Paraxial protocadherin mediates cell sorting and tissue morphogenesis by regulating C-cadherin adhesion activity

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Little is known about how protocadherins function in cell adhesion and tissue development. Paraxial protocadherin (PAPC) controls cell sorting and morphogenetic movements in the *Xenopus laevis* embryo. We find that PAPC mediates these functions by down-regulating the adhesion activity of C-cadherin. Expression of exogenous C-cadherin reverses PAPC-induced cell sorting and gastrulation defects. Moreover, loss of endogenous PAPC results in elevated C-cadherin adhesion activity in the dorsal mesoderm and interferes with the normal blastopore closure, a defect that can be rescued by a dominant-negative C-cadherin mutant. Importantly, activin induces PAPC expression, and PAPC is required for activin-induced regulation of C-cadherin adhesion activity and explant morphogenesis. Signaling through Frizzled-7 is not required for PAPC regulation of C-cadherin, suggesting that C-cadherin regulation and Frizzled-7 signaling are two distinct branches of the PAPC pathway that induce morphogenetic movements. Thus, spatial regulation of classical cadherin adhesive function by local expression of a protocadherin is a novel mechanism for controlling cell sorting and tissue morphogenesis.

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Introduction

Protocadherins are transmembrane glycoproteins that contain six or more conserved cadherin-repeats (EC domains) in their extracellular domains. They constitute a large subfamily of the cadherin superfamily (Nollet et al., 2000; Suzuki, 2000; Yagi and Takeichi, 2000). Classical cadherins are Ca^{2+}-dependent, homophilic cell–cell adhesion molecules with five EC domains. Their adhesion activities rely on two common features: the conserved Trp2 in the first cadherin-repeat domain (EC1), which is necessary for homophilic binding, and the conserved catenin-binding motifs in the cytoplasmic domain, which are required for signaling and linkage to the actin cytoskeleton (Gumbiner, 2005). Much less is known about the adhesion properties of protocadherins. Protocadherins do not have the Trp2 residue in the extracellular domain or the catenin-binding motifs in the cytoplasmic domain (Nollet et al., 2000). It is not even entirely clear whether they function as adhesion molecules or have evolved to perform different cellular functions. Some protocadherins exhibit weak cell aggregation activity when overexpressed in L cells, whereas others do not (Sano et al., 1993; Sago et al., 1995; Yoshida et al., 1998; Hirano et al., 1999; Yoshida, 2003). It is not clear whether the weak cell aggregation mediated by a few of these protocadherins reflects true cell adhesion function at physiological levels of expression. More direct and thorough studies, like those that have been performed on classical cadherins, are needed to establish the adhesion properties of a protocadherin.

Xenopus laevis paraxial protocadherin (PAPC) is a protocadherin that has been shown to play an essential role in the convergence and extension movements of paraxial mesoderm and in the establishment of somite boundaries during the early development of *X. laevis* embryos (Kim et al., 1998, 2000). It is first expressed in Spemann's organizer at the onset of gastrulation, and is later expressed in the paraxial trunk mesoderm. By stage 14, PAPC is expressed in stripes and prefigures the forming somites. PAPC also induces the sorting out of blastomeres, which was taken as evidence that it functions as a homophilic cell-adhesion molecule. Recently, two groups reported that PAPC interacts with *X. laevis* Frizzled-7 (Xfz7) and can activate RhoA and JNK signaling via the noncanonical Wnt pathway to regulate tissue separation or convergent extension (Medina et al., 2004; Unterseher et al., 2004).

C-cadherin is a classical cadherin that mediates cell–cell adhesion between *X. laevis* blastomeres. It is expressed both

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Abbreviations used in this paper: COMO, control morpholino; DMZ, dorsal marginal zone; DN, dominant-negative; IL2Rα, interleukin 2 receptor α; PAPC, paraxial protocadherin; PAPCMO, PAPC morpholino; UTR, untranslated region; VMZ, ventral marginal zone.

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maternally and zygotically in all cell types throughout the early stages of *X. laevis* embryonic development (Choi et al., 1990; Ginsberg et al., 1991; Levi et al., 1991), and plays essential roles in the maintenance of embryo integrity (Heasman et al., 1994) and in morphogenetic cell movements (Brieher and Gumbiner, 1994; Lee and Gumbiner, 1995). Inhibition of C-cadherin adhesion activity by dominant-negative (DN) C-cadherins causes failure of blastopore closure (Lee and Gumbiner, 1995). Furthermore, the adhesion activity of C-cadherin at the blastomere surface is down-regulated during activin-induced elongation of animal cap explants, a process believed to mimic the convergence and extension cell movements during gastrulation (Brieher and Gumbiner, 1994). Disrupting the down-regulation of C-cadherin adhesion activity by an activating antibody blocks animal cap elongation (Zhong et al., 1999). These findings demonstrate that dynamic regulation of C-cadherin adhesion activity plays a pivotal role in embryonic tissue morphogenesis. However, the mechanism by which C-cadherin activity is regulated during morphogenesis is unknown.

Because PAPC, like C-cadherin, is a cadherin with a role in cell sorting and convergence and extension morphogenetic cell movements during *X. laevis* gastrulation, we chose it as an interesting model protocadherin to investigate. We first undertook a thorough examination of the adhesion properties of PAPC, including an analysis of domains required for its function. We also investigated the mechanism by which PAPC mediates cell sorting in the embryo and its relationship to C-cadherin–mediated adhesion. Finally, we asked how PAPC and C-cadherin cooperate to regulate tissue morphogenesis in the *X. laevis* embryo.

Results

PAPC does not mediate homophilic cell adhesion in several different cell types

CHO cells do not express endogenous cadherins and have been successfully used for studying the adhesion activities of classical cadherins (Brieher et al., 1996; Chappuis-Flament et al., 2001; Niessen and Gumbiner, 2002). We generated stable CHO cell lines that express full-length PAPC (FL-PAPC), a cytoplasmic tail–deleted form of PAPC (M-PAPC; Kim et al., 1998), or GFP as control, and examined their cell aggregation properties and their capacity to adhere to a substrate of purified PAPC protein. We tested M-PAPC as well as FL-PAPC because M-PAPC has been reported to have stronger cell sorting activity than FL-PAPC (Kim et al., 1998). Both FL-PAPC and M-PAPC were expressed on the surface of CHO cells, as demonstrated by accessibility to trypsinization, surface biotinylation, and immunofluorescence staining of intact cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200602062/DC1). Surprisingly, in cell aggregation assays, neither FL-PAPC-CHO cells nor M-PAPC-CHO cells aggregated to any extent compared with mock control cells (GFP-CHO; Fig. 1 A). For comparison, a stable cell line expressing C-cadherin, C-CHO, aggregated over time in the same experiment (Fig. 1 A). We also performed cell attachment flow assays using a purified PAPC protein as an adhesion substrate (PAPC-EC.Fc, which is the extracellular domain of PAPC fused with human IgG Fc, see Fig. S2). Although C-cadherin–expressing cells (C-CHO) adhered strongly to a C-cadherin substrate (C-cad-EC.Fc), neither FL-PAPC-CHO cells nor M-PAPC-CHO cells adhered to the PAPC substrate (Fig. 1 B). In addition to PAPC-EC.Fc, we also used another adhesion substrate, purified soluble PAPC with a C-terminal 6×His-tag (PAPC-EC.His; Fig. S2), which forms higher-order oligomers rather than dimers (unpublished data) and may have a conformation different from PAPC-EC.Fc. However, PAPC-expressing cells did not adhere to PAPC-EC.His either (unpublished data). Therefore, PAPC does not mediate homophilic cell adhesion in CHO cells.

It is possible that CHO cells lack the necessary cytoplasmic factors for PAPC-mediated adhesion. Therefore, we also prepared multiple stable PAPC-expressing cell lines using different kinds of cell types, including human epithelial A431 cells (Fig. 1 C), *X. laevis* XTC cells (Fig. 1 D), MBA-MD231, SW480, and MCF7 cells, and several others (unpublished data). None of these PAPC-expressing cell lines exhibited cell adhesion activity to purified PAPC substrates, but they were all able to adhere strongly to either purified E- or C-cadherin substrates via their endogenous cadherins. We also examined the adhesion activity of PAPC in *X. laevis* blastomeres, in which it has been shown to mediate cell sorting (Kim et al., 1998). FL-PAPC, M-PAPC, or GFP was expressed in embryos by mRNA injection, and blastomeres dissociated from isolated animal caps were tested for adhesion to either PAPC-substrates or the C-cadherin substrate. Blastomeres expressing PAPC did not adhere to either PAPC substrate, whereas the same blastomeres were able to adhere strongly to a 10 times less concentrated C-cadherin substrate (Fig. 1 E). Adhesion assays were also performed on blastomeres from the dorsal trunk mesoderm of stage 12 embryos, in which endogenous PAPC is expressed, but these blastomeres did not adhere to PAPC substrates either (Fig. 1 F). In summary, both the blastomere adhesion assays and the cell culture adhesion assays indicate that PAPC does not function effectively as a homophilic cell–cell adhesion molecule.

