Xenopus paraxial protocadherin inhibits Wnt/β-catenin signalling via casein kinase 2β

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**INTRODUCTION**

The Wnt signalling pathway has a major role in early embryogenesis and later controls self-renewal of tissues in the adult organism (Clevers, 2006). Misregulation of this pathway can perturb embryogenesis, can lead to defects in organ formation and growth, and has been found in multiple human cancers (Tolwinski & Wieschaus, 2004).

In early *Xenopus* embryogenesis, Wnt/β-catenin signalling controls the establishment of the dorsoventral body axis and the anterior/posterior patterning of the neural tissue (De Robertis & Kuroda, 2004). Recently, it was shown that ectopic expression of casein kinase 2 beta (CK2β), which is part of the CK2 holoenzyme. The CK2α/β complex stimulates Wnt/β-catenin signalling, and the physical interaction of CK2β with PAPC antagonizes this activity. By this mechanism, PAPC restricts the expression of Wnt target genes during gastrulation. These experiments identify a novel function of protocadherins as regulators of the Wnt pathway.

Keywords: CK2; PAPC; protocadherin; Wnt pathway; *Xenopus*

**RESULTS AND DISCUSSION**

To identify interaction partners and proteins involved in signalling events downstream of *Xenopus* PAPC, we performed a yeast two-hybrid (Y2H) screen using the cytoplasmic domain of *papc* (*papcc*) as bait and screened 3.5 × 10⁶ independent clones of a *X. laevis* oocyte cDNA library. CK2β, which is the regulatory subunit of the CK2 holoenzyme, was identified as an interaction partner of PAPC. CK2β has an important role in assembly and stabilization of the tetrameric enzyme complex and modulates activity and substrate specificity of the CK2α subunit. In addition, there is evidence that CK2β has activities independent of the CK2 holoenzyme complex (Guerra et al., 2003; Bibby & Litchfield, 2005; Olsen & Guerra, 2008).

In the Y2H system, we tested the specificity of the PAPC/CK2β interaction and determined which portion of the cytoplasmic domain of PAPC is essential for its interaction with CK2β (Fig 1). CK2β strongly interacted with PAPCc, but not with the closely related protocadherin PCNS (protocadherin in neural crest and somites), despite an amino-acid identity of 57% in the intracellular domains (Rangarajan et al., 2006). This finding demonstrates that the interaction of the cytoplasmic domain of PAPC and CK2β is specific (Fig 1A). By using deletion constructs of PAPCc, we were able to show that amino acids 900–920 of the PAPC protein are required for the interaction with CK2β (Fig 1B).

The physical interaction of PAPC and CK2β was validated in *Xenopus* embryos by co-immunoprecipitation (co-IP). Embryos
were microinjected with synthetic mRNA coding for a Flag-tagged version of PAPCc or PAPCcΔ899 and Myc-tagged CK2β. Immunoprecipitation with an anti-Flag antibody specifically pulled down CK2β in complex with the cytoplasmic domain of PAPC. The co-IP experiments demonstrated that the PAPCcΔ899 deletion, consisting of the amino acids 715–899 of PAPC, interacted only very poorly with CK2β, in contrast to the interaction with the intracellular domain of PAPC (715–920). In the Y2H data (Fig 1C), the interaction of PAPC and CK2β was further corroborated by the observation that PAPC induced membrane localization of CK2β. GFP-tagged CK2β expressed in Xenopus animal cap tissue was localized in the cytoplasm but moved to the membrane when full-length (FL-) PAPC was coexpressed. In contrast, a PAPC protein lacking the intracellular domain (M-PAPC) was not able to recruit CK2β to the membrane (Fig 1D).

