki, 1996). In a recent study, neural fold protocadherin (NFPC) mRNA has been shown to mediate strong adhesion in ectoderm when microinjected into Xenopus embryos (Bradley et al., 1998).

Xenopus *papc* is expressed in the paraxial mesoderm of the trunk in regions comparable to those abnormal in *spt*$^{-}$ zebrafish, and when overexpressed can mediate cell adhesion activity in aggregation assays of embryonic cells (S. H. Kim et al., unpublished observations). Thus, *papc* seemed a reasonable candidate for either encoding the *spt* gene or a close *spt* downstream target, prompting us to clone its zebrafish homologue. The observation that a deficiency allele of *spt* still contained *papc* DNA sequences (S. L. A. and A. Y., unpublished observations) eliminated the first possibility, and here we provide evidence that the second is the case. As this work was in progress, a cDNA encoding a T-box transcription factor was found to encode *spt* (Griffin et al., 1998).

In this paper we present studies on the role of zebrafish *papc* during mesodermal morphogenesis. During gastrulation, *papc* is initially expressed in dorsal mesodermal cells, but expression subsequently disappears from midline cells that will give rise to notochord. *papc* is not expressed in *spt* mutant gastrulae, demonstrating that *papc* lies downstream of *spt*. *spt* and *papc* transcripts are ectopically expressed in axial mesodermal cells in *flh*$^{-}$ embryos, indicating that *flh* functions to repress expression of *spt* and *papc* in the midline. In microinjection studies, a dominant-negative secreted form of *papc* leads to phenotypes consistent with decreased convergence movements in the mesodermal layer, particularly in cells that give rise to somitic muscle. We propose that the PAPC cell adhesion molecule is a downstream effector of the spadetail transcription factor, and that PAPC may be a component of the molecular machinery that executes some of the morphogenetic cell movements during gastrulation.

## MATERIALS AND METHODS

### Cloning of *papc* cDNA

To isolate zebrafish *papc* cDNA, we first tried RT-PCR cloning with degenerate primers using total RNA, isolated from 60-80% epiboly embryos. Degenerate primers were designed as in Sano et al. (1993) with a slight modification based upon the Xenopus *papc* sequence ((S. H. Kim et al., unpublished observations). The primers F5′-CCG-AATTCAA(A/G)(C/G)(C/G)NNTNGA(C/T)T(A/T)(C/T)GA-3′; R5′-CCGGATCCNNNGGNGC(A/G)TT(A/G)TC(A/G)TT-3′ generated three different protocadherin fragments (zPCR 1-3) that did not correspond to zebrafish *papc* homologues. To clone zebrafish *papc* a somite stage λgt10 cDNA library, a kind gift of H. Okamoto (Inoue et al., 1994), was screened at low stringency with a Xenopus *papc* probe (nucleotides 2186-3014), which encodes the transmembrane and cytoplasmic domains. A full-length clone was isolated and its 3156 nucleotides were sequenced in both strands.

### Embryos and in situ hybridization

Embryos were obtained from the AB or ABC lines at the University of Oregon zebrafish colony and raised in embryo medium as described by Westerfield (1995). The origin and maintenance of mutant lines, *flh*$^{n1}$ and *spt*$^{b104}$, are described in Talbot et al. (1995) and Amacher and Kimmel (1998), respectively. Live embryos were photographed using Nomarski optics after orienting in 3% methylcellulose.

Digoxygenin-UTP-labeled or fluorescein-UTP-labeled RNA probes were synthesized using T7 polymerase from *Xba*I-linearized *myoD* template (Weinberg et al., 1996), an *Eco*RI-linearized *spt* template (Griffin et al., 1998), and an *Xba*I-linearized *gsc* template (Stachel et al., 1993) and using T3 polymerase from an *Apa*I-linearized *papc* template. Two-color in situ hybridizations were performed as described (Jowett and Yan, 1996); the fluoresceinated probe was detected using Fast Red (Sigma) or using BCIP alone (no NBT), which gives an aquamarine color. Single probe hybridizations, mounting and photography were done as described previously (Amacher and Kimmel, 1998). Embryos were sectioned after whole-mount in situ hybridization as described (Halpern et al., 1995).

### Functional analyses

Full-length *papc* cDNA was subcloned into pCS2+ expression vector (a gift of R. Rupp) and designated pCS2 *FL-papc*. To generate a dominant-negative form of *papc* mRNA (*DN-papc*), pCS2 *FL-papc* was digested with *Xho*I and religated (pCS2 *DN-papc*). pCS2 *FL-papc* and pCS2 *DN-papc* were linearized with *Apa*I and transcribed with SP6 RNA polymerase using the Ambion Message Machine Kit (Ambion). All mRNAs were quantified by gel electrophoresis and spectrophotometry.

Analysis of the adhesion activity of zebrafish *papc* in Xenopus embryos injected into single blastomeres at the 32-cell stage was carried out as described (S. H. Kim et al., unpublished observations). Zebrafish embryos were injected at the 2-4 cell stage using air pressure. After injection, embryos were raised in embryo medium (Westerfield, 1995) and fixed at the 8-9 somite stage. Injected embryos were analyzed morphologically (before fixation) and by in situ hybridization with *myoD* probe. In some experiments 500 pg of nuclear *lacZ* mRNA was co-injected with *DN-papc* mRNA. Embryos were fixed with 4% paraformaldehyde for 1 hour and processed to detect lacZ activity using salmon-gal (Biosynth International), before in situ hybridization.

### Protein secretion assay

*FL-papc-HA* and *DN-papc-Flag* were generated by PCR, adding the carboxy-terminal amino acid sequences YPYDVPDYA and DYKDDDDK, respectively. Embryonic human kidney cells (293T) were cultured in D-MEM containing 10% fetal calf serum and transfections carried out with Lipofectamin (GIBCO BRL). After 48 hours, condition medium and cell pellets were collected and analyzed using the ECL plus Western Blotting kit (Amersham) according to the manufacturer's instructions.