PAPC mediates cell sorting and influences gastrulation movements by down-regulating C-cadherin adhesion activity

The lack of intrinsic cell adhesion activity for PAPC appeared inconsistent with the reported cell sorting activity of PAPC in *X. laevis* embryos (Kim et al., 1998). Therefore we tried to reproduce the cell sorting assays as described by Kim et al. (1998), using both cell dispersal assays and reaggregation assays. In cell dispersal assays, with GFP mRNA alone injected into a single blastomere at the 32-cell stage, labeled cells extensively interspersed with surrounding unlabeled cells at a later stage of development (Fig. 2 A). In contrast, cells derived from FL-PAPC or M-PAPC mRNA-injected blastomeres (with a GFP lineage tracer) formed tight patches and maintained sharp boundaries with their unlabeled neighbors (Fig. 2, B and C), confirming the cell sorting activity of both FL-PAPC and M-PAPC. Moreover, in dissociation and reaggregation assays, blastomeres dissociated from FL-PAPC or M-PAPC mRNA-injected embryos (with a GFP lineage tracer) nicely sorted out

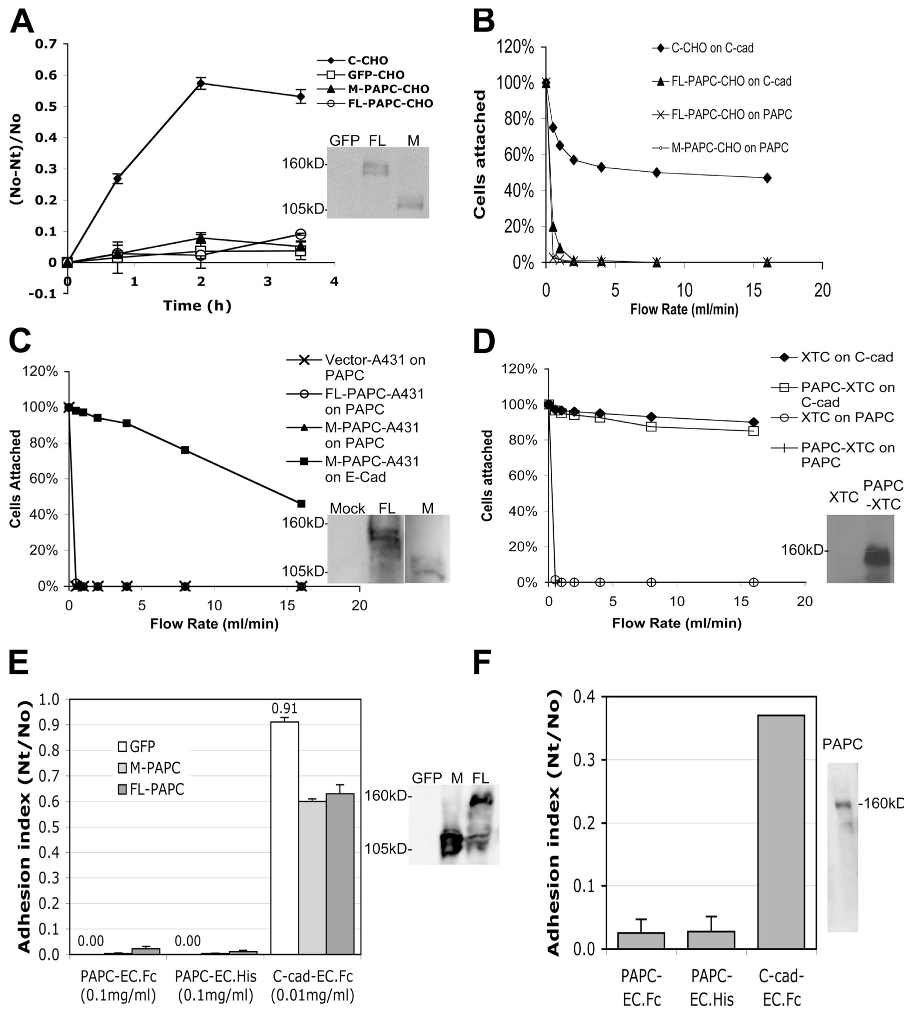


Figure 1. **PAPC does not mediate homophilic cell adhesion.** (A) Cell aggregation assay of CHO cells stably expressing FL-PAPC (FL-PAPC-CHO), M-PAPC (M-PAPC-CHO), C-cadherin (C-CHO, as positive control), or GFP (GFP-CHO, as negative control). (B) Cell attachment flow assay of stable FL-PAPC-CHO and M-PAPC-CHO cells on PAPC-EC.Fc substrate. Flow assay of C-CHO on C-cad-EC.Fc substrate was performed as positive control, and assay of FL-PAPC-CHO on C-cad-EC.Fc was used as negative control. (C) Cell attachment flow assay of stable FL-PAPC-A431 and M-PAPC-A431 cells on PAPC-EC.Fc substrate. Mock-transfected A431 (Vector-A431) cells were used as negative control, and adhesion of M-PAPC-A431 on human E-cad-EC.Fc was the positive control. (D) Cell attachment flow assay of stable PAPC-expressing XTC cells (PAPC-XTC) and parental XTC cells on PAPC-EC.FC substrate. The adhesion of XTC and PAPC-XTC on C-cadherin (C-cad-EC.Fc) substrate was positive control. (E) Blastomere adhesion assay with animal cap cells that ectopically express GFP, FL-PAPC (FL), or M-PAPC (M). 1.5 ng RNA was injected into embryos. Adhesion substrates were coated with 0.1 mg/ml PAPC-EC.Fc, 0.1 mg/ml PAPC-EC.His, or 10 µg/ml of C-cad-EC.Fc. (F) Blastomere adhesion assay with dorsal trunk mesodermal blastomeres from stage 12 embryos. Adhesion substrates were coated as in E. Expression of PAPC in cells used for adhesion assays was shown by anti-PAPC Western blot on the right of each graph. Error bars are the SEM.

from blastomeres obtained from uninjected embryos (Fig. 2, E, F, H, and I), whereas blastomeres from embryos in which GFP mRNA alone was injected uniformly mixed with uninjected blastomeres (Fig. 2, D and G).

Notably, FL-PAPC has the same activity in inducing cell sorting as M-PAPC because the same amount of mRNA was injected in every experiment. This appears different from an earlier study that found M-PAPC mRNA to be seven times more efficient in inducing cell sorting (Kim et al., 1998). However, in that study, protein expression levels were not measured because anti-PAPC antibodies were not yet available. Indeed, the FL-PAPC construct used in the previous study produces seven times less protein in embryos than the M-PAPC construct (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200602062/DC1). This original FL-PAPC construct differs from the M-PAPC construct because it retains both the 5′ and 3′ untranslated regions (UTRs). For our experiments, we removed the 3′ (and 5′) UTR, which results in similar protein expression levels for FL-PAPC and M-PAPC (Fig. S3 D–F) and, thus, higher cell-sorting activity for FL-PAPC (Fig. S3, G–I). Therefore, the cytoplasmic tail is not required for the cell-sorting activity of PAPC. Furthermore, expression of the membrane-bound cytoplasmic domain of PAPC had no detectable affect on cell sorting or on M-PAPC–induced cell sorting (Fig. S4).

It is important to note that the PAPC-expressing cells sorted to the outside of the aggregates in clusters. This was apparent in a surface view (Fig. 2, E and F), but was also confirmed by bisection of the aggregates (Fig. 2, H and I). According to Steinberg's differential adhesion theory (Steinberg, 1970; Foty and Steinberg, 2004), cells with weaker adhesion strength tend to sort to the periphery of coaggregates. This suggests that PAPC-expressing cells have weaker adhesion strength than uninjected cells. Indeed, in blastomere aggregation assays, FL-PAPC– or M-PAPC–expressing cells only formed small aggregates (Fig. 2, K and L) compared with control GFP-expressing cells (Fig. 2 J), showing that PAPC-expressing cells exhibit less overall cell adhesion activity. Therefore, both FL-PAPC and M-PAPC may induce cell sorting by down-regulating the overall cell adhesion strength.

Knowing that PAPC does not mediate cell adhesion itself and yet can induce cell sorting, we hypothesized that PAPC changes the adhesion activity of other adhesion molecules. The best candidate is C-cadherin, because C-cadherin is expressed throughout the *X. laevis* embryo in early stages of development and has been shown to be necessary for blastomere adhesion (Heasman et al., 1994). In addition, it has been shown that C-cadherin activity can be down-regulated by growth factors such as activin (Brieher and Gumbiner, 1994). To examine