In Xenopus blastula and gastrula embryos, active Wnt signalling is essential to establish the dorsoventral body axis. As CK2 is expressed in these early stages of embryogenesis and is able to promote Wnt signalling, we asked whether the interaction of CK2β and PAPC would modulate the activity of the Wnt/β-catenin pathway (Dominguez et al, 2004, 2005). Overexpression of CK2β in combination with the CK2α subunit in Xenopus embryos induced expression of Wnt target genes and the formation of secondary body axes (Dominguez et al, 2004). pappc mRNA is not present in the animal cap, and we therefore asked whether ectopic expression of FL-PAPC or the intracellular domain of PAPC could influence CK2-mediated activation of nodal-related 3 (xnr3) transcription. PCR analysis revealed that the injection of synthetic ckk2a/ck2β mRNA into the animal pole of four-cell-stage embryos induced expression of the Wnt target gene xnr3 in animal cap explants (Fig 2A). The FL-PAPC, as well as PAPCc, inhibited CK2-induced xnr3 expression. In contrast, a PAPC deletion mutant (pappcΔ899flag) showing only weak CK2β interaction in Y2H and co-IP was not able to inhibit xnr3 induction (Fig 2A; supplementary Fig S1 online). The inability of PAPCc and M-PAPC to inhibit CK2β/β-catenin xnr3 expression or CK2β membrane recruitment was due to impaired PAPCc and...
M-PAPC expression. Western blot analysis demonstrated that all PAPC constructs were expressed at similar levels (S2). To further emphasize that the inhibition of Wnt/β-catenin signalling is mediated through PAPC, we ectopically expressed CK2α and CK2β in the ventral mesoderm and analysed the induction of the Wnt target genes xnr3 and siamois (x sia) by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in ventral halves of bisected gastrula embryos. CK2α/β strongly induced both Wnt targets and PAPCcflag, and FL-PAPC-HA inhibited xnr3 and siamois expression (Fig 2B).

From the experiments in animal caps and ventral marginal zones, we conclude that the interaction of PAPC with CK2β inhibits the CK2-mediated expression of xnr3 and siamois, arguing that PAPC could function as a negative regulator of Wnt/β-catenin signalling in vivo. To test this hypothesis, we investigated whether the expression of endogenous Wnt target genes could be enhanced by the knockdown of PAPC function.

We injected PAPC morpholino antisense oligonucleotides (PAPC-MO1 and 2) (Medina et al, 2004) into the dorsal-marginal zone of four-cell-stage embryos and determined the expression levels of xnr3 in the dorsal half of early gastrula embryos by qRT-PCR (Fig 3A). At early gastrula stage, we detected xnr3 expression on the dorsal side of uninjected Xenopus embryos but not on the ventral side, indicating that endogenous Wnt signalling is active dorsally. PAPC knockdown led to an increase of xnr3 expression on the dorsal side, and this upregulation could be counteracted by co-injection of a kinase-inactive form of CK2 (ck2αk1) (Dominguez et al, 2005). In line with our results in animal caps, overexpression of PAPC led to a reduction of endogenous xnr3 expression (S3). These results demonstrate not only that overexpression of PAPC inhibits CK2-mediated Wnt signalling but also that endogenous PAPC functions as a negative regulator of Wnt activity in early Xenopus embryos. Our finding that the elevated level of Wnt signalling observed in PAPC morphants could be reduced by simultaneous overexpression of a kinase-inactive form of CK2 suggests that PAPC suppresses Wnt signalling through its interaction with CK2β.

During gastrulation, pasc is expressed in the involuting mesendoderm, whereas xnr3 is expressed in the epithelial layer of the organizer (Glinka et al, 1996; Kim et al, 1998). As there is no overlap of the expression domains of pasc and xnr3, we tested whether PAPC could restrict the xnr3 expression domain in vivo (Fig 3B). Knockdown of PAPC enhanced endogenous Wnt signalling, and we asked whether the spatial expression pattern of xnr3 is altered in these embryos. PAPC-MO1 and 2 were microinjected into the dorsal marginal zone of four-cell-stage embryos. In situ hybridization of sagittally hemi-sectioned gastrula embryos revealed an enlargement of the xnr3 expression domain on PAPC knockdown. In all, 68% (n = 87) of the PAPC-MO-injected embryos displayed an expansion of xnr3 expression into the involuted mesendoderm, the region where PAPC is normally expressed. Surprisingly, we also found that xnr3 expression was expanded in the ectoderm in which PAPC is not expressed. This non-cell-autonomous effect of the PAPC morpholino...
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**Fig 3** Loss of *Xenopus* paraxial protocadherin (PAPC) upregulates the Wnt/β-catenin signalling pathway. (A) Expression of *xnr3* on the dorsal side of gastrula embryos. Embryos were injected in dorsal blastomeres with synthetic mRNA for *papc* flag (800 pg) or with 40 ng PAPC morpholino antisense oligonucleotides (PAPC-MO1 and 2) alone or in combination with mRNA for a kinase-inactive form of casein kinase 2 (CK2; 800 pg *ck2αKI*). Real-time PCR was performed for *xnr3* on semi-sectioned gastrula embryos (wt, wild-type, un.injected embryos). (B) Upper panel: *in situ* hybridization for *xnr3* (blue) in sagittally sectioned gastrula embryos. Embryos were injected with 40 ng PAPC-MO1 and 2 into the dorsal marginal zone and fixed at gastrulation. Lower panel: Summary of the *xnr3* *in situ* hybridization analysis. The expression domains of *xnr3* were classified in comparison with un injected embryos as follows: wild type (wt pattern); larger expression domain of *xnr3* extending into the PAPC expression domain and into the ectoderm (expanded pattern); smaller expression domain than observed in wt embryos (reduced pattern); or no expression detectable. (C) Embryos were injected into the ventral or dorsal marginal zone with a Wnt/β-catenin luciferase reporter plasmid containing the Wnt-responsive region of the siamois promoter (300 pg) alone or in combination with PAPC-MO1 and 2 or with synthetic *Xenopus wnt8* mRNA. Luciferase activity (*luc*) was measured at early gastrula (stage 10.25).

