RESEARCH COMMUNICATION

***Xenopus* Paraxial Protocadherin regulates morphogenesis by antagonizing Sprouty**

Yingqun Wang,^{1,4} Patricia Janicki,¹
Isabelle Köster,¹ Corinna D. Berger,¹
Christian Wenzl,² Jörg Großhans,²
and Herbert Steinbeisser^{1,3}

¹Institute of Human Genetics, University Heidelberg, 69120 Heidelberg, Germany; ²Center for Molecular Biology (ZMBH), University Heidelberg, 69120 Heidelberg, Germany

***Xenopus* Paraxial Protocadherin (xPAPC) has signaling functions that are essential for convergent extension (CE) movements and tissue separation during gastrulation. PAPC modulates components of the planar cell polarity (PCP) pathway, but it is not clear how PAPC is connected to β -catenin-independent Wnt-signaling. By yeast two-hybrid screen, we found that the intracellular domain of PAPC interacts with Sprouty (Spry), an inhibitor of CE movements. Upon binding to PAPC, Spry function is inhibited and PCP signaling is enhanced. Our data indicate that PAPC promotes gastrulation movements by sequestration of Spry and reveal a novel mechanism by which protocadherins modulate β -catenin-independent Wnt-signaling.**

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Paraxial Protocadherin (PAPC) was identified in *Xenopus* as a gene expressed in the Spemann organizer (Kim et al. 1998). Expression of xPAPC in the gastrula embryo is regulated by nodal, Wnt/ β -catenin and Wnt-5a signaling (Wessely et al. 2004; Schambony and Wedlich 2007). PAPC modulates in a yet unknown manner C-cadherin-mediated cell adhesion through its extracellular domain and thereby promotes cell sorting (Chen and Gumbiner 2006). The intracellular domain of PAPC exerts signaling functions and is implicated in the regulation of convergent extension (CE) movements and separation behavior of the involuting mesoderm and the neuroectoderm (Kim et al. 1998; Medina et al. 2004; Unterseher et al. 2004). *Zebrafish* PAPC (zPAPC) gene is regulated by the transcription factor spadetail and zPAPC function is required for CE movements in the fish gastrula (Yamamoto et al. 1998). In *Xenopus*, the regulation of CE movements and tissue separation by PAPC depends on

its ability to modulate the activity of Rho GTPase and c-jun N-terminal kinase, which are effectors of the planar cell polarity (PCP) pathway (Medina et al. 2004; Unterseher et al. 2004). However, the mechanism by which PAPC modulates PCP signaling remains unclear. Recently it was reported that *Xenopus* Sprouty 1 and 2 proteins act as inhibitors of the PCP pathway and are part of the morphogenetic machinery that regulates gastrulation (Sivak et al. 2005). In this study, we provide evidence that PAPC interacts with Sprouty and antagonizes its inhibitory effects on PCP, therefore providing novel insight into the link between protocadherin and PCP signaling.

Results and Discussion

In an effort to identify potential proteins involved in signaling downstream from xPAPC, we performed a yeast two-hybrid screen using the cytoplasmic domain of xPAPC (xPAPCc) as bait. Independent clones (3.5×10^6) of *Xenopus laevis* oocyte cDNA library were screened (Fig. 1A). Among the positive clones isolated was xSprouty1 (xSpry1). Sprouty is an inhibitor of receptor tyrosine kinase (RTK) signaling (Mason et al. 2006). In *Drosophila*, Sprouty (dSpry) inhibits FGF-mediated activation of mitogen-activated protein kinase (MAPK) in the larval trachea (Hacohen et al. 1998). *Xenopus* xSpry1 and xSpry2, in contrast, do not inhibit MAPK-mediated transcription of FGF-target genes and do not interfere with mesoderm specification but instead block morphogenetic movements by interfering with PCP pathway (Nutt et al. 2001; Sivak et al. 2005).

dSpry as well as xSpry1 and xSpry2 interacted with xPAPCc (Fig. 1B). In contrast, xSpred1, a protein related to xSpry1, failed to do so (Fig. 1B). Interestingly, Spred proteins inhibit MAPK signaling in *Xenopus* embryos but do not interfere with morphogenetic processes regulated by PCP pathway (Sivak et al. 2005). Mutations of putative phosphorylation sites in the xPAPCc peptide, which were identified by the Scansite computer program as putative 14–3–3-binding sites, weakened (S741A) or abolished (S955A) the interaction with xSpry1 (Fig. 1A,B). Amino acid exchanges that mimic phosphorylation did not impair xSpry1 binding (S741E, S955E) (Fig. 1B). We have evidence that xPAPCc is phosphorylated in embryos and that the phosphorylation is reduced when S741 and S955 residues are mutated (Supplemental Fig. S2H).