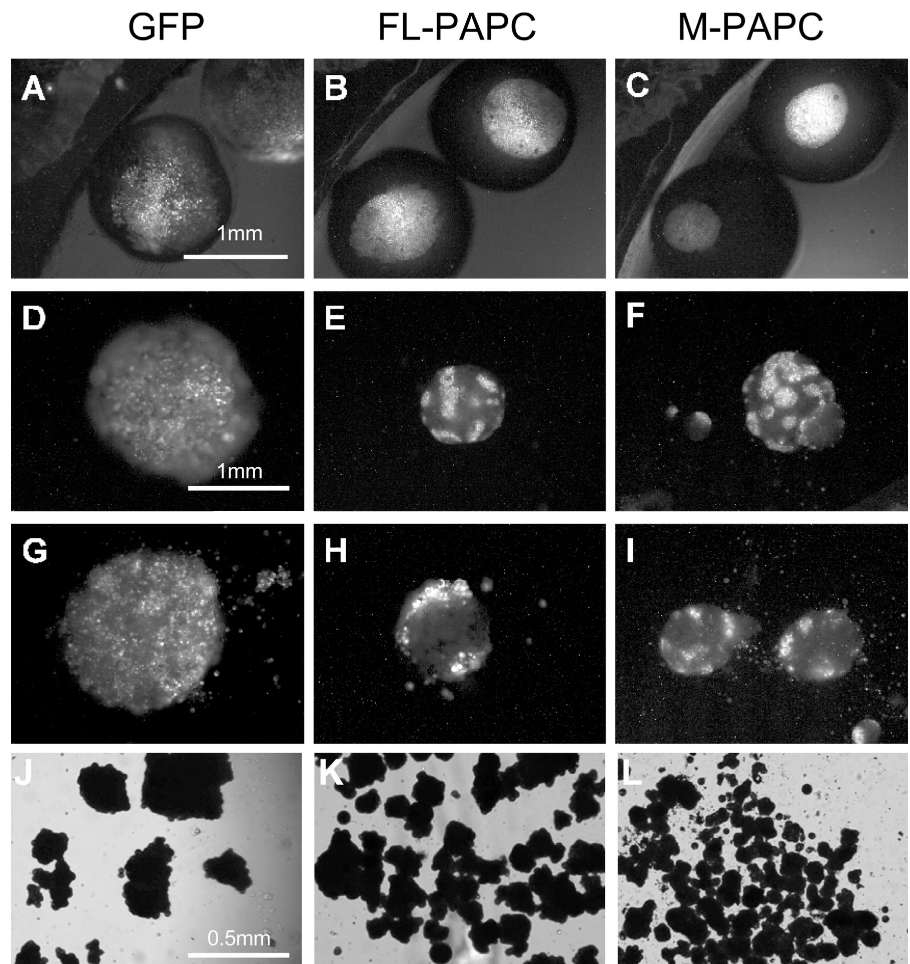


Figure 2. **FL-PAPC and M-PAPC induce cell sorting and decreased adhesion with similar activity.** (A–C) Cell dispersal assays. 500 pg of control GFP mRNA (A), FL-PAPC mRNA (B), or M-PAPC mRNA (C) was coinjected with 200 pg of NLS-GFP mRNA into one animal blastomere at the 32-cell stage. Pictures of the injected embryos were taken under fluorescence-microscope at stage 14. (D–I) Cell dissociation and reaggregation assays. Dissociated animal cap blastomeres from control GFP mRNA- (D and G), FL-PAPC mRNA- (E and H), or M-PAPC mRNA- (F and I) injected embryos were mixed with those from uninjected embryos and allowed to form coaggregates overnight. All injections were traced by coinjecting NLS-GFP mRNA. (D–F) Overview of the aggregates. (G–I) Bisectional view of the aggregates. (J–L) Blastomere aggregation assays. As in D–I, blastomeres expressing GFP (J), FL-PAPC (K), or M-PAPC (L) were allowed to aggregate in the presence of calcium for 1 h on a rocker. At the end of the assay, pictures of the aggregates were taken.

whether PAPC regulates C-cadherin adhesion, we performed blastomere adhesion assays under conditions used to detect activin-regulation of C-cadherin adhesion activity. Blastomeres obtained from FL-PAPC or M-PAPC mRNA-injected embryos exhibited significantly decreased levels of C-cadherin–mediated adhesion, which was equivalent to only ∼40% of the control level exhibited by blastomeres from GFP mRNA-injected embryos (Fig. 3 A).

A decrease in C-cadherin adhesion could be caused either by decreased C-cadherin protein level at the cell surface or by decreased intrinsic adhesion activity of C-cadherin. PAPC expression did not change the overall levels of C-cadherin protein in whole embryos (Fig. 3 B, lane 1–2) or in animal cap blastomeres used for adhesion assays (Fig. 3 B, lane 3–4). To determine whether the surface level of C-cadherin was changed by PAPC expression, we treated dissociated blastomeres with trypsin-EDTA to remove cell surface C-cadherin. PAPC expression did not alter the amount of trypsin-accessible C-cadherin (Fig. 3 B, lane 5–8). Previous work has shown that a specific C-cadherin–activating antibody, AA5, can reverse activin-regulation of C-cadherin–mediated adhesion at the cell surface, demonstrating intrinsic regulation of C-cadherin adhesion activity by activin (Zhong et al., 1999). AA5 similarly reversed PAPC-regulation of C-cadherin–mediated adhesion (Fig. 3 C). Therefore, PAPC functions by decreasing intrinsic C-cadherin adhesion activity at the cell surface. The effect of PAPC expression on C-cadherin–mediated adhesion is specific because blastomere adhesion to fibronectin or to antibodies against a nonspecific, exogenously expressed cell surface protein, human interleukin 2 receptor α (IL2Rα), is not affected by PAPC expression (Fig. 3, E and F).

To determine whether the down-regulation of C-cadherin activity is the cause of PAPC-induced cell sorting, we asked whether increasing C-cadherin expression levels could reverse PAPC-induced cell sorting. In cell dispersal assays with increasing amounts of C-cadherin mRNA coinjected with M-PAPC (and GFP) mRNA, the GFP-labeled cell population gradually changed from a tight patch to a loose patch and, eventually, to total mixing with their uninjected neighbors (Fig. 4 A). Therefore, overexpression of C-cadherin reverts PAPC-induced cell sorting. This result provides additional evidence that the down-regulation of C-cadherin adhesion by PAPC, rather than added PAPC-mediated adhesion, causes cell sorting. If PAPC increased cell adhesion, coexpression of C-cadherin should bolster cell sorting instead of blocking it.

Overexpression of M-PAPC in the animal hemisphere of the *X. laevis* embryo consistently caused failure of blastopore closure during gastrulation (defect rate 30/30; Fig. 4 B, middle column), which is a phenotype similar to that caused by DN C-cadherin expression (Lee and Gumbiner, 1995).

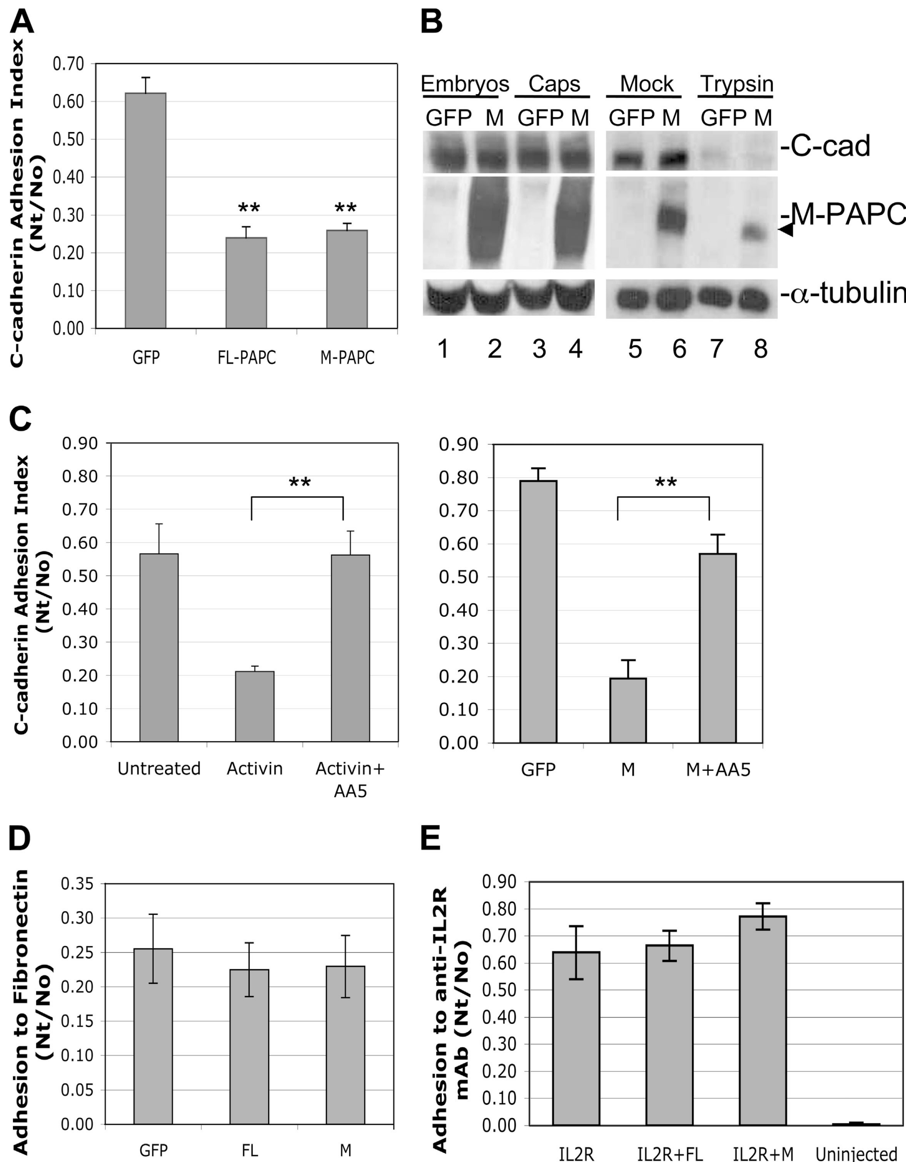


Figure 3. **Both FL-PAPC and M-PAPC down-regulate C-cadherin adhesion activity.** (A) Blastomere adhesion assay of GFP mRNA- (control), FL-PAPC mRNA-, or M-PAPC mRNA-injected embryos (1.5 ng/embryo) on 4 μg/ml C-cad-EC. Fc–coated substrates. **, $P < 0.001$ (by *t*-test) compared with the control GFP-expressing blastomeres. (B) M-PAPC expression does not change either total or cell surface C-cadherin protein levels in blastomeres. Embryos were injected as in A. C-cadherin levels in total embryo lysates (lanes 1–2), stage 9 animal cap explants (lanes 3–4), and dissociated animal cap cells that were either mock treated (lanes 5–6) or trypsin/EDTA treated (lanes 7–8) were determined by Western blotting with anti–C-cadherin mAb (6B6). Expression of M-PAPC was confirmed by anti-PAPC blotting, and anti–α-tubulin blots served as loading control. Arrowhead, a fragment of M-PAPC. (C) C-cadherin–activating antibody AA5 (1 μg/ml F*ab* fragment) reverts the down-regulation of C-cadherin–mediated adhesion induced by either activin-treatment (left) or M-PAPC expression (right). **, $P < 0.01$. (D) Blastomere adhesion to fibronectin is not changed by PAPC-expression. (E) Blastomere adhesion to anti-IL2R mAb BB10 is not changed by PAPC-expression. FL, FL-PAPC; M, M-PAPC; IL2R, IL2Rα. Error bars are the SEM.