Expression of *xnr3* outside the PAPC domain could be due to suppressed BMP-4 activity.

The knockdown experiments demonstrate that endogenous PAPC negatively regulates the expression of *xnr3* in gastrula embryos. To show that the regulation of *xnr3* by PAPC is exerted through the modulation of Wnt signalling, we monitored the endogenous activity of the Wnt/β-catenin pathway in gastrula embryos by a luciferase reporter system (Fig 3C).

A Wnt-responsive promoter fragment of the *siamois* gene that drives the expression of luciferase (Brannon et al., 1997) was injected into the marginal zone of dorsal blastomeres of four-cell-stage embryos in combination with PAPC-MO or as positive control with *wnt8* synthetic mRNA. At early gastrula (stage 10.25), we measured luciferase activity in embryo homogenates. Ectopic expression of *wnt8* increased Wnt reporter activity by a factor of two. A comparable upregulation was observed when PAPC was knocked down on the dorsal side of the embryo.

Additional evidence that PAPC inhibits Wnt/β-catenin signalling through CK2 came from experiments in which PAPC function was knocked down and simultaneously CK2/β levels were raised. Low doses of PAPC-MO and synthetic mRNA for CK2/β were injected either alone or in combination into dorsal blastomeres of *Xenopus* embryos. PAPC-MO or CK2/β mRNA alone elevated *xnr3* mRNA levels only moderately and had no effect on *siamois* expression. Co-injection of PAPC-MO and CK2/β mRNA strongly enhanced *xnr3* and *siamois* expression (Fig 4A).

**CONCLUSION**

These experiments demonstrate that PAPC functions in the dorsal marginal zone as an intracellular inhibitor of Wnt/β-catenin signalling, restricting the expression domain of Wnt target genes such as *xnr3* and *siamois*. This inhibitory effect of PAPC is dependent on the binding of the regulatory subunit CK2β to the cytoplasmic domain of PAPC. As PAPC is a transmembrane

antisense oligonucleotide (PAPC-MO) might be explained by the finding that nr3 can inhibit the BMP-4 protein by binding of the nr3 pro-region (Haramoto et al., 2006). Inhibition of BMP signalling allows dorsalization of the mesoderm and neuralization of the ectoderm (Wilson & Hemmati-Brivanlou, 1995). Therefore,
protein, it can recruit CK2β to the membrane, which would prevent a positive regulation of Wnt signalling by the CK2 holoenzyme (Fig 4B).

The recruitment of the CK2 subunits to distinct compartments of the cell was proposed as a regulatory mechanism for CK2 kinase activity. A physical separation of α- and β-subunits mediated by different interaction partners could regulate CK2 activity in a cell-type-specific manner (Montenarh, 2010).

Our data demonstrate for the first time that protocadherins can inhibit Wnt/β-catenin signalling. Interestingly, the WiT49 tumour cell line, in which a major part of the protocadherin locus is hypermethylated and γ-protocadherins were knocked down by siRNA, shows elevated levels of Wnt signalling (Dallosso et al, 2009).

Canonical Wnt/β-catenin signalling is inhibited by the PCP branch of the Wnt cascade (Torres et al, 1996). The mechanisms that underlie this antagonism, however, are still not fully understood. In cells expressing PAPC, PCP signalling is augmented through binding of ANR5 and Sprouty 1 (Chung et al, 2007; Wang et al, 2008), and the Wnt/β-catenin pathway is inhibited by sequestering CK2β. These results suggest that PAPC could be a component of the Wnt cascade, which could mediate the antagonism between canonical and non-canonical Wnt signalling.