Next, we set out to confirm the interaction of xPAPCc and xSpry1 in vivo by coimmunoprecipitation (co-IP) assays. Synthetic mRNAs for Myc-xSpry1, Flag-xPAPCc, and for the Flag-tagged intracellular domain of *Xenopus* Axial Protocadherin (Flag-xAXPCc) were injected into four-cell stage *Xenopus* embryos. Myc-xSpry1 coimmunoprecipitated with Flag-xPAPCc but not with Flag-xAXPCc using Flag antibody (Fig. 1C). Similarly, Myc-xSpry1 was not coimmunoprecipitated with Flag-xPAPCc-S741A/S955A (Flag-xPAPCc^{mut}) harboring point mutations (Fig. 1D). In the reciprocal experiment, Myc antibody specifically coimmunoprecipitated Myc-xSpry1 and Flag-xPAPCc but not Flag-xAXPCc or Flag-xPAPCc^{mut} (Fig. 1E; data not shown). These experiments corroborated the data obtained in the yeast two-

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Corresponding authors.

³E-MAIL Herbert.Steinbeisser@med.uni-heidelberg.de; FAX 6221-565153

⁴E-MAIL Yingqun.Wang@med.uni-heidelberg.de; FAX 6221-568884.

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antagonizing xSpry1. xPAPC expression is induced in animal caps exposed to the TGF- β growth factor activin (Hukriede et al. 2003; Chen and Gumbiner 2006), and such explants elongate due to PCP-dependent CE movements (Kim et al. 1998; Chen and Gumbiner 2006). Activin-induced elongation (95%, $n = 20$) (Fig. 3A) was inhibited in 80% of the explants ($n = 20$) by the expression of high amounts of xSpry1 (100 pg of RNA per embryo). Injection of 30 pg of xSpry1 mRNA or 20 ng of xPAPC antisense morpholino oligonucleotides (MO), which partially blocks translation of the endogenous PAPC mRNA under these conditions (Medina et al. 2004), did not inhibit elongation of the animal cap explants (90% elongated, $n = 20$). When 20 ng of xPAPC MO were injected together with 30 pg of xSpry1 mRNA, 90% of the animal caps failed to elongate ($n = 20$). This indicated that reduction of endogenous xPAPC activity enhanced the inhibitory effect of xSpry1 on CE movements. The partial knockdown of xPAPC sensitized the animal caps for the inhibition by xSpry1, supporting the idea of an antagonism between xPAPC and xSpry1 (Supplemental Table S1). This antagonism could be confirmed experimentally in DMZ tissue. Explanted DMZs, so-called Keller explants (Keller 2002), express both PAPC and xSpry1 and display CE movements. Elongation was inhibited by overexpression of xSpry1 but partially rescued by coexpression of xPAPC or xPAPCc (Fig. 3B). In contrast, xPAPCmut and

xPAPCmut, which did not interact with xSpry1 in yeast and in co-IP experiments and did not induce membrane recruitment of GFP-xSpry1, failed to rescue elongation in DMZs injected with xSpry1 (Fig. 3B). Overexpression of xPAPC or xPAPCmut inhibited DMZ elongation only weakly, very likely due to the modulation of cadherin-mediated adhesion (Chen and Gumbiner 2006). xPAPCc and xPAPCmut had no adverse effect on DMZ elongation. Altogether, these results demonstrate that xPAPC and xSpry1 have antagonistic activities that contribute to the regulation of CE movements. These data also support the view that the inhibition of xSpry1 function by xPAPC is achieved by binding of its intracellular domain to xSpry1.

Hallmarks of active PCP signaling are the membrane localization of Dishevelled (Dsh) (Wallingford et al. 2000) and protein kinase C δ (PKC δ) (Kinoshita et al. 2003). We therefore examined whether the localization of these PCP components could be modulated by the antagonism between xPAPC and xSpry1. GFP-Dsh and GFP-PKC δ were expressed in *Xenopus* animal cap cells, and GFP localization was analyzed by confocal microscopy and quantified (Fig. 4A,B; Supplemental Fig. S4). Frizzled-7 (Fz7) but not xPAPC-induced membrane localization of GFP-Dsh and GFP-PKC δ . This is consistent with the yeast two-hybrid assay showing that xPAPCc did not bind Dsh (Fig. 1B). The recruitment of Dsh and PKC δ to the membrane by Fz7 was inhibited by xSpry1.