The same was observed for FL-PAPC mRNA injection (unpublished data). This phenotype is specific because injection of GFP mRNA did not cause any defect at the same stage (defect rate 0/50; Fig. 4 B, left column). Co-injection of C-cadherin mRNA, along with PAPC mRNA, rescued the blastopore closure defect significantly (defect rate 6/51; Fig. 4 B, right column). These results suggest that ectopic PAPC expression exerts its overall gastrulation phenotype by down-regulating C-cadherin activity.

To determine whether endogenous PAPC functions to inhibit C-cadherin adhesion activity, we also did loss-of-function studies. It is known that at the early stage of gastrulation, PAPC expression is limited to the dorsal marginal zone (DMZ; Kim et al., 1998). We found that the blastomeres obtained from the DMZ of stage 10.5 embryos exhibit significantly lower C-cadherin adhesion level than those from the ventral marginal zone (VMZ; Fig. 5 A, first two columns). We effectively knocked down endogenous PAPC expression using PAPC-specific morpholinos (PAPCMO; Fig. 6 B, lanes 5–6). Knocking down of endogenous PAPC expression results in a significant increase in the level of C-cadherin–mediated adhesion of the DMZ blastomeres to that of the ventral blastomeres (Fig. 6 A, third column), suggesting that PAPC is responsible for the lower level of C-cadherin–mediated adhesion in the DMZ of control morpholino (COMO)–injected embryos. Moreover, the C-cadherin–activating mAb AA5 can also increase the adhesion of the DMZ blastomeres (Fig. 6 A, last column), indicating that the lower level of C-cadherin–mediated adhesion in the DMZ blastomeres is caused by specific down-regulation of the adhesion activity of the C-cadherin protein.

In the whole embryo, loss of PAPC expression by PAPCMO injection leads to a blastopore closure defect in stage 12.5 embryos (Fig. 5, B and C, PAPCMO), indicating defects in morphogenetic movements of gastrulation. This defect is specifically caused by the loss of PAPC, because it can be rescued by a morpholino-resistant form of FL-PAPC (Fig. 5, B and C, PAPCMO+FL). We then asked whether this gastrulation defect occurred because of the lack of down-regulation

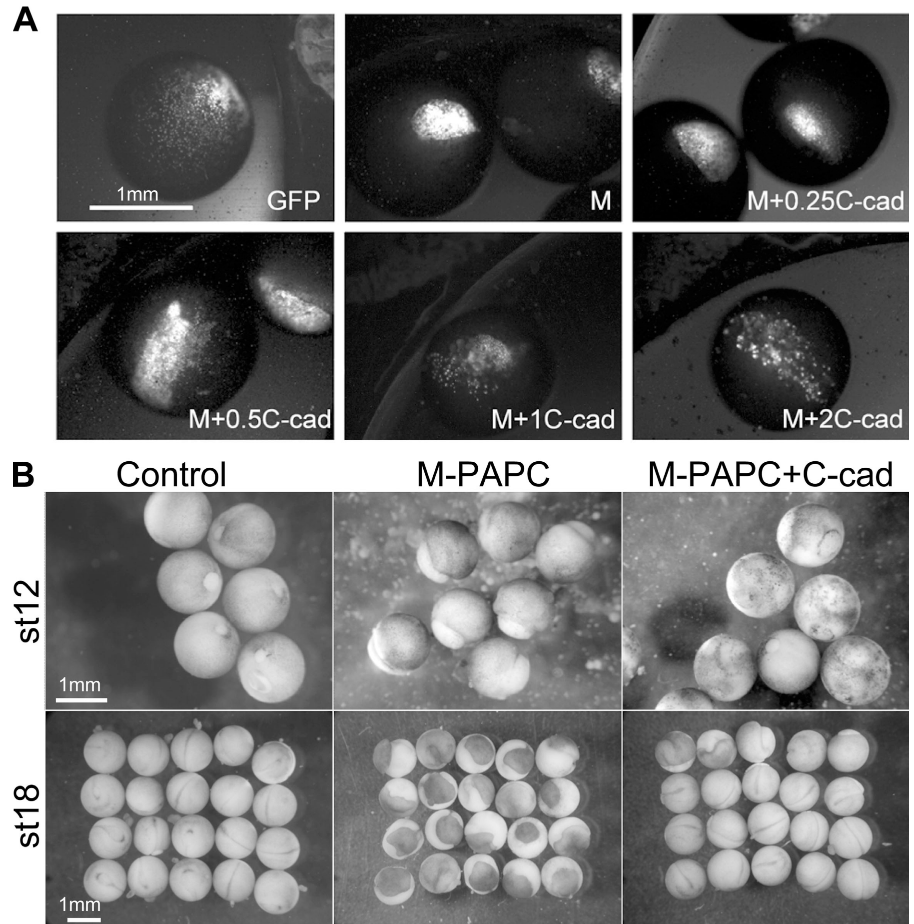


Figure 4. **Overexpression of C-cadherin reverts M-PAPC–induced cell sorting and gastrulation defects.** (A) Coexpression of C-cadherin reverts M-PAPC–induced cell sorting. Cell dispersal assays were performed by coinjecting different doses of C-cadherin mRNA, along with 300 pg of M-PAPC mRNA and 180 pg of NLS-GFP mRNA, as tracer. As control, 200 pg of NLS-GFP mRNA alone was injected (GFP). The C-cadherin mRNA doses were 0 pg (M), 75 pg (M + 0.25C-cad), 150 pg (M + 0.5C-cad), 300 pg (M + 1C-cad), and 600 pg (M + 2C-cad). (B) Exogenous C-cadherin expression rescues M-PAPC–induced blastopore-closure defects. 1 ng of GFP mRNA (Control), 0.5 ng of M-PAPC mRNA alone (M-PAPC), or 0.5 ng M-PAPC mRNA plus 1 ng of C-cadherin mRNA (M-PAPC + C-cad) were injected at the 4-cell stage into the animal poles of all four blastomeres. At stage 12 (top row), control embryos showed normal, nearly closed blastopores (30/30); M-PAPC mRNA-injected embryos failed to close their blastopores and showed an exogastrula phenotype (0/10 normal); coinjection of C-cadherin mRNA rescued the blastopore-closure defect (30/31 normal). In another experiment, embryos were allowed to develop to stage 18 (bottom row). GFP mRNA-injected embryos appeared normal (20/20), and M-PAPC mRNA-injected embryos failed to close their blastopores and exhibited exogastrula phenotype (0/20 normal), whereas coinjection of C-cadherin mRNA significantly rescued the defect (15/20 normal).

of C-cadherin adhesion activity by PAPC. Indeed, a DN C-cadherin mutant, the cytoplasmic tail of C-cadherin (Lee and Gumbiner, 1995), was able to rescue the PAPCMO-defect to the same extent as the morpholino-resistant FL-PAPC (Fig. 5, B and C, PAPCMO + Ctail). Both decreased the blastopore size from ~67% of total embryo diameter in PAPCMO-embryos to ~37% of embryo diameter in rescued embryos. These results strongly suggest that PAPC functions in vivo to down-regulate C-cadherin adhesion activity and that this function of PAPC is required for proper morphogenetic cell movements during gastrulation.

PAPC expression is induced by activin and is necessary for activin-induced regulation of adhesion and morphogenesis

Activin, which is a TGFβ family growth factor, is a mesoderm inducer that induces elongation of animal cap explants, a process mimicking the convergence and extension movements that normally occur during gastrulation (Symes and Smith, 1987). Activin down-regulates C-cadherin activity without changing the level of C-cadherin, and this down-regulation is necessary for induction of animal cap elongation (Brieher and Gumbiner, 1994; Zhong et al., 1999). We therefore examined whether PAPC plays a role in activin regulation of adhesion and induction of morphogenesis. Activin treatment of animal caps induced PAPC expression in 1–2 h compared with untreated animal caps (Fig. 6 A), which is similar to the time required for the down-regulation of C-cadherin adhesion activity by activin (Brieher and Gumbiner, 1994). Injection of PAPC morpholinos (PAPCMO) significantly reduced both activin-induced PAPC expression and endogenous PAPC expression compared with a control morpholino (COMO; Fig. 6 B). In PAPCMO-injected embryos, activin-treatment failed to down-regulate C-cadherin adhesion activity (Fig. 6 C, columns 3 and 4), in contrast to significant decrease of C-cadherin adhesion activity in COMO-injected embryos (Fig. 6 C, columns 1 and 2). Furthermore, coinjection of a morpholino-resistant form of FL-PAPC with PAPCMO (Fig. 6 C, column 5) resulted in significant down-regulation of C-cadherin adhesion, even without activin treatment, which is similar to injection of FL-PAPC mRNA alone (Fig. 6 C, column 6). The level of down-regulation by PAPC is comparable to the down-regulation caused by activin. These results demonstrate that PAPC is necessary as well as sufficient to mediate activin-induced down-regulation of C-cadherin adhesion activity in *X. laevis* blastomeres.