## RESULTS

### Cloning of zebrafish *papc*

Initial attempts to isolate the zebrafish homologue of Xenopus PAPC made use of a PCR strategy, and DNA fragments for at least three different zebrafish protocadherins were isolated (indicated as ZPCR 1-3 in Fig. 1). However, these sequences had amino acid deletions or insertions in the amplified region with respect to Xenopus PAPC, suggesting that they were not true orthologues. We isolated a zebrafish *papc* homologue using a low stringency hybridization approach with a probe spanning the cytoplasmic domain of Xenopus *papc*. The zebrafish *papc* cDNA clone is 3156 base pairs long and encodes a complete open reading frame of 950 amino acids (GenBank accession #AF042191). It was isolated by virtue of two short regions of high conservation to the probe in the cytoplasmic domain (Fig. 1A), one of which has a stretch of 67 nucleotides sharing 80% identity between Xenopus and zebrafish *papc*. In addition, unlike the sequences of our initial PCR fragments and of other protocadherins available in the database, the regions corresponding to extracellular cadherin repeat 4 in both zebrafish and Xenopus PAPC align without amino acid insertions or deletions, as shown in Fig. 1B. When the entire protein sequences of zebrafish and Xenopus PAPC

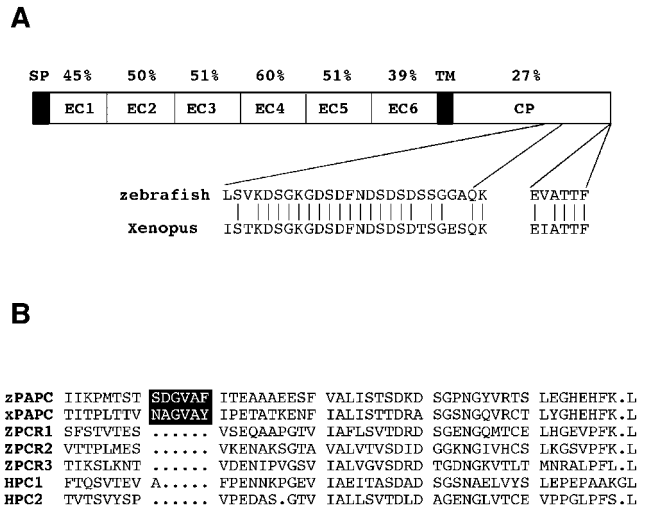


**Fig. 1.** Primary structure of zebrafish Paraxial Protocadherin. (A) PAPC has an extracellular region containing six extracellular cadherin domains (EC 1-6), a transmembrane region (TM), a cytoplasmic domain (CP) and a signal peptide (SP). The percentages above the boxes represent the amino acid identity between zebrafish and Xenopus PAPC protein sequences. In the cytoplasmic region, total identity is low but, as indicated, there are short highly conserved regions. (B) Amino acid comparison of comparable regions of zPAPC, xPAPC, ZPCR1-3, and human protocadherins 1 and 2. Note that the PAPC homologues lack deletions or insertions of amino acids. The accession numbers for the new sequences reported here are AF042191 for zPAPC, AF043902 for ZPCR1, AF043903 for ZPCR2 and AF043904 for ZPCR3.

are aligned, an overall identity of 43% (and 63% similarity using the GCG program) is observed. In comparison, zebrafish PAPC is 29% and 31% identical to human protocadherins 1 and 2 (Sano et al., 1993), respectively.

Several lines of evidence indicate that our cDNA clone corresponds to a protocadherin and not to a classical cadherin. First, *papc* is predicted to encode a transmembrane protein with six extracellular cadherin (EC) domains instead of the five domains present in classical cadherins. Second, PAPC lacks signature amino acid sequences present in EC3 and EC5 of classical cadherins (Suzuki, 1996). Finally, the cytoplasmic domain of PAPC lacks a β-catenin binding site present in all classical cadherins (Ozawa et al., 1989) but absent from all protocadherins (Suzuki, 1996). Zebrafish *papc* was cloned because of a highly conserved stretch of 26 amino acids in the middle of the cytoplasmic domain, which is rich in Ser and Asp (Fig. 1A). This intriguing conserved region might provide a binding site for intracellular components regulating *papc* function. To summarize, we have isolated a zebrafish cDNA clone that appears to be a close homologue of Xenopus *papc* (S. H. Kim et al., unpublished observations). This conclusion is based on sequence comparisons and on close parallels in expression patterns during development, as described in the following section.

### *papc* is expressed in gastrulating mesoderm

Before gastrulation starts, at 30-40% epiboly, *papc* transcripts are found on the dorsal side of the marginal zone (Fig. 2A). During early gastrulation (6 hours, shield stage, all stages according to Kimmel et al., 1995), *papc* expression expands to encompass the entire blastoderm margin, including the ventralmost cells (Fig. 2B). As gastrulation proceeds, *papc* expression decreases in dorsal midline cells corresponding to the future notochord (Fig. 2C). By midgastrulation, *papc* transcripts are completely absent from the dorsal midline and the band of *papc* expression widens as the mesoderm continues to involute in the marginal zone (Fig. 2D, arrowhead). At the end of gastrulation a sharp anterior border becomes apparent, separating trunk mesoderm from the more anterior head domain (arrowhead in Fig. 2E), and the *papc* expression domain includes ventral mesodermal and future tailbud cells (Fig. 2F). Histological analyses show that *papc* expression is confined to gastrula mesoderm; at shield stage *papc* mRNA expression spans the dorsal midline region, but at later stages it is repressed in the notochord, persisting in paraxial mesoderm (Fig. 2K-M).