xPAPC and xPAPCc but not M-PAPC or xPAPCmut rescued the recruitment of Dsh and PKC δ (Fig. 4A,B). Since previous studies have shown that xPAPC can activate RhoA, a downstream effector of the PCP pathway (Medina et al. 2004; Unterseher et al. 2004), we further characterized the antagonism between xPAPC and xSpry1 on the level of RhoA activation. Overexpression of xSpry1 in *Xenopus* embryos inhibited RhoA activation. xPAPC but not xPAPCmut antagonized the xSpry1 effect and rescued RhoA activation (Fig. 4C). Knockdown of PAPC function by xPAPC MO inhibited RhoA activation (Medina et al. 2004; Unterseher et al. 2004). Importantly, xPAPC but not xPAPCmut could rescue RhoA activation in PAPC-depleted embryos (Fig. 4D). Taken together, these results suggest that xPAPC and xSpry1 act antagonistically in PCP pathway downstream from Fz7 and upstream of PKC δ , Dsh, and RhoA. The serine residues 741 and 955 are essential for both xSpry1 binding and Rho activation.

In *Xenopus*, PAPC is able to antagonize xSpry1 activity, and the intracellular domain of xPAPC but not xPAPCmut is able to bind *Drosophila* sprouty (Fig. 1B; data not shown). Therefore, we used the *Drosophila* larva as a genetic test system for the inhibition of dSpry by xPAPC. *sprouty* mutants in *Drosophila* larvae display enhanced branching of the tracheae and an increased number of terminal cells due to elevated FGF signaling (Hacohen et al. 1998). Therefore, we tested whether xPAPC is able to antagonize Spry in *Drosophila* larvae, which should result in a higher number of terminal cells. Transgenic fly lines were generated that allow inducible expres-

Figure 3. Antagonistic functions of xPAPC and xSpry1 in CE movements. (A) Schematic outline of the animal cap experiment. Synthetic mRNAs were injected into four blastomeres of four-cell-stage embryos. At stage 8.5, animal caps were explanted, exposed to activin for 3 h, and cultured to stage 20. Activin-treated animal caps from control embryos or embryos injected with 100 pg or 30 pg of xSpry1, 20 ng of xPAPC MO, or 30 pg of xSpry1 and 20 ng of xPAPC MO. (B) Modulation of CE movements in DMZ explants. mRNAs for xSpry1, xPAPC, xPAPCc, xPAPCmut, and xPAPCcmut (400 pg each) were injected into dorsal blastomeres at the four-cell stage. At stage 10+, DMZs were explanted and the elongation was scored at stage 20. DMZs were grouped in fully elongated (green), partially elongated (blue), and not elongated (red).

Figure 4. xPAPC stimulates membrane localization of PCP components Dsh and PKC δ and activates RhoA by antagonizing xSpry1. (A,B) mRNAs were injected at the four-cell stage, caps were excised at stage 9, and the localization of Dsh-GFP (A) or PKC δ -GFP (B) was analyzed by confocal microscopy. The amounts of mRNA injected were 300 pg for Dsh-GFP or PKC δ -GFP, 100 pg for Fz7, 1 ng for xSpry1, and 800 pg for xPAPC, M-PAPC, xPAPCc, or xPAPCmut. (C,D) Myc-tagged xRhoA DNA was injected alone or in combination with xSpry1, xPAPC, or xPAPCmut mRNA or xPAPC MO. Activated RhoA was precipitated by GST-RBD and detected on a Western blot with Myc antibody. Whole-embryo lysate was used as loading control.

sion of xPAPC (UAS-PAPC), M-PAPC (UAS-M-PAPC), or xAXPC (UAS-AXPC). Following expression of the protocadherin constructs in the tracheae by breathless-Gal4 (btl-Gal4) (Shiga et al. 1996), the number of terminal cells labeled by GFP was determined for the different genotypes. Wild-type larvae contain two terminal tracheal cells per segment (Fig. 5F), but three terminal cells were seen in tracheae in which FGF signaling was elevated by expression of an activated allele of FGF receptor (UAS- λ -btl) (Fig. 5A; Lee et al. 1996). Expression of PAPC in wild-type larvae (*Spry*^{+/+}) caused a higher number of terminal cells. About 30% of larvae had three or four terminal cells in at least one segment (Fig. 5B; Supplemental Fig. S5). This phenotype was rarely seen for the other genotypes (expression of M-PAPC or AXPC) (Fig. 5C,D; Supplemental Fig. S5). To test whether the effect of PAPC is based on an antagonism of Spry, we reduced the gene dose of *spry* to 50%. The percentage of larvae with segments containing more than two terminal cells increased to >60% (Supplemental Fig. S5). Since no marked chromosomes were used in this cross we could not discriminate between wild-type and *Spry* heterozygous larvae. To quantify the genetic interaction of PAPC and Spry more precisely, we used a more stringent way of analysis and employed marked chromosomes for this cross. This allowed us to score the *spry*^{+/+} and *spry*^{+/−} larvae separately. We determined the percentage