We also asked whether PAPC expression is required for activin-induced elongation of animal cap explants. Animal caps excised from COMO-injected embryos fully elongated (20/20) in response to activin treatment (Fig. 6 D, 1), whereas explants from PAPCMO-injected embryos fell into two groups: no elongation (18/30; Fig. 6 D, 2b) and partial elongation without significant narrowing (12/30; Fig. 6 D, 2a). As shown in Fig. 6 D

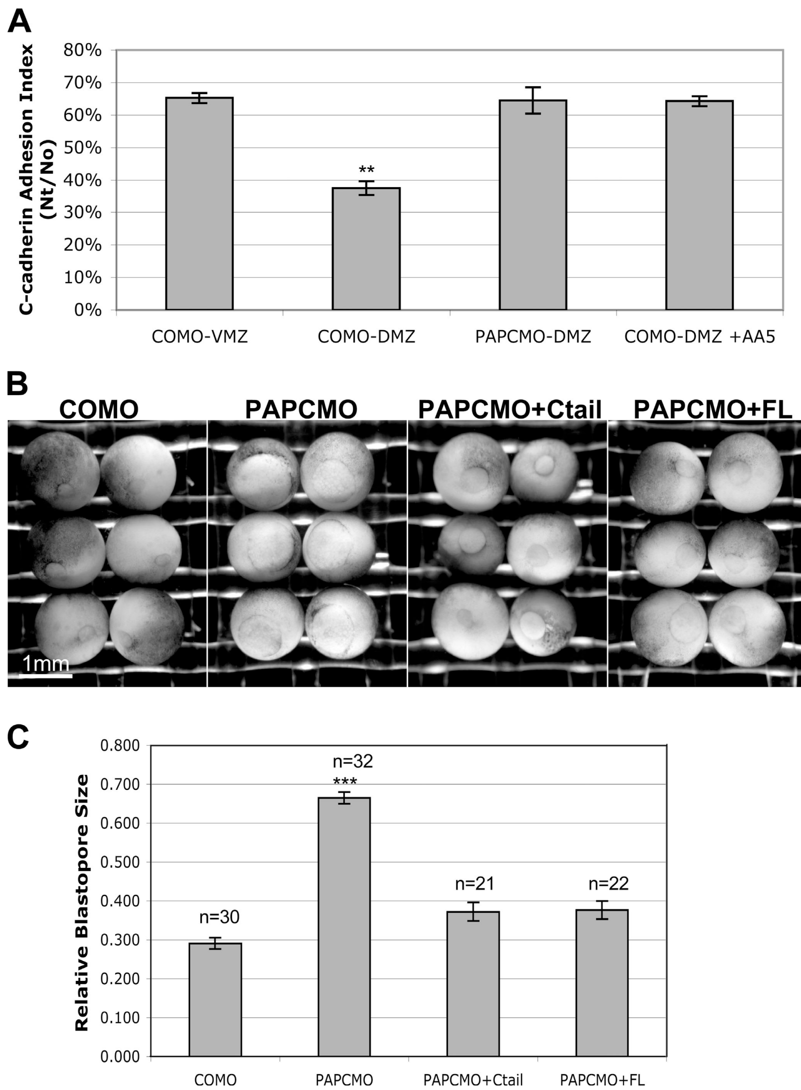


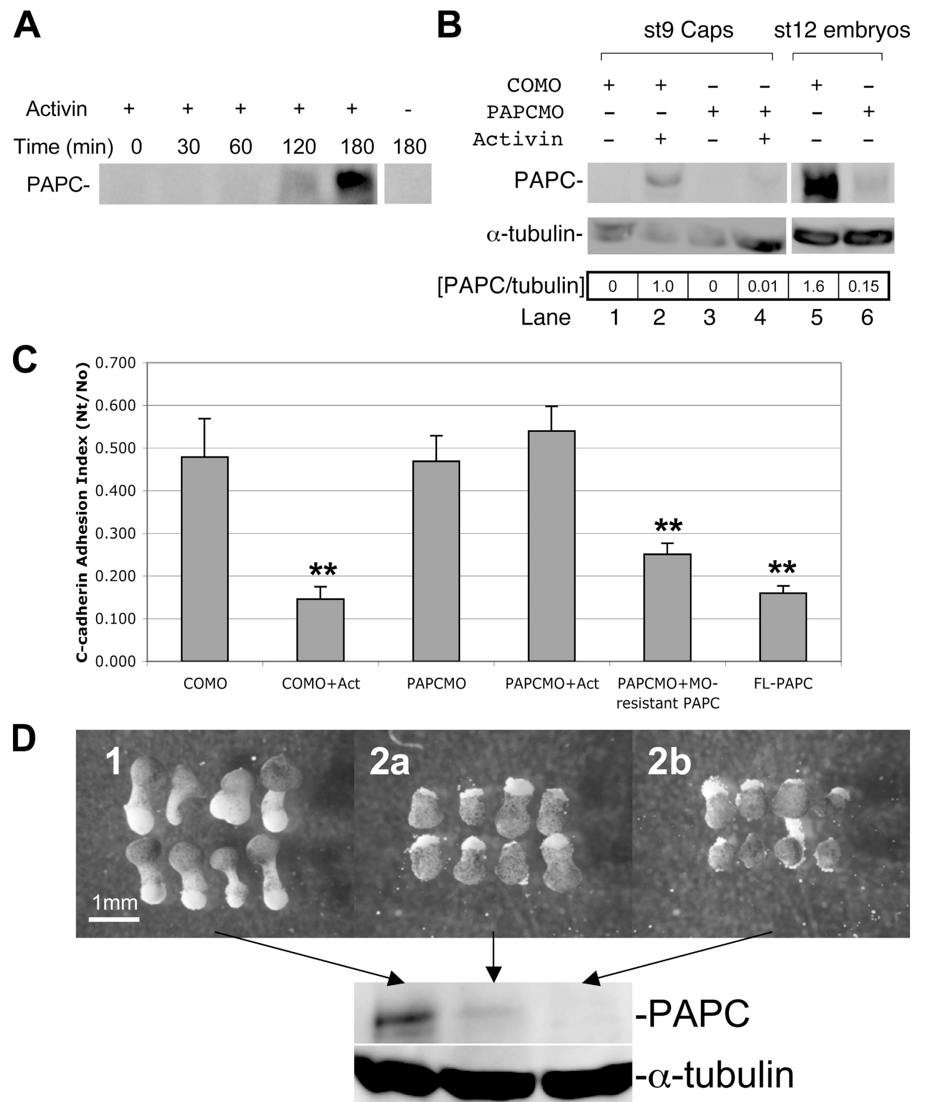
Figure 5. **Loss of endogenous PAPC expression results in increased C-cadherin–mediated adhesion in DMZ cells and gastrulation defects that can be rescued by decreased C-cadherin adhesion.** (A) Effects of loss of PAPC on C-cadherin–mediated adhesion in DMZ cells. 80 ng of control (COMO) or PAPC morpholinos (PAPCMO) were injected into the DMZ of 4–8–cell stage embryos. At stage 10.5, blastomeres were isolated from the VMZ or DMZ of five COMO- or PAPCMO-injected embryos and then tested for adhesion to purified C-cad-EC.Fc. Half of the blastomeres from the DMZ of control embryos were treated with 1 μg/ml AA5 F*ab* fragment before adhesion assay. **, $P < 0.001$ compared with the rest samples. (B and C) Rescue of PAPC morpholino-induced gastrulation defects by DN C-cadherin. 80 ng COMO, PAPCMO, or PAPCMO supplemented with either 25 pg C-cadherin cytoplasmic tail RNA (Ctail) or 50 pg FL-PAPC(-UTR) RNA (FL) was injected into the DMZ of 4-cell stage embryos. At stage 12.5, pictures of six randomly picked embryos from each group of embryos were taken (B). Meanwhile, the relative blastopore size (the ratio of the blastopore diameter versus the embryo diameter) of each injected embryo was measured and graphed (C). Error bars are the SEM. ***, $P < 0.0001$ compared with the rest groups of embryos. *n*, the number of embryos in each group.

(bottom), the explants from COMO-injected embryos had high levels of PAPC, whereas the “partial-elongation” group of explants from PAPCMO-injected embryos had lower, but detectable, levels of PAPC expression, and the “no-elongation” group of explants from PAPCMO-injected embryos had no detectable PAPC expression. Hence, strong inhibition of PAPC expression blocked elongation, whereas partial reduction in its expression partially blocked elongation. On the other hand, animal caps from PAPC mRNA-injected embryos did not elongate in the absence of activin treatment (unpublished data). Therefore, PAPC expression is necessary, but not sufficient, for activin-induced animal cap elongation.

PAPC-regulation of C-cadherin is independent of Frizzled-7 signaling

Two recent studies reported that PAPC functionally interacts with *X. laevis* Frizzled-7 (Xfz7) –mediated Wnt/planar cell polarity pathway to control tissue separation behavior (Medina et al., 2004) and convergent extension movements (Unterseher et al., 2004). Because M-PAPC, unlike FL-PAPC, does not induce tissue separation when coexpressed with Xfz7 (Medina et al., 2004), but is still capable of decreasing C-cadherin–mediated adhesion suggests that the mechanism of PAPC-dependent tissue separation is different from that of PAPC-inhibition of C-cadherin adhesion activity. Nonetheless, we decided to directly test whether Xfz7 mediates the C-cadherin down-regulation activity of PAPC. Two methods were used to disrupt Xfz7 function: Xfz7 morpholinos (Xfz7MO) and cytoplasmic domain–deleted DN form of Xfz7 (DN-Xfz7). Both Xfz7MO and DN-Xfz7 had been successfully used previously to interfere with Xfz7 function in *X. laevis* embryos (Sumanas and Ekker, 2001; Sumanas et al., 2000). Xfz7MO- or DN-Xfz7–injected embryos developed severe gastrulation defects, and failed to form a normal axis at the neurula stage (Fig. 7 A), indicating that both the morpholinos and the DN construct effectively interfered with Xfz7 function. However, coinjection of Xfz7MO or DN-Xfz7 mRNA with PAPC mRNA into embryos had no affect on the ability of PAPC to decrease C-cadherin adhesion activity compared with COMO coinjection (Fig. 7 B). Moreover, Xfz7MO or DN-Xfz7 coinjection did not block PAPC-mediated cell sorting (Fig. 7 C). Thus, interference with Xfz7 function does not affect

Figure 6. **Requirement for PAPC in the activin-induced regulation of C-cadherin adhesion activity and animal cap morphogenesis.** (A) Activin induces PAPC protein expression in animal cap explants. Stage 8 animal caps were treated with 5 ng/ml activin for 0, 30, 60, 120, and 180 min and immediately processed for anti-PAPC Western blot analysis. (B) PAPC morpholinos (PAPCMO) suppress activin-induced PAPC expression and endogenous PAPC expression. Lanes 1–4: stage 9 animal caps analyzed by anti-PAPC Western blotting. 80 ng COMO or PAPCMO were injected into the animal hemisphere at the 2–4–cell stage, and animal caps were excised, dissociated, and treated with or without 5 ng/ml activin for 1.5 h. Lanes 5–6: whole embryos analyzed by anti-PAPC Western blotting. 80 ng of COMO or PAPCMO were injected into the dorsal side at the 4-cell stage, and incubated to stage 12. The level of PAPC was normalized to the level of α-tubulin. (C) PAPCMO block activin-induced down-regulation of C-cadherin activity. Blastomere adhesion assays on 4 μg/ml C-cad-EC.Fc–coated substrates were performed with the blastomeres described in B (lanes 1–4). In addition, blastomeres from embryos injected with PAPCMO plus morpholino-resistant FL-PAPC mRNA or with FL-PAPC mRNA alone were also assayed in parallel. **, $P < 0.01$ compared with COMO-injected, untreated blastomeres. Error bars are the SEM. (D) PAPCMO blocked activin-induced animal cap elongation. 80 ng of COMO or PAPCMO were injected into the animal hemisphere of 2–4 cell stage embryos. (1) The control COMO-injected caps fully elongated (30/30). The PAPCMO-injected caps were divided into two groups according to their phenotypes: (2a) partial elongation (12/30) and (2b) no elongation (18/30). Five caps from each of the groups were processed for Western blot analyses with anti-PAPC and anti–α-tubulin antibodies.



PAPC-mediated down-regulation of C-cadherin adhesion. Furthermore, we did not observe any change of C-cadherin adhesion activity because of Xfz7 expression or the sorting out of Xfz7-expressing cells (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200602062/DC1). Therefore, PAPC regulates C-cadherin adhesion activity and cell sorting independent of Xfz7 signaling.

Discussion

PAPC does not function by directly mediating cell–cell adhesion in *X. laevis* embryos

Previous studies on protocadherins, including *X. laevis* PAPC, have either assumed or suggested that they function by mediating cell–cell adhesion (Nollet et al., 2000; Suzuki, 2000). This notion has been based primarily on the presence of cadherin EC domains, but in some cases also based on limited evidence for adhesive function. In the case of PAPC, the evidence for adhesion was that it caused cell sorting out, which is a common consequence of adhesion molecule function (Kim et al., 1998). To our surprise, we found no evidence that PAPC functions as a bona fide homophilic cell–cell adhesion molecule. First, cells that express PAPC at their surfaces, either in tissue culture or from *X. laevis* embryos, exhibit no detectable adhesion to purified PAPC proteins. Second, there is no detectable aggregation of PAPC-expressing cells, indicating no adhesive interactions between PAPC molecules, even when both are presented on the surface of living cells. Third, PAPC- expressing blastomeres exhibit less aggregation activity than non–PAPC-expressing blastomeres and sort to the outside of coaggregates with non–PAPC-expressing blastomeres, suggesting a decrease, rather than an increase, in the cell-adhesive strength of PAPC-expressing cells. Furthermore, overexpression of C-cadherin counteracts, rather than reinforces, PAPC-mediated cell sorting, consistent with the notion that PAPC does not mediate cell adhesion. Although we cannot exclude the possibility that PAPC has weak homophilic binding activity undetectable in our adhesion assays or that PAPC mediates adhesion in some cell systems other than the ones we tested, it is clear that its cell sorting activity in the *X. laevis* embryo is not mediated by PAPC-mediated increase in cell–cell adhesion.

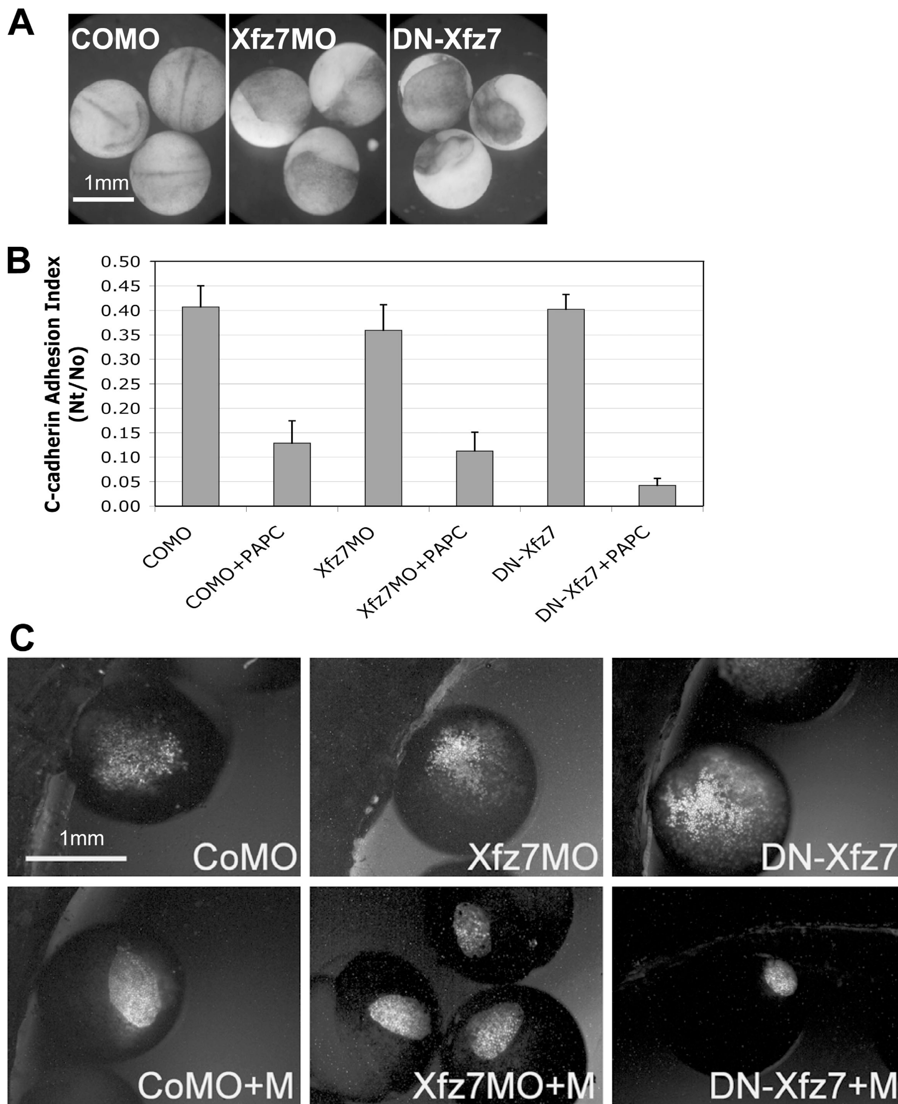


Figure 7. **PAPC-regulation of C-cadherin adhesion and cell sorting is independent of Frizzled-7.** (A) Frizzled-7 morpholinos (Xfz7MO) and DN Frizzled-7 (DN-Xfz7) cause sever gastrulation defects. 40 ng of control morpholino (COMO), 40 ng of Xfz7MO, or 2.4 ng of DN-Xfz7 mRNA was injected into 2-cell stage embryos, which were allowed to develop to stage 20. (B) PAPC down-regulates C-cadherin activity even in the presence of Xfz7-MO or DN-Xfz7. Embryos were injected as in A. At the 4-cell stage, half of the injected embryos were further injected with 1.2 ng of FL-PAPC mRNA. Blastomere adhesion assays were performed on 4 μg/ml C-cad-EC.Fc–coated substrates. Error bars are the SEM. (C) Xfz7-MO and DN-Xfz7 have no affects on M-PAPC–induced cell sorting. (top row) Cell dispersal assays with 10 ng of COMO, 10 ng of Xfz7MO, or 500 pg of DN-Xfz7mRNA (all with NLS-GFP mRNA as tracer) injected into one blastomere of 32-cell stage embryos. (bottom row) Cell dispersal assays with 40 ng of COMO, 40 ng of Xfz7MO, or 4 ng of DN-Xfz7mRNA injected into 2-cell stage embryos, followed by injection of 500 pg of M-PAPC mRNA plus 200 pg of NLS-GFP mRNA (as tracer) into one blastomere at the 32-cell stage.

PAPC down-regulates C-cadherin adhesion activity

A key finding of this study is that PAPC down-regulates C-cadherin adhesion activity to cause cell sorting and contribute to morphogenetic movements. PAPC expression causes a significant decrease in blastomere adhesion to purified C-cadherin protein (Fig. 3). In addition, PAPC-induced cell sorting in embryos is reversed by coexpression of C-cadherin, consistent with the view that decreased C-cadherin adhesion in PAPC-expressing cells is the cause of cell sorting (Fig. 4 A). Furthermore, PAPC-induced gastrulation defects in embryos phenocopies the adhesion defect caused by DN C-cadherin (Lee and Gumbiner, 1995), and overexpression of C-cadherin rescues PAPC-induced gastrulation defects (Fig. 4 B). More importantly, knocking-down of endogenous PAPC results in increased C-cadherin adhesion activity in corresponding tissue, and loss-of-PAPC-function defects can be rescued by decreasing C-cadherin–mediated adhesion using a DN C-cadherin construct (Fig. 5).