During somitogenesis, *papc* has a second, very dynamic, phase of expression. As each new somite forms, the anterior border of expression is displaced towards the posterior (Fig. 2G-I), until *papc* mRNA fades away as somite formation is completed in the tailbud (Fig. 2J, arrowhead). Four bilateral pairs of bands appear in paraxial mesoderm, and posterior to them the segmental plate (consisting of unsegmented somitic precursor cells) is stained uniformly and less intensely. Histological sections indicate that the first band is located in the anterior border of the newest somite formed and the second in the forming somite as it emerges from the segmental plate (Fig. 2N). The two posterior, stronger bands are located within the segmental plate mesoderm (Fig. 2N). To confirm that the *papc* bands of expression form before somite formation, we examined the extent of overlap of *papc* and *myoD*. *myoD* is prominently expressed in each formed somite, as well as one or two fainter bands in the newest forming somite and in the segmental plate (Weinberg et al., 1996). Double-label in situ hybridization shows that the two strong *papc* bands are indeed found within the segmental plate (Fig. 2O). Thus, *papc* bands are detected in regions that have been proposed to form somitomeres, preceding overt segmentation (Meier, 1979). Several genes expressed in striped patterns in the segmental plate have been described recently (Müller et al., 1996; Jen et al., 1997; Palmeirim et al., 1997) and *papc* can be added to this growing list. Although *papc* has an interesting late phase of expression during somitogenesis, this study will focus on the genetic regulation of *papc* during gastrulation by *spadetail* and *floating head* and on the potential role of PAPC in mediating morphogenetic movements.

### *papc* requires *spt* during gastrulation

The *spt* gene is required for the convergence of mesodermal cells towards the dorsal side during gastrulation (Kimmel et al., 1989; Ho and Kane, 1990). To test whether PAPC, a cell adhesion molecule expressed in gastrulating mesoderm, might participate in these cell movements, we examined its expression in $spt^{-}$ embryos that carry a null mutation ($spt^{b104}$, Amacher and Kimmel, 1998; Griffin et al., 1998). As shown in Fig. 3, expression of *papc* is strikingly affected in $spt^{-}$ embryos. During early gastrulation, mesodermal *papc* transcripts are greatly reduced in 25% of embryos obtained by intercrossing *spt* heterozygous fish (Fig. 3A′,B′). At the completion of gastrulation and during early somitogenesis, a

few *papc*-positive cells are found in *spt*⁻ embryos, located in regions where adaxial cells would be formed (adaxial cells are slow muscle precursors; Devoto et al., 1996); importantly, no expression is found in any other cells including the prospective tailbud region (compare Fig. 3C-C′ and D-D′). The bands of *papc* expression formed during somitogenesis are also under *spt* regulation (Fig. 3D-D′). However, as