of larval segments showing three or more terminal cells (120 segments counted = 15 larvae). In *spry* heterozygous larvae, we found that three to five terminal cells were present in 38% of all segments that expressed FL-PAPC (Fig. 5E). In contrast, 4%–8% of larval segments expressing M-PAPC and AXPC had more than two terminal cells (Fig. 5E). This experiment demonstrated that the higher number of terminal cells seen upon expression of PAPC was enhanced when in addition the gene dose of *spry* was reduced. It supports the idea that PAPC sequesters Spry and thereby antagonizes its function. This inhibition is specific because AXPC is unable to do so. The M-PAPC mutant lacking C terminus cannot antagonize Spry, indicating that the cytoplasmic domain of xPAPC is crucial for this function. Since there is no PAPC homolog in *Drosophila*, one can conclude from these experiments that the domain of dSpry protein that is essential for the interaction with PAPC is conserved between *Xenopus* and *Drosophila* for reasons other than PAPC interaction. The experiments in *Xenopus* and *Drosophila* also demonstrated that PAPC is able to antagonize the different functions of Spry. The inhibitory effects of Spry on PCP in *Xenopus* as well as on MAPK pathway in *Drosophila* could be counteracted by xPAPC.

In *Xenopus*, Spry inhibits the PCP pathway in the cytoplasm via an unknown mechanism. As a result, Dsh and PKC δ are not localized to the plasma membrane and RhoA is not activated. Recently, it was reported that *Xenopus* ANR5 protein, which can modulate gastrulation movements, interacts with xPAPC, corroborating the role of PAPC in intracellular signaling (Chung et al. 2007). Our results presented here show that gastrulation movements can be modulated by sequestration of the inhibitor Spry by the cytoplasmic domain of xPAPC. This mechanism provides a direct link between protocadherins and the PCP pathway and contributes a novel regulatory module to β -catenin-independent Wnt signaling.

Materials and methods

DNA constructs

For the yeast two-hybrid assay, the cytoplasmic domain of xPAPC (residues 715–979) was amplified by PCR and cloned into the LexA-based bait plasmid pNLX3 to generate pNLX3-xPAPCc. xSpry2, xSprd1, or xDsh was cloned into the Gal4-AD-based prey plasmid pACT2 to generate pACT2-xSpry2, pACT2-xSprd1, or pACT2-xDsh. For expression in *Xenopus*, the cytoplasmic domain of xPAPC or xAXPC (residues 838–1016) was cloned into pCS2 + Flag to generate Flag-xPAPCc or Flag-xAXPC. Mutant constructs pNLX3-xPAPCc-S741A, pNLX3-xPAPCc-S955A, pNLX3-xPAPCc-S741E, pNLX3-xPAPCc-S955E, Flag-xPAPCc-S741A/S955A (xPAPCcmut), and xPAPC-S741A/S955A (xPAPCmut) were generated by PCR site-directed mutagenesis. All of the engineered constructs were confirmed by sequencing. Myc-xSpry1 was kindly provided by E. Nishida (Hanafusa et al. 2002).

Xenopus embryo manipulation

xPAPC antisense MOs MoPAPC I and II (1:1), which target both xPAPC alleles, were injected (Medina et al. 2004). Capped mRNAs were synthesized from linearized plasmids using the mMessage mMachine Kit (Ambion). In vitro fertilization, embryo culture and staging, microinjections, and Keller explants were performed as described (Nieuwkoop and Faber 1975; Medina et al. 2004; Unterseher et al. 2004). For confocal microscopy, animal caps were excised at stage 9, fixed in 4% formaldehyde, and subjected to microscopic analysis using a Nikon C1Si spectral imaging confocal laser scanning system on a Nikon TE2000-E inverted microscope.