The affect of PAPC on blastomere adhesion is specific to C-cadherin and not caused by an overall interference with the capacity for cell adhesion. Neither the integrin-mediated adhesion to fibronectin nor attachment via IL2Rα to anti-IL2Rα antibodies is affected by PAPC expression. Most important is the finding that a specific anti–C-cadherin–activating mAb can reverse the affects of PAPC on C-cadherin adhesion, demonstrating that the adhesive change is intrinsic to the C-cadherin protein. Therefore, PAPC specifically down-regulates C-cadherin adhesion activity, resulting in changes in cell sorting behavior.

We find that both M-PAPC, the cytoplasmic domain deletion mutant of PAPC, and FL-PAPC have the same activity in cell sorting and regulation of adhesion. This differs from a previous study in which M-PAPC was found to have higher cell-sorting activity than wild type FL-PAPC (Kim et al., 1998). Our results using anti-PAPC antibodies indicate that this was caused by differences in the levels of PAPC protein expression. Removal of the 3′UTR from the FL-PAPC mRNA resulted in higher protein expression and similar sorting and adhesion regulation activity as M-PAPC. Therefore, the cytoplasmic domain of PAPC is not required for its function in regulation of adhesion and induction of cell sorting.

The cytoplasmic domain of PAPC is probably involved in other signaling functions of PAPC. Recent studies found that FL-PAPC interacts and cooperates with *X. laevis* Frizzled-7 (Xfz7) and activates RhoA and JNK in regulation of tissue separation, as well as convergent extension movements (Medina et al., 2004; Unterseher et al., 2004). The cytoplasmic domain appears to be required because M-PAPC cannot induce tissue separation together with Xfz7 (Medina et al., 2004). Furthermore, an earlier study has found that FL-PAPC, but not M-PAPC, promotes elongation of animal cap explants that are treated with low activin, suggesting a requirement for the cytoplasmic domain in induction of morphogenetic movements (Kim et al., 1998). The cytoplasmic domain of PAPC contains a region of 25 amino acid residues (aa 816–840) that is highly conserved across species and present in other protocadherins. This region might be important for mediating interactions with unknown cytoplasmic factors involved in Xfz7-mediated signal transduction events.

Although Xfz7 can mediate signaling events induced by PAPC (Medina et al., 2004; Unterseher et al., 2004), it does not appear to be involved in PAPC regulation of C-cadherin adhesion activity. The regulation of C-cadherin by PAPC does not require the cytoplasmic domain, in contrast to Xfz7-mediated PAPC-control of tissue separation and convergent extension movements (Fig. 8 A). Moreover, interference with Xfz7 expression or function has no affect on the ability of PAPC to down-regulate C-cadherin adhesion activity or to induce cell sorting (Fig. 7). Furthermore, overexpression of Xfz7 does not affect C-cadherin–mediated adhesion nor induce cell-sorting behavior in embryos (Fig. S5). Therefore, PAPC regulates C-cadherin adhesion activity and cell sorting independent of Xfz7.

The molecular mechanism by which PAPC down-regulates C-cadherin activity is not yet understood. One possibility is that PAPC could interact with C-cadherin directly and influence its adhesive conformation and activity, but in preliminary experiments, we have not yet observed significant amounts of C-cadherin coimmunoprecipitated with PAPC from detergent lysates of *X. laevis* embryos or PAPC/C-cadherin–expressing CHO cells (unpublished data). Moreover, stable expression of PAPC in C-cadherin–expressing CHO cells does not appear to significantly change C-cadherin adhesion activity, suggesting that a more complicated mechanism is involved in regulation in *X. laevis* embryo blastomeres. For example, it is possible that PAPC interacts with another membrane protein that either links PAPC to C-cadherin or transduces a signal from PAPC to regulate C-cadherin. If we are able to identify such a membrane protein in future studies, it will be interesting to determine whether it can reconstitute PAPC regulation of C-cadherin in CHO cells, as it does in *X. laevis* blastomeres.

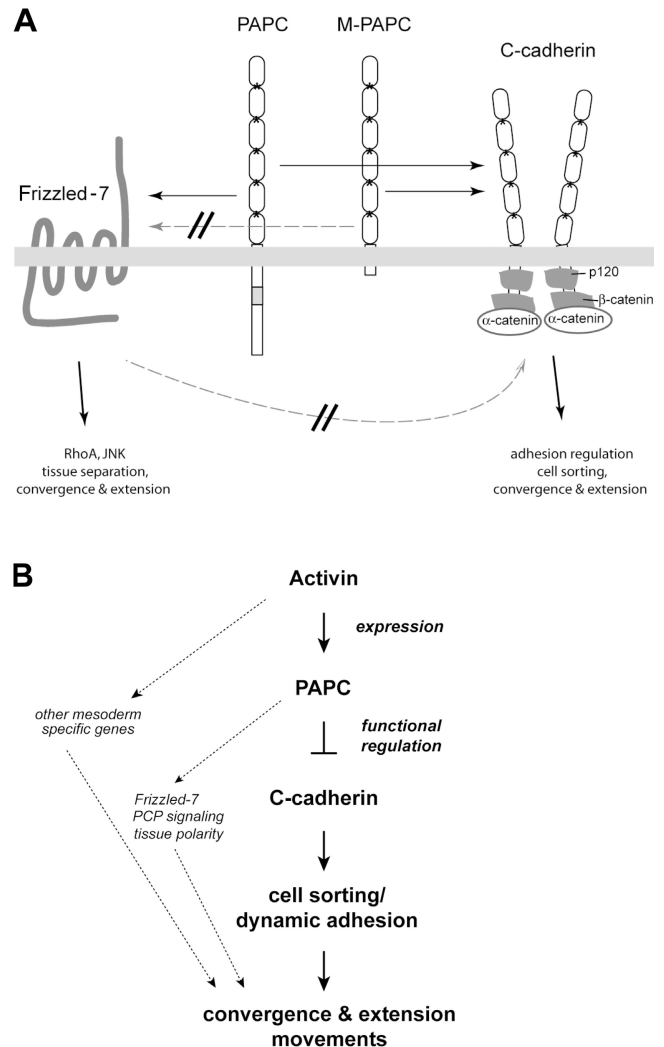


Figure 8. **Models for the role of PAPC in activin-induced regulation of C-cadherin adhesion and tissue morphogenesis.** (A) Relationship between PAPC, Frizzled-7 signaling, and regulation of C-cadherin–mediated adhesion. The membrane-bound PAPC extracellular domain, as well as wild-type PAPC, down-regulates C-cadherin adhesion activity either directly or indirectly, and the regulation of C-cadherin adhesion activity contributes to convergence and extension cell movements. PAPC also interacts with Xfz7 and participates in the activation of RhoA and JNK by Xfz7-mediated signaling to affect tissue separation and convergent extension. Full-length PAPC is required for Xfz7-mediated tissue separation. (B) A signaling cascade that mediates the activin-induced tissue morphogenesis. The findings from this study are shown in bold. Additional signaling steps shown in other studies to be important for activin-induced morphogenesis are indicated by dotted arrows.

PAPC mediates activin-induced down-regulation of C-cadherin adhesion activity and convergent extension movements

Activin is a TGFβ family member that induces mesodermal gene expression in *X. laevis* embryos. Activin treatment triggers *X. laevis* animal cap explants to elongate, a process mimicking convergence and extension movements during gastrulation. Little is known about the mechanism of activin-induced animal cap elongation. Activin has been reported to decrease C-cadherin adhesion activity in animal cap explants (Brieher and Gumbiner, 1994), and reversing this down-regulation with a C-cadherin–activating antibody blocks activin-induced animal cap elongation (Zhong et al., 1999). In the present study, we demonstrate that activin induces PAPC expression and that PAPC expression is necessary for activin-regulation of C-cadherin adhesion activity, as well as activin induction of animal cap elongation. PAPC has also been reported to be required for animal cap elongation induced by another TGFβ family growth factor, BVg1 (Medina et al., 2004).

However, PAPC expression alone is not sufficient to induce animal cap elongation (unpublished data). These results suggest that additional signals resulting from activin induction are required to elicit morphogenetic cell movements.

We propose a model for how activin or other TGFβ family members present in the embryo regulate cell adhesion and induce morphogenesis (Fig. 8 B). Activin induces PAPC expression and PAPC down-regulates C-cadherin adhesion activity. Dynamic regulation of C-cadherin–mediated cell–cell adhesion is required for convergence and extension cell movements (Zhong et al., 1999). PAPC probably also contributes to morphogenesis via Frizzled-7–mediated planar cell polarity pathway, perhaps via cell polarization. Because PAPC expression alone is not sufficient to induce animal cap elongation, activin probably induces expression of additional factors that participate in the generation of convergent extension movements.

Do most protocadherins function as adhesion molecules?

Protocadherins represent a huge subfamily of molecules in the cadherin superfamily in vertebrates, and have been implicated in several biological processes, especially in the nervous system (Nollet et al., 2000; Suzuki, 2000; Yagi and Takeichi, 2000). Protocadherins have neither the known conserved cadherin interfaces for homophilic adhesion, including the Trp2 residue, that mediate adhesion of classical cadherins nor do they have the catenin-binding motifs required for cytoskeletal interactions in their cytoplasmic domains (Nollet et al., 2000).

To date, the protocadherins that have been studied either exhibit no adhesion activity or have been suggested to mediate weak adhesion based on limited evidence (Sano et al., 1993; Sago et al., 1995; Yoshida et al., 1998; Hirano et al., 1999; Yoshida, 2003). Whether the weak interaction between some of these protocadherins represents bona fide cell–cell adhesion or is actually involved in other functions such as signal transduction remains unclear. The protocadherin α proteins (Pcdhα) bind the secreted protein reelin and mediate reelin signaling via the nonreceptor tyrosine kinase Fyn that binds to their cytoplasmic domains (Kohmura et al., 1998). Therefore, protocadherins can function as receptors for extracellular ligands that mediate signal transduction into the cell.