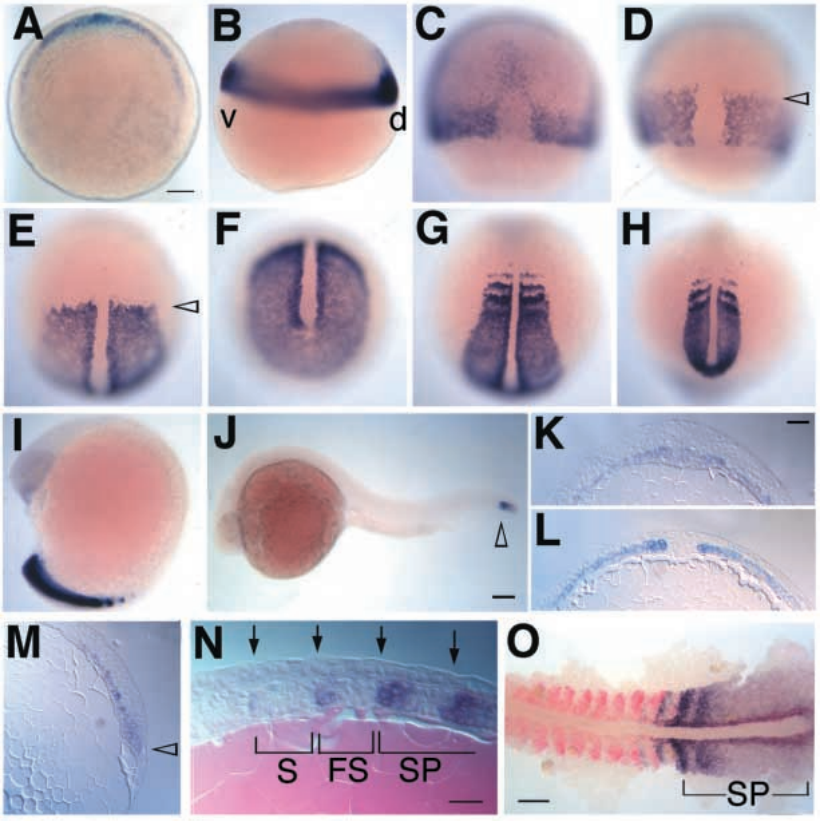


**Fig. 2.** *papc* expression in wild-type embryos. (A) Animal pole view at 30-40% epiboly stage (5 hours), showing dorsal expression of *papc* (blue) overlapping with that of *goosecoid* (which is weakly stained in aquamarine color) at the top of the panel. (B) Lateral view at early gastrula (shield stage, 6 hours); *papc* is detected in the entire marginal zone. d, dorsal; v, ventral. (C) 60% epiboly stage (7 hours); *papc* transcripts decrease in the dorsal midline. A weak *papc* signal is also seen in presumptive head mesendoderm. (D) 70-75% epiboly stage (8 hours); *papc* expression domain is broader along the animal-vegetal axis (anterior edge indicated by arrowhead); expression in the dorsal midline is undetectable. (E) At the end of gastrulation (bud stage, 10 hours), the anterior and mediolateral borders of *papc* expression become sharp. The arrowhead marks the anterior border of *papc* expression. (F) Bud stage, posterior view, the mesoderm ventral to the blastopore is positive for *papc* RNA. Signals are strongest next to the midline, where the adaxial cell population is located. (G) Dorsal view at 3-somite stage showing *papc* expression in paraxial mesoderm and anterior segmental bands. (H) Posterior and (I) lateral view at 14-somite stage; *papc* transcripts are detected in tail presomitic mesoderm. (J) Lateral view of a 24 hour embryo. Expression is detected only in the tip of the tail (arrowhead). (K-N) Sections of embryos hybridized in whole mount. (K) Section through the blastoderm margin at shield stage; expression is restricted to the involuting mesoderm and spans the midline. (L) Transverse section at bud stage (10 hours) showing *papc* expression in paraxial, but not axial, mesoderm. (M) Parasagittal section of the dorsal side of a late shield-stage embryo. Signal is detected in involuted and involuting hypoblast and the surface epiblast of the blastopore (arrowhead) in the marginal zone. (N) Parasagittal section at the 4-somite stage. The four bands of *papc* expression are indicated by arrows; weak expression is detectable at the anterior edge of the last formed (S) and forming somite (FS); the two strong bands are located in the segmental plate (SP). Anterior is to the left. (O) Double staining with *myoD* (red) and *papc* (blue) at 8-9 somite stage. Expression of the two genes overlaps in the last formed and the forming somite but not in the segmental plate (SP). Embryo was deyolked manually and flattened. Maternal transcripts were detectable by in situ hybridization at early cleavage stages (data not shown). Bars, 100 μm (A; also applies to B-I); 50 μm (K-M); 25 μm (N); 100 μm (O).

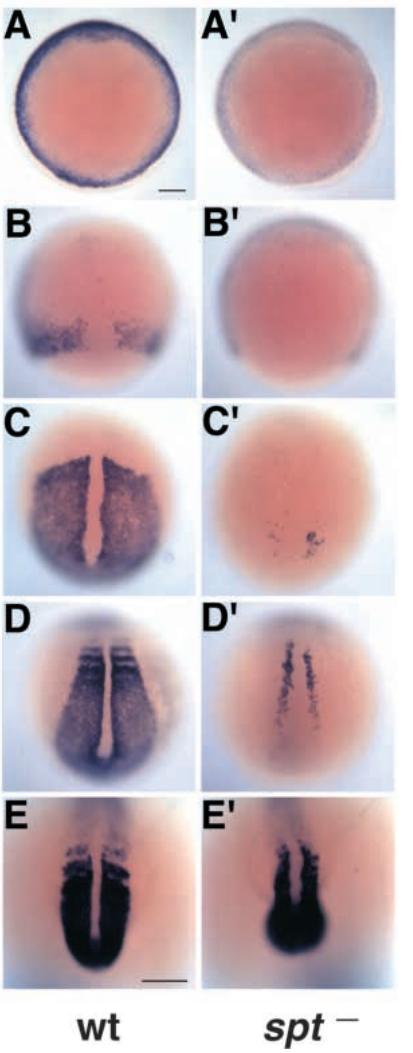


**Fig. 3.** *papc* expression is defective in *spt* mutant embryos. (A-E) Wild-type embryos. (A′-E′) *spt*$^{b104}$ homozygous mutant embryos. (A,A′) Shield stage showing greatly reduced *papc* in *spt*⁻ embryos at the onset of gastrulation. (B,B′) 80% epiboly showing lack of *papc* expression in the marginal zone of the *spt* mutant. (C,C′) Bud (10 hour) stage, (D,D′) 4-6 somite stage; expression is detected only in a few adaxial-like cells in *spt* mutants. (E,E′) 18 somite stage; the expression of *papc* recovers in tail somites, in the segmental plate and in the tailbud of *spt* mutants. A-D and A′-D′ are dorsal views, E and E′ are posterior views. Bars in A (also applies to B-D) and E, 100 μm.

development proceeds, *papc* transcripts appear in the tail somites, paraxial mesoderm and in the tailbud (Fig. 3E-E′).

This genetic analysis indicates that *papc* is a downstream target gene of the spadetail T-box transcription factor. In *spt* mutants *papc* is not expressed in gastrulating trunk mesodermal cells affected by the mutation. However, in regions of the embryo where *spt* mutants have more normal development, such as in tail somites (Kimmel et al., 1989) and islands of putative adaxial cells (Weinberg et al., 1996), *papc* is transcribed. The late expression of *papc* during tail somitogenesis reflects the existence of a second, *spt* independent, pathway in the regulation of mesodermal development (Griffin et al., 1998).

### *papc* parallels *spt* expression

If the *spt* transcription factor is an upstream regulator of *papc*, one expectation is that both genes should be expressed in similar regions. To test this, a detailed comparison of the expression patterns of *spt* (Griffin et al., 1998) and *papc* was performed. During gastrulation stages, the parallels between *spt* and *papc* expression are striking. Both are expressed in involuting mesoderm and later repressed in dorsal midline cells (notochord progenitors) with similar time courses (compare in Fig. 4A,B and C,D). At the end of gastrulation the patterns start to differ partially, with *papc* mRNA extending more anteriorly than *spt* (Fig. 4E,F). This difference in anterior expression borders becomes more marked when somitogenesis begins, as *spt* mRNA does not form bands of expression in paraxial mesoderm, but *papc* mRNA does (compare Fig. 4G,H). During gastrulation stages, when *spt* is required for convergence movements, the parallels between *spt* and *papc* expression patterns are remarkable. These observations, taken together with the genetic analyses shown in Fig. 3, suggest that *papc* encodes a molecule acting closely downstream of *spt*.

### *papc* and *spt* are repressed by *flh*

*floating head* is a homeobox gene expressed in the dorsal midline; *flh* mutants lack a notochord, and the axial mesoderm adopts a paraxial mesodermal (muscle) fate (Talbot et al., 1995; Halpern et al., 1995; Melby et al., 1996). The loss-of-function of a second gene, *spt*, prevents the formation of muscle in the midline and, surprisingly, restores anterior notochord formation in *flh*;*spt* double mutant embryos (Amacher and Kimmel, 1998). This finding suggested that in some way *flh* antagonizes *spt* function in axial midline cells. One possibility, not previously tested, is that *spt* is turned off in the axial midline by *flh* function. We found that this is indeed the case by examining *spt* expression in *flh* mutants and showing that *spt* is expressed ectopically in the *flh*$^{-}$ midline (Fig. 4A′,C′,E′,G′). Since *papc* is a downstream target of *spt*, one would expect that *papc* would also be turned on by the ectopic *spt* product in the *flh*$^{-}$ midline, and this is indeed the case (Fig. 4B′,D′,F′,H′). These findings show that both *spt* and *papc* are repressed by the *flh* organizer-specific homeobox gene in axial mesodermal cells, and further strengthen the view that *papc* is a close downstream target of *spt*.

### *DN-papc* inhibits convergence of somitic precursors

Xenopus *papc* mRNA promotes cell adhesion activity in injected embryos (S. H. Kim et al., unpublished observations). Similarly, injection of zebrafish full-length *papc* mRNA (*FL-papc*) into single Xenopus animal blastomeres was sufficient to create adhesive patches of clonally related cells at gastrula stages (data not shown). When *FL-papc* mRNA was microinjected into wild-type zebrafish embryos no obvious malformations were observed (Fig. 6D, Table 1). Attempts to

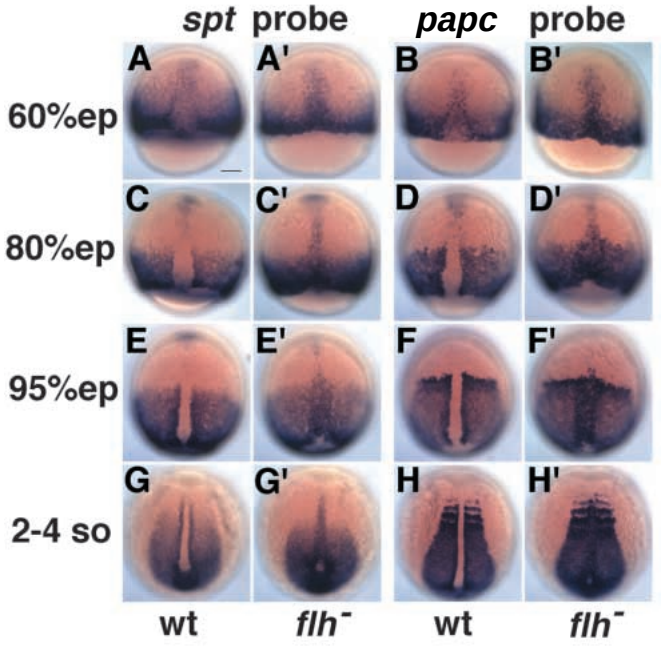


**Fig. 4.** *spt* and *papc* are expressed ectopically in the midline of *flh* mutant embryos. Probes and embryo genotypes used are shown at the top and the bottom of the figure, respectively. The stage is indicated on the left; ep, epiboly; so, somite. The *spt* expression pattern in wild-type embryos is very similar to that of *papc* except that the anterior border of expression differs at early somite stages. In addition, in the case of *papc*, the anterior border is segmented. In *flh* mutant embryos, expression of *spt* and *papc* is not excluded from the midline, even in the somite stage. Bar, 100 μm.

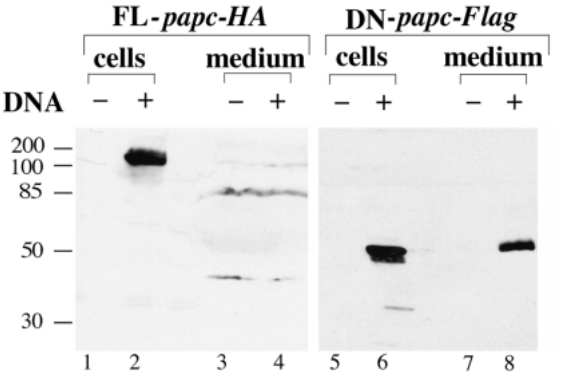


**Fig. 5.** A dominant-negative PAPC is secreted by cultured cells. 293T cells were transfected with or without *Fl-papc-HA* (lanes 1-4) or *DN-papc-Flag* (lanes 5-9). Cell pellets or supernatants corresponding to $1.5\times10^4$ cells were analyzed by western blot. Note that *DN-papc* (lane 8) is secreted into the culture medium but *FL-papc* is not (lane 4). Lanes 1-4 and 5-8 show different background bands because they were probed with different epitope tag antibodies. The positions of marker proteins is indicated.

**Table 1. Widening or loss of somites caused by dominant-negative *papc* mRNA and its rescue by full-length *papc***

| mRNA (pg) | Wider somites (%) | Reduced somites (%) | Normal (%) | Total (*n*) |
|---|---|---|---|---|
| *DN-papc* (25-100)[a] | 32 | 14 | 54 | 173 |
| *lacZ* (500) | 0 | 0 | 100 | 52 |
| *FL-papc* (100)[b] | 1 | 6 | 93 | 70 |
| Rescue experiments[c] | | | | |
| *DN-papc* (50) | 25 | 13 | 62 | 57 |
| *DN-papc* (50) +*FL-papc* (200) | 5 | 2 | 93 | 60 |

Embryos were injected into a single site at the 2-4 cell stage and analyzed at the 8-9 somite stage with a *myoD* probe. About 50% of embryos with wider somites showed unilateral defects. Most of the embryos with reduced somites were unilaterally affected.

[a]These phenotypes include embryos from 3 independent experiments, injected with *DN-papc* at 100 pg/embryo (*n*=50), 50 pg (*n*=84) or 25 pg (*n*=37). No significant differences in phenotype frequencies were noted at these concentrations; the phenotype may depend principally on the site of delivery of the mRNA. The notochord was in general of normal width but was wider in 15% of the embryos in which somites were affected by *DN-papc*.

[b]Embryos injected with *FL-papc* showed unilateral lack of somitic tissue in some cases; this might be caused by increased cell adhesion in paraxial mesoderm by *FL-papc*.

[c]The phenotypic effects of *DN-papc* were largely rescued by co-injection with full-length *papc*. 200 pg of *FL-papc* represents only a small molar excess over 50 pg *DN-papc* because its mRNA is three times longer. Data were pooled from two independent experiments; no embryos with widened notochords were observed in these experimental series.

rescue the *spt*⁻ phenotype by injecting FL-*papc* mRNA into mutant embryos failed to demonstrate recovery of trunk somites (data not shown). To test whether *papc* functions in mesodermal morphogenesis in zebrafish, we constructed a truncated form of *papc* mRNA that encodes only three amino-terminal extracellular cadherin domains. Transfection into 293T human kidney cultured cells showed that this truncated construct secretes a stable protein product into the culture medium (Fig. 5, lane 8). Secreted forms of extracellular domains of cadherins have been described previously (Brieher et al., 1996) and a similar construct of Xenopus *papc* was shown to act in a dominant-negative fashion to counteract PAPC homophilic adhesion (S. H. Kim et al., unpublished observations).

Injection of zebrafish dominant-negative *papc* mRNA (*DN-papc*) resulted in embryos in which somites were expanded laterally or disrupted in the trunk (Fig. 6A-C). These phenotypes were specific for paraxial mesoderm, because the notochord (axial mesoderm) usually had normal width and morphology (Fig. 6A-C). To analyze the *DN-papc* phenotype in detail, embryos were injected into a single site at the 2-4 cell stage with synthetic mRNA and monitored at the 8-9 somite stage by in situ hybridization with *myoD*. The *myoD* probe is useful because it marks each somite as well as the adaxial cells that flank the notochord on either side (Weinberg et al., 1996). As can be seen in Fig. 6D-F and Table 1, two phenotypes were observed. In 32% of injected embryos, somitic *myoD* expression was spread out laterally, suggesting reduced convergence of somitic precursors towards the midline (Fig. 6E,H,I). The second phenotype, observed in 14% of injected embryos, was the reduction or lack of trunk somites, usually on one side of the embryo (Fig. 6C). This phenotype is reminiscent of that of *spt*⁻ embryos, in which cell convergence movements to the dorsal midline are severely impaired and somites fail to form in the trunk. However, in the case of somite disruption by *DN-papc* mRNA, a few isolated *myoD*-positive cells were usually detected in paraxial mesoderm at a distance from the midline (Fig. 6F). These phenotypes were observed at 12 hours of development; however, by 24 hours paraxial cells were able to converge to the midline, forming somites of relatively normal width (data not shown). A similar regulation or recovery of the trunk somite defects is seen in *spt*⁻ embryos (Kimmel et al., 1989).

To investigate the specificity of phenotypes caused by injected *DN-papc* mRNA, several controls were performed. Injection of large amounts of *lacZ* mRNA (500 pg) had no phenotypic effects (Fig. 6A and Table 1). When *lacZ* mRNA was injected together with 50 pg of *DN-papc* mRNA, the lineage tracer was always detected on the side in which the *myoD* expression pattern was affected (*n*=53; Fig. 6H,I), whereas *myoD* expression was normal on the injected side in embryos receiving *lacZ* mRNA only (Fig. 6G). The specificity of the dominant-negative phenotypes of *DN-papc* mRNA was further investigated in co-injection experiments with *FL-papc* mRNA. The results showed that the full-length product could rescue the observed phenotypes; as seen in Table 1 (bottom two lines), the frequency of both the wide somite and the somite-loss phenotype caused by *DN-papc* mRNA was reduced by co-injection of *FL-papc* mRNA. These microinjection experiments using a dominant-negative approach to inhibit *papc* function suggest that *papc* encodes a transmembrane cell adhesion molecule involved in convergence movements of paraxial mesoderm during gastrulation.

## DISCUSSION

We have isolated a zebrafish cDNA, *papc*, encoding a structural transmembrane protein that is expressed at the right time and place to play a role in mesodermal morphogenesis during gastrulation. *papc* encodes a typical member of the protocadherin family of cell adhesion molecules (Suzuki, 1996) expressed transiently in paraxial mesodermal precursors undergoing morphogenesis.

### *papc* is downstream of *spt*

The *spadetail* gene controls movements of cells in lateral mesoderm. In *spt* mutants lateral mesodermal cells fail to converge towards the dorsal midline and at later stages somites are missing (Kimmel et al., 1989; Ho and Kane, 1990). One characteristic of *spt* mutants is that although somites are absent in the trunk, they are present in the tail. The molecular nature of the *spt* gene has been recently identified by Griffin et al. (1998); it encodes a transcription factor of the T-box family, cloned independently by Ruvinsky et al. (1998). The *spt* gene is a zebrafish homologue of a Xenopus gene isolated independently by four groups and named *Xombi*, *VegT*, *Antipodean* and *Brat* (Lustig et al., 1996; Zhang and King, 1996; Stennard et al., 1996; Horb and Thomsen, 1997). In microinjection studies, *Xombi* mRNA can induce the formation of ectopic blastopore lips, indicating that *Xombi* may have a morphogenetic function (Lustig et al., 1996). Another T-box gene, *Brachyury*, has also been implicated in the movement of