Figure 5. xPAPC antagonizes dSpry in the tracheae of *Drosophila* larvae. Btl-Gal4 flies were crossed with UAS- λ -btl (A), UAS full-length (FL) PAPC (B), UAS-M-PAPC (C), and UAS-AXPC (D). Morphology of the tracheae and number of terminal cells were analyzed in third instar larvae. (A–D) Bright-field microscopy. (A'–D') GFP fluorescence visualizing the trachea. (A''–D'') Overlay of GFP and bright-field. (E) Analysis of terminal cells in segments of larvae heterozygous for *spry* (*sty*[266]) expressing FL-PAPC, M-PAPC, or AXPX in the trachea. (F) Schematic drawing of trachea and terminal cells in one larval segment.

Whole-mount *in situ* hybridization

xPAPC mRNA was detected in gastrula embryos as described in Medina et al. (2004). The digoxigenin-labeled xSpry antisense RNA probe was synthesized by T7 RNA polymerase from the Myc-xSpry1 plasmid linearized with HindIII.

Cell culture and immunofluorescence

Transfection of HEK293 cells was performed with linear polyethyleneimine MW ~25,000 (Polysciences, Inc.). After transfection cells were grown in DMEM Ready Mix or serum-free DMEM high-glucose medium (both from PAA Laboratories) for 18 h. Medium was then exchanged for fresh DMEM Ready Mix, serum-free medium, or serum-free medium containing 5 ng/mL bFGF (Invitrogen) and 0.1 mg/mL BSA or 5 μ M SU5402 (Calbiochem) or both. Cells were washed with ice-cold PBS and fixed in 4% formaldehyde. For immunofluorescence, cells were stained with α -PAPC 11A6 mAb (1:100) (kindly provided by B.M. Gumbiner) and α -mouse-Alexa488 Ab (1:500) (Molecular Probes). Fluorescent images were acquired using a Nikon Eclipse 80i upright microscope, a Nikon DS-Qi1Mc CCD camera, and the NIS-Elements 2.30 program.

Yeast two-hybrid assay

For the two-hybrid screen, the yeast strain L40 was cotransformed with pNLX3-xPAPC and a *X. laevis* oocyte cDNA library (Matchmaker, Clontech) by standard protocols. Positive clones were selected on medium lacking leucine, tryptophan, and histidine and were checked with a filter assay for β -galactosidase activity. Prey plasmids were then isolated and retransformed into yeast with pNLX3-xPAPC or with pNLX3 as a negative control to confirm the specificity of the interaction.

Co-IP

Xenopus embryos were coinjected with 500 pg of Myc-xSpry1 and 500 pg of Flag-xPAPC, Flag-xPAPCmut, or Flag-AXPC mRNA at the four-cell stage. Protein was extracted from embryos grown to gastrula stage

and subjected to immunoprecipitation as described (Medina et al. 2004). Antibodies used were α -Myc Ab-1 (Oncogene) and α -Flag M2 (Sigma).

Phosphatase treatment of protein extracts

Lysates from gastrula-stage embryos were immunoprecipitated with α -Flag Ab, resuspended in λ protein phosphatase buffer, and incubated for 1.5 h at 30°C in the presence or absence of λ protein phosphatase. Flag-xPAPC and Flag-xPAPCmut were detected on a Western blot with the Flag Ab.

RhoA activity assay

Xenopus embryos were injected in the animal pole at the four-cell stage with Myc-tagged xRhoA DNA (50 pg) alone or in combination with 600 pg of xSpry1, 40 ng of xPAPC MO, 800 pg of xPAPC, or 800 pg of xPAPCmut mRNA. Protein was extracted from 40 embryos grown to stage 10.5 and subjected to GST-RBD-binding assay as described (Medina et al. 2004; Kitzing et al. 2007).

Experiments with *Drosophila*

Constructs and GFP to mark the trachea were expressed from UAS transgenes with breathless-Gal4 (*btl*-Gal4). *spry* heterozygous larvae were recognized by the absence of the segregating balancer chromosome marked with a uniformly expressed actin-GFP transgene. λ -btl encodes a constitutively active btl protein. For analysis of trachea morphology, third instar larvae, raised at 29°C, were anesthetized on ice, mounted in halocarbon oil, and photographed with bright-field and fluorescence optics. The number of terminal cells in each segment of 15 larvae per genotype was counted. The following stocks were used: UAS- λ -btl (III. chr.), *btl*-Gal4, UAS-GFP (II. chr.), and *sty*[226] (obtained from S. Luschig, Bayreuth). PAPC, M-PAPC, and AXPX were cloned from pCS plasmids into corresponding sites of the vector pUAST.

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