METHODS
Yeast two-hybrid assay. The yeast strain L40 was co-transfected with pNLX3, containing deletion constructs of PAPCβ, or pNLX3-xPCNSc and pACT2-CK2β or pACT2-xSpry1. The transfection was tested on selection medium lacking Trp and Leu; interaction was tested on medium lacking Trp, Leu and His and checked for β-galactosidase activity after 72 h.

Xenopus embryo manipulations. All experiments on living organisms were conducted in accordance with relevant guidelines and regulations. Xenopus eggs were fertilized in vitro and de-jellied embryos were microinjected in 1 × MBSH buffer (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.41 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 10 mM HEPES pH 7.4, 10 μg/ml penicillin). The embryos and animal caps were cultured in 0.1 × MBSH and 0.5 × MBSH, respectively. Embryos were staged according to Nieuwkoop & Faber (1994).

Co-immunoprecipitation. Xenopus embryos were co-injected with synthetic mRNA of Myc-CK2β (600 pg) in combination with papc-flag or papcΔ899flag (600 pg of each) at the four-cell stage and cultured until late gastrula. Protein was extracted in Nonident P40 lysis buffer and incubated with 2 μg rabbit anti-Flag (OctA-probe: Santa Cruz) or 2 μg rabbit anti-IgG antibody (Dianova). To the supernatant of 30 embryos, 20 μl magnetic beads (Adamtech) were added. The immunoprecipitates were resuspended in PAG Elution Buffer (Adamtech) and separated by 15% SDS–PAGE. For western blotting, a mouse anti-Myc antibody (Cell Signaling Technology) and a mouse anti-Flag antibody (Sigma-Aldrich) were used.

Membrane recruitment assay. 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.

RT–PCR. Total RNA was prepared using Trizol reagent (Invitrogen) or MasterPure RNA Purification Kit (Epicentre Biotechnologies).
cDNA was synthesized using random hexamer primers and H minus M-MuLV reverse transcriptase (Fermentas).

PCR was performed using Taq polymerase (Euro Clone). Odc primers: 5’-TTGATCCGCTTTCTTCGACC-3’, 5’-GTCATAGTGAGCCTTCGAT-3’, 57°C for 24 cycles; xnr3 primers: 5’-TGAATCTACTGTAGCAATGTTCC-3’, 5’-GACATCTGTTATCC-3’, 60°C for 30 cycles.

Real-time PCR was performed using SybrGreen mix (Thermo Scientific) 60°C for 40 cycles. Samples were normalized to odc. Odc primers: 5’-TGCAATGTGAAGCCAGTTCC-3’, 5’-GCCCATC ACGTTTGTC-3’; xnr3 primers: 5’-CTAAAGGCTCTTCGCTAA A-3’, 5’-AAAAAGGAGGGCAAATACCC-3’; siamois primers: 5’-TCTGTAGAACCTTACTCTGTTTGG-3’, 5’-AACCTTACGTTT TGCTGACC-3’.

Statistics. The figures show the mean of n ≥ 3 replicates; standard errors are given and the significance (P value) was determined by paired Student’s t-test (Microsoft Excel) *P < 0.05, **P ≤ 0.01, ***P < 0.001.

In situ hybridization. Embryos were fixed in MEMFA and hemi-sectioned, and whole-mount in situ hybridization was performed as described (Sive Hazel et al, 2000). The pdor-xNR3 (Smith et al, 1995) was linearized with EcoRI and was digoxigenin-labelled.

Luciferase assay. Four-cell stage embryos were injected into the ventral or dorsal marginal zone with 300 pg luciferase reporter plasmid (p01234-Luc; Brannon et al, 1997) and 50 pg TK Renilla, alone or in combination with 40 ng PAPC-MO1 and 2 or synthetic xmt8 mRNA. Triplicates of five embryos were lysed according to the manufacturer’s protocol (Promega), and 20 μl of cell lysate was used for luciferase detection.

Supplementary information is available at EMBO reports online (http://www.emboreports.org).

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Author contributions: H. Steinbeisser co-initiated, coordinated and co-wrote the study. Y. Wang performed the Y2H screen, an animal cap PCR, recruitment assay and co-initiated the study. D. Weber performed the western blot analysis and supported the qRT–PCRs. A. Kietzmann performed rest of experiments described, co-initiated the study and co-wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES