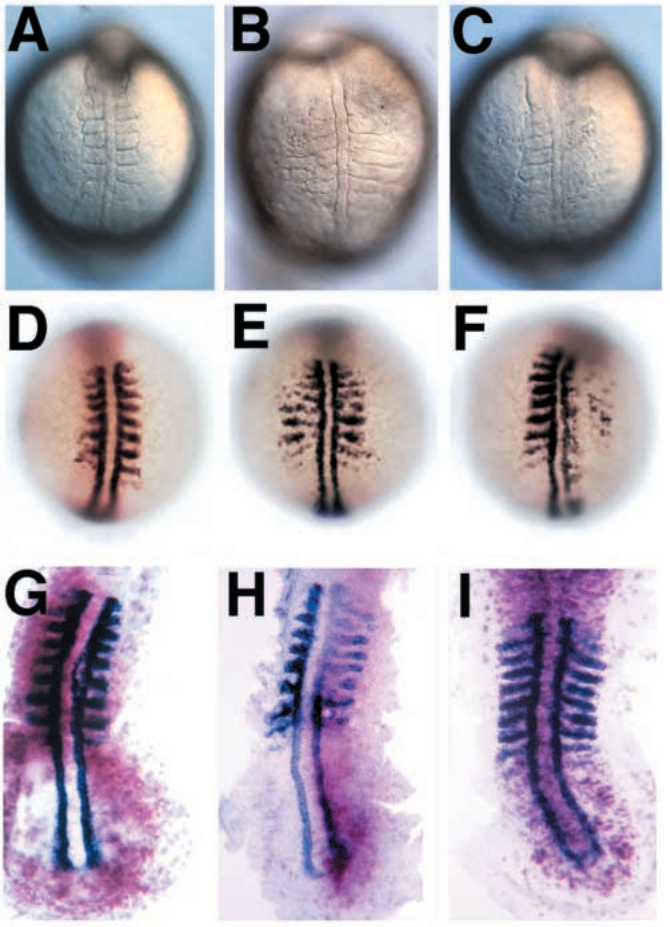


**Fig. 6.** Dominant-negative *papc* inhibits convergence movements of paraxial mesoderm. (A) Control embryo injected with *lacZ* mRNA. (B) Embryo with widened somites caused by injection of *DN-papc* mRNA. (C) Embryo injected with *DN-papc* showing unilateral lack of trunk somites. (D) Embryo injected with *FL-papc* mRNA (100 pg) and stained with *myoD* probe; somites and the adaxial muscle cells flanking the notochord are normal. (E) *DN-papc* mRNA injection causes lateral expansion of *myoD* expression; axial mesoderm is normal. (F) Reduction of *myoD* expression caused by *DN-papc* injection, a few *myoD*-positive cells are seen in lateral positions but not in the paraxial region. (G) Embryo injected with *lacZ* mRNA. *lacZ* is in red and *myoD* in blue. Somites are normal and *lacZ* activity is seen on both sides. (H) Widened somites on the injected side of embryo injected with *DN-papc* mRNA. (I) Embryo that received mRNA bilaterally showing expanded somites on both sides. The amount of mRNA injected was 50 pg for *DN-papc* and 500 pg for *lacZ* in all cases.

mesodermal cells as they pass through the primitive streak of the mouse gastrula (Wilson et al., 1993). Thus, transcription factors of the T-box family stand out as major regulators of mesodermal cell movements but it is not known how they cause such profound effects on cell movements during gastrulation. *papc* now provides a possible link between *spt*, a transcriptional regulator, and the cell surface.

In zebrafish, loss of *spt* function extinguishes *papc* expression in the mesodermal mantle throughout gastrulation and until early segmentation stages (except for a few putative adaxial cells, which are known to be less affected in *spt* mutant embryos; Weinberg et al., 1996). Therefore, *papc* lies genetically downstream of *spt* during early gastrulation. At later stages, the expression of these two genes diverges: *papc* is expressed in a banded pattern in forming somites whereas *spt* is not (compare Fig. 4G,H), and *papc* is not expressed in Rohon-Beard neurons, which are a site of expression of the Xenopus *spt* homologue (Zhang and King, 1996). Thus, at later stages additional factors regulate *papc* expression. Preliminary attempts to rescue the *spt*$^{-}$ phenotype by injection of full-length *papc* mRNA did not restore the formation of trunk somites, scored by *myoD* staining at the 8-somite stage. In future it will be interesting to test whether cell convergence movements can be rescued in *spt*$^{-}$ cells by injection of *papc* mRNA followed by cell transplantation into wild-type embryos (Ho and Kane, 1990).

During gastrulation the expression patterns of *papc* and *spt* in wild-type embryos are remarkably similar, suggesting that *papc* is activated closely downstream of *spt*. *papc* transcripts are markedly decreased in the trunk of *spt* mutants, but are present in the tail, a pattern that mirrors the regions affected in *spt* mutants. During late segmentation stages *papc* is expressed in the tailbud, and it has been proposed (Griffin et al., 1998) that a good candidate to take on *spt* function in the tail is *tbx6*, a T-box gene expressed in tail mesoderm in zebrafish (Hug et al., 1997) and mouse (Chapman and Papaionnou, 1998).

### *papc* is involved in mesodermal convergence

Studies in the Xenopus animal cap system have shown that a secreted dominant-negative form of *xPAPC* inhibits the convergence and extension movements induced by high doses of activin without affecting cell differentiation (as determined with the *myoD*, α-*actin*, *Xbra*, *collagen 2* and *Xwnt-8* molecular markers; S. H. Kim et al., unpublished observations). In addition, full-length *xPAPC* enhances convergence and extension movements and characteristic changes in cell shape in animal caps treated with low doses of activin. This cell movement-promoting activity does not affect the differentiation of tissue types (S. H. Kim et al., unpublished observations). Thus, Xenopus PAPC is not merely a cell adhesion molecule but is also able to trigger cell movements. During convergence and extension mesodermal cells associate closely with their neighbors, but cell attachments must be formed and subsequently released as intercalating cells move past each other in directional movements towards the dorsal midline (Keller et al., 1992). It has been proposed that Xenopus PAPC may promote cell convergence by mediating transient associations between neighboring cell surfaces (S. H. Kim et al., unpublished observations).