An important finding of our study is that a protocadherin can modify cell adhesion by regulating the adhesion activity of a classical cadherin. This could be a potential general mechanism for how protocadherins affect cell adhesion. In fact, Angst et al. speculate that Pcdhα may regulate N-cadherin function in neurons (for review see Angst et al., 2001), but it would be interesting to test whether Pcdhα regulates N-cadherin–mediated adhesion. Two other *X. laevis* protocadherins, neural fold protocadherin and axial protocadherin, also induce cell sorting like PAPC (Bradley et al., 1998; Kim et al., 1998; Kuroda et al., 2002), but their intrinsic adhesion activities have not been directly tested. One possibility is that they, like PAPC, regulate adhesion activities of other adhesion molecules. To determine whether any of these protocadherins function as bona fide adhesion molecules, direct careful examination of their adhesive functions will be required.

Materials and methods

Constructs

The plasmids pCS2+/FL-PAPC, pCS2+/M-PAPC, and pCS2+/DN-PAPC were provided by E. DeRobertis (University of California, Los Angeles, Los Angeles, CA; Kim et al., 1998). The 3′- and 5′- UTR of the FL-PAPC cDNA were removed to generate pCS2+/FL(-5′ and -3′) for FL-PAPC mRNA production. For eukaryotic expression, the FL-PAPC coding sequence was amplified by PCR and inserted into the NheI–XhoI site of pCDNA6-V5-His/A vector (Invitrogen), and the M-PAPC coding sequence excised from pCS2+/M-PAPC was inserted into the EcoRI–NotI site of pCDNA-V5-His/A. The coding sequence for the membrane-bound cytoplasmic domain of PAPC (TMC; aa 680–979) was amplified by PCR and inserted to the XhoI–XbaI site of the pCS2+/DN-PAPC to replace the DN-PAPC coding sequence for expression.

For production of recombinant soluble PAPC proteins, PAPC extracellular domain (PAPC-EC; aa 1–685) was amplified by PCR and inserted into the HindIII–XbaI site of the pEE14-Fc vector described previously (Brieher et al., 1996), resulting in a soluble PAPC protein with a C-terminal human IgG Fc fusion. To prepare 6×His-tagged PAPC-EC construct, PAPC-EC was first cloned into the NheI–XbaI site of pCDNA6-V5-His/A vector. The whole PAPC-EC coding sequence plus the V5-His tag sequence was then excised with NheI and PmeI and inserted into the XbaI–SmaI site of pEE14 vector.

pCS2+/NLS-GFP encodes a nucleus-localized GFP and was a gift from L. Davidson (University of Pittsburgh, Pittsburgh, PA). pT3TS/Xfz7 encodes the full-length Xfz7 and pT3TS/DN-Xfz7 encodes a DN-Xfz7 that lacks the cytoplasmic domain. Both were gifts from M. Marsden (University of Waterloo, Ontario, Canada) and were originally constructed by S. Sumanas (University of Minnesota, Minneapolis, MN; Sumanas et al., 2000). pSP64T/C-cad and pSP36T/Ctail (Lee and Gumbiner, 1995) were used to make C-cadherin mRNA and C-cadherin cytoplasmic tail RNA (DN form), respectively.

All constructs were confirmed by DNA sequencing.

Transfections

All transfections were performed with Lipofectamine or Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction. For stable transfections, cells cotransfected with pCS2+ constructs and pCDNA3 (containing G418-resistant gene) were selected against 0.8 mg/ml G418; cells transfected with pEE14 constructs were selected against 25 μM methionine sulfoximine; cells transfected with pCDNA6 constructs were selected against 5 μg/ml blasticidine.

Protein purification and antibodies

Recombinant C-cad-EC.Fc, C-cad-EC.His, and human E-cad-EC.Fc were purified from conditioned media, as previously described (Brieher et al., 1996; Niessen and Gumbiner, 2002). PAPC-EC.Fc and PAPC-EC.His were similarly purified. After initial purification by protein A or Ni-NTA affinity chromatography, all proteins were further purified on a HiTrap Q ion-exchange column (Invitrogen). Anti-PAPC mAbs, 11A6 and 28F12, were generated against purified PAPC-EC at the Monoclonal Antibody and Hybridoma Facility at Memorial Sloan-Kettering Cancer Center. Anti–C-cadherin mAbs, 6B6, and the activating antibody AA5 have been previously described (Brieher and Gumbiner, 1994; Zhong et al., 1999).

Trypsinization and biotinylation assays

Cells were mock treated or treated with 100 μg/ml trypsin and 2 mM EDTA at 4°C for 20 min. Digestion was terminated by washing 3× with PBS containing 2 mg/ml soybean trypsin inhibitor, and lysed directly in SDS-PAGE sample buffer. The trypsinization of blastomeres was carried out as previously described (Brieher and Gumbiner, 1994). Cell surface biotinylation was performed with Sulfo-NHS-ss-Biotin (Pierce Chemical Co.) following the manufacturer's instruction. Biotinylated cells were lysed in PBS/1% NP-40/protease inhibitors (Roche), and biotinylated proteins were pulled down with streptavidin beads.

Cell adhesion assays

The cell aggregation assay was previously described (Nose et al., 1988). The cell attachment flow assay was performed as previously described (Chappuis-Flament et al., 2001; Niessen and Gumbiner, 2002), with the following minor modifications: glass capillary tubes were first coated with 5 mg/ml purified goat anti–human IgG (Fc specific; Jackson ImmunoResearch Laboratories, Inc.) before loading of the Fc-fused adhesion substrates (0.1 mg/ml). Blastomere aggregation assays were performed as

previously described (Zhong et al., 1999). Blastomere adhesion assays were performed as previously described (Niessen and Gumbiner, 2002), with the following modifications: (a) 15 μl of 0.1 mg/ml purified PAPC-EC.Fc, 0.1 mg/ml PAPC-EC.His, 3–10 μg/ml C-cad-EC.Fc, 50 μg/ml fibronectin, or 0.5 mg/ml anti-IL2R mAb BB10 was used for substrate coating, and 1% BSA was used for substrate blocking; and (b) *X. laevis* animal caps (at least five) were excised at stage 9 to obtain blastomeres. As needed, the dissociated blastomeres were treated with 5 ng/ml activin for 1 h and/or with 1 μg/ml AA5 F*ab* for 30 min. The adhesion strength of blastomeres was measured by the ratio of the number of blastomeres remaining attached after shaking (Nt) versus the number before shaking (No). At least four independent experiments were performed for each sample. The SEM was plotted as error bars.

In vitro transcription and morpholinos

Capped mRNAs were synthesized using the Riboprobe in vitro transcription systems (Promega). Two PAPC morpholinos (Medina et al., 2004) and two Xfz7 morpholinos (Sumanas and Ekker, 2001) have been described, which were ordered from Gene Tools, LLC. In each case, a 1:1 mix of the two morpholinos was used for injection. The same amount of standard control morpholino (Gene Tools, LLC) was injected to control embryos.

***X. laevis* embryo manipulations**

All experimental protocols that involved the use of *X. laevis* were approved by the Animal Care and Use Committee at the University of Virginia. *X. laevis* eggs and embryos were obtained and handled by standard techniques (Newport and Kirschner, 1982). Standard Nieuwkoop staging of embryos was used (Nieuwkoop and Faber, 1967). Microinjection of mRNAs or morpholinos was performed at the 2–4–cell stage, as previously described (Lee and Gumbiner, 1995). Typically, 1–2 ng mRNA or 40–80 ng of morpholinos were injected into the animal pole of the embryos. For animal cap elongation assays, animal caps were excised at stage 8, treated with 5 ng/ml activin for 75 min in 1× Modified Barth's Saline (Gurdon, 1977), rinsed, and further incubated in 1× Modified Barth's Saline at 16°C overnight.

Blastomere reaggregation assay and dispersal assay

Both assays were performed as previously described (Kim et al., 1998). NLS-GFP mRNA (200–400 pg/embryo) was coinjected with PAPC mRNA as a lineage tracer. In brief, for reaggregation assays, RNAs (with tracer) were injected into 4-cell stage embryos. At stage 9, animal caps were excised and cap-blastomeres dissociated from injected and uninjected embryos were mixed at a 1:2 ratio and rocked overnight in Ca^{2+}-containing media. The aggregates, with or without bisection, were examined under fluorescence microscope. For dispersal assays, sample mRNA, together with tracer, was injected into one blastomere at the animal hemisphere of 32-cell stage embryos. After stage 13, the injected embryos were observed under fluorescence microscope for distribution of GFP-labeled blastomeres.

Image acquisition

All images were acquired at room temperature. Light images of embryos were acquired with a digital camera (model G6; Canon; at ∼4× optical zoom) mounted on a dissecting microscope (Stemi SVII; Carl Zeiss MicroImaging, Inc.) with lens magnification set between 0.8 and 1.6×. Fluorescence images of embryos were acquired with a color LCD camera (SPOT Insight; Diagnostic Instruments) mounted on an inverted microscope (Diaphot; Nikon) with an objective lens (Plan 4; Nikon) and a Fitz filter. Immunofluorescence microscopy was performed on an Axioplan2 microscope with a Neoplan 20× objective lens and Cys3 filter (all from Carl Zeiss MicroImaging, Inc.). Images were acquired with a digital cameral (model C4742-95; Hamamatsu) and with Openlab 4.0 (Improvision) software.

Online supplemental material

Fig. S1 shows surface expression of PAPC in CHO cells. Fig. S2 shows the purity of PAPC adhesion substrates. Fig. S3 shows that the 3′UTR of FL-PAPC inhibits its protein expression. Fig. S4 shows the membrane-bound cytoplasmic domain of PAPC does not induce cell sorting or affect M-PAPC–induced cell sorting. Fig. S5 shows that Xfz7 expression does not decrease C-cadherin–mediated adhesion or induce cell sorting. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200602062/DC1.

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