To test whether *papc* participates in convergence movements in zebrafish, we adopted a dominant-negative strategy using a secreted form of *papc*. A striking phenotype caused by microinjection of zebrafish *DN-papc* mRNA into the 2 or 4-cell embryo is the formation of laterally widened somites (Fig. 6B,E). A similar wide-somite phenotype has been found in zebrafish gastrulation mutants such as *knypek* and *trilobite*, which are proposed to be defective in convergence movements (Solnica-Krezel et al., 1996; Hammerschmidt et al., 1996). A second phenotype observed with *DN-papc*-injected embryos was the lack of somites and *myoD* expression (except for isolated *myoD* positive cells in paraxial mesoderm) on the injected side of the embryo (Fig. 6C,F). A lack of somites in

the trunk region is seen in *spt* mutants (Kimmel et al., 1989; Ho and Kane, 1990). Both phenotypes may be explained by decreased cell convergence movements toward the dorsal midline. Together with the remarkable correspondence between the regions in which cell movements and *papc* expression are affected in *spt*$^{-}$ embryos, the dominant-negative results suggest that *papc* may play an important role in mesodermal morphogenesis.

Protocadherins belong to a large gene family (Suzuki, 1996). In zebrafish we have isolated five additional protocadherins that are expressed in distinct patterns during early development (A. Y., unpublished observations). This raises the question of whether the dominant-negative effects are specific for *papc*. Although one can not eliminate cross-reaction with other protocadherins, three lines of evidence indicate that the effects may be specific for *papc*. First, the area affected by *DN-papc* correlates with the region of endogenous *papc* expression. Second, the dominant-negative effects can be rescued by full-length *papc* mRNA. Lastly, in Xenopus *PAPC*-mediated cell adhesion can be inhibited by *DN-PAPC*, but not by a dominant-negative form of another protocadherin expressed in Axial mesoderm (*AXPC*). Conversely, adhesion patches mediated by Xenopus *AXPC* can be disrupted by *DN-AXPC* but not by *DN-PAPC*, indicating that they affect distinct homotypic protocadherin interactions (S. H. Kim et al., unpublished observations). The definitive loss-of-function phenotype of *papc* must await the identification of a zebrafish mutant or the targeted mutation of its mouse homologue.

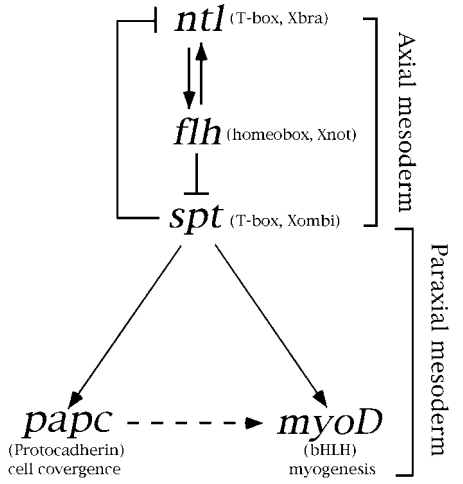


**Fig. 7.** Model of genetic interactions involved in axial and paraxial mesoderm specification in zebrafish. All solid arrows shown here are deduced from loss-of-function studies in mutant fish. The mutual positive regulatory interaction between *ntl* and *flh* was deduced by Halpern et al. (1997). The genetic interaction between *spt* and *flh* was suggested by Amacher and Kimmel (1998); in this study we show that this repression occurs at the level of gene expression. The antagonistic action of *spt* on *ntl* function was proposed by Amacher and Kimmel (1998). A previous study showed that *spt* activity was required for *myoD* expression during gastrulation (Weinberg et al., 1996) and in this study we show that *spt* activity is required for *papc* expression. Gain-of-function microinjection studies in Xenopus further support some of these gene interactions. *Xombi* induced *myoD* (Horb and Thomsen, 1997) as well as *papc* (S. H. Kim et al., unpublished observations). *Xnot-2* repressed *papc* (S. H. Kim et al., unpublished observations) and promoted notochord formation (Gont et al., 1996). The *DN-papc* injection analyses presented in this paper suggest that *papc* may mediate the function of *spt* in the biological processes of cell convergence or cell-type specification and are indicated by a dashed line.

### Genetic interactions between *spt*, *flh* and *papc*

*spadetail* was the first zebrafish gene proposed to regulate cell movements in mesoderm (Kimmel et al., 1989). Since then, the genetic pathways that determine axial versus paraxial mesoderm and their epistatic relationships have been studied extensively (Halpern et al., 1993, 1995, 1997; Talbot et al., 1995; Odenthal et al., 1996; Amacher and Kimmel, 1998). Fig. 7 summarizes these genetic interactions. In *flh* mutants the notochord is absent (Talbot et al., 1995) and in gain-of-function studies *Xnot* recruits additional mesoderm into notochord fates at the expense of paraxial fates in Xenopus (Gont et al., 1996). We now show that *spt* and *papc* are ectopically expressed in the midline when *flh* function is lost (Fig. 4). Thus, an important function of the wild-type *flh* homeobox gene is to repress transcription of *spt* in the midline. In *flh* mutants, expression of *spt* in axial cells leads to the adoption of paraxial (*myoD*-positive) cell fates and consequently to the loss of notochord. When both *flh* and *spt* functions are removed in double mutants, ectopic muscle can no longer form in the midline and instead anterior notochord, including *ntl*-positive cells, can develop (Amacher and Kimmel, 1998). *spt* is required to activate *myoD* (Weinberg et al., 1996) and *papc* expression. As with any genetic model, the interactions indicated in Fig. 7 may be direct or indirect; in future it would be interesting to investigate whether the *papc* promoter contains T-box binding sites.

*paraxial protocadherin* introduces a new player in the pathway of mesodermal patterning. Many genes expressed specifically in the vertebrate gastrula, particularly in Spemann's organizer, have been described in recent years (reviewed by Lemaire and Kodjabachian, 1996; Harland and Gerhart, 1997). Since these genes encode regulatory transcription or secreted factors, one must wonder at which point the building of an embryo through morphogenetic movements of coherent cell sheets begins. Unlike these regulatory transcription and growth factors, PAPC is a transmembrane protein that may be part of the actual structural machinery that effects cell movements during gastrulation. The PAPC cell adhesion molecule may provide a link between transcription factors and morphogenesis.

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