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

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***Xenopus* paraxial protocadherin (PAPC) regulates cadherin-mediated cell adhesion and promotes the planar cell polarity (PCP) pathway. Here we report that PAPC functions in the *Xenopus* gastrula as an inhibitor of the Wnt/ β -catenin pathway. The intracellular domain of PAPC interacts with casein kinase 2 beta (CK2 β), which is part of the CK2 holoenzyme. The CK2 α / β complex stimulates Wnt/ β -catenin signalling, and the physical interaction of CK2 β 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*
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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 (CK2) on the ventral side of *Xenopus* embryos stimulates the Wnt/ β -catenin pathway and induces secondary body axes (Dominguez *et al*, 2004). The CK2 holoenzyme consists of a dimer of CK2 β and two molecules of CK2 α , or the isoform CK2 α' , and functions mainly as a serine/threonine kinase (Allende and Allende, 1995). Essential components of the Wnt/ β -catenin pathway such as Dishevelled and β -catenin are substrates for the CK2 complex (Willert *et al*, 1997; Song *et al*, 2003). Specific phosphorylation by CK2 activates Dishevelled and stabilizes β -catenin in the cytoplasm, which is essential for nuclear entry and Wnt target gene regulation (Wu *et al*, 2009; Bernatik *et al*,

2011). Here we describe a novel interaction between CK2 β and the *Xenopus* paraxial protocadherin (PAPC). With more than 70 genes, the protocadherins make up the largest subfamily of the cadherin superfamily. Protocadherins not only mediate homophilic adhesion but are also involved in intracellular signalling (Redies *et al*, 2005). During *Xenopus* gastrulation, PAPC controls cadherin-mediated adhesion, convergent extension movements and tissue separation (Medina *et al*, 2004; Unterseher *et al*, 2004). The intracellular domain of PAPC interacts with ANR5 and Sprouty 1 and thereby promotes the planar cell polarity (PCP) pathway (Chung *et al*, 2007; Wang *et al*, 2008). Here we present evidence that the interaction of PAPC and CK2 β reduces the level of Wnt/ β -catenin signalling, resulting in the repression of Wnt target genes. These experiments identify PAPC as a regulator of β -catenin-dependent and -independent pathways.

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* (*papc_c*) as bait and screened 3.5×10^6 independent clones of a *X. laevis* oocyte cDNA library. CK2 β , which is the regulatory subunit of the CK2 holoenzyme, was identified as an interaction partner of PAPC. CK2 β has an important role in assembly and stabilization of the tetrameric enzyme complex and modulates activity and substrate specificity of the CK2 α subunit. In addition, there is evidence that CK2 β 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_c/CK2 β interaction and determined which portion of the cytoplasmic domain of PAPC is essential for its interaction with CK2 β (Fig 1). CK2 β strongly interacted with PAPC_c, 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 CK2 β is specific (Fig 1A). By using deletion constructs of PAPC_c, we were able to show that amino acids 900–920 of the PAPC protein are required for the interaction with CK2 β (Fig 1B).

The physical interaction of PAPC and CK2 β was validated in *Xenopus* embryos by co-immunoprecipitation (co-IP). Embryos

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Fig 1 | The intracellular domain of PAPC interacts with CK2 β . (A) Interaction in the Y2H system. The intracellular domain of PAPC or PCNS was used as bait and CK2 β or xSpry1 was used as prey. The interaction of bait and prey was visualized by growth on selection plates and synthesis of β -galactosidase. (B) Interaction of PAPCc and deletion mutants with CK2 β . CK2 β was used as prey and the intracellular domain of PAPC (PAPCc: residues 715–979) or deletion mutants—residues 837–899, 900–979, 837–979, PAPCcΔ899: residues 715–899, 715–920, 715–951, 715–966—were used as bait. (C) Co-immunoprecipitation of CK2 β and the intracellular domain of PAPC. Immunoprecipitation from *Xenopus* embryos expressing PAPCcflag or PAPCcΔ899flag and mycCK2 β using a rabbit anti-Flag antibody or rabbit IgG as control. Western blot analysis was performed using a mouse anti-Flag or a mouse anti-Myc antibody. (D) PAPC recruits CK2 β to the membrane in animal cap cells. GFP-CK2 β mRNA (400 pg) was injected into four-cell-stage embryos either alone or with mRNAs encoding full-length PAPC (600 pg, *fl-papc*) or PAPC lacking the intracellular domain (600 pg, *m-papc*). Animal caps were excised at stage 9, and the localization of GFP-CK2 β was determined by confocal microscopy (scale bar, 25 μ m). —, no protein interaction; +/—, poor interaction; +, marginal interaction; ++, moderate interaction; + + +, strong interaction; CK2 β , casein kinase 2 β ; GFP, green fluorescent protein; PAPC, *Xenopus* paraxial protocadherin; PCNS, protocadherin in neural crest and somites; wt, wild type; xSpry1, *Xenopus* Sprouty1; Y2H, yeast two-hybrid.

were microinjected with synthetic mRNA coding for a Flag-tagged version of PAPCc or PAPCcΔ899 and Myc-tagged CK2 β . Immunoprecipitation with an anti-Flag antibody specifically pulled down CK2 β 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 PAPCc, interacted only very poorly with CK2 β , confirming the Y2H data (Fig 1C). The interaction of PAPC and CK2 β was further corroborated by the observation that PAPC induced membrane localization of CK2 β . GFP-tagged CK2 β 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 CK2 β 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 CK2 β and PAPC would modulate the activity of the Wnt/ β -catenin pathway (Dominguez et al, 2004, 2005). Overexpression of CK2 β in combination with the CK2 α subunit in *Xenopus* embryos induced expression of Wnt target genes and the formation of secondary body axes (Dominguez et al, 2004). *papc* 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 *ck2 α /ck2 β* 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 (*papc* Δ 899flag) showing only weak CK2 β 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 CK2 α / β -induced *xnr3* expression or CK2 β membrane recruitment was not due to impaired PAPCc and

Fig 2 | PAPC antagonizes the function of CK2. (A) Upper panel: scheme of animal cap assay. Embryos were animal injected at the four-cell stage with synthetic mRNA. Animal caps were explanted at stage 8 and gene expression was monitored at gastrulation (stage 10.25) by PCR. Lower panel: analysis of *nodal-related 3* expression after injection of synthetic *ck2 α / β* mRNA (500 pg each) alone or in combination with *fl-papc*, the intracellular domain of PAPC (800 pg, *papc.flag*) or a construct lacking the predicted interaction site with CK2 (800 pg, *PAPCc Δ 899flag*). *Ornithindecarboxylase* (*Odc*) served as loading control. (B) Upper panel: scheme of the experiment. Embryos were injected ventrovegetally with synthetic mRNA. Embryos were hemi-sectioned at early gastrula stage and real-time PCR was performed. Lower panel: analysis of *xnr3* and *siamois*. Embryos were injected with synthetic CK2 α / β mRNA (900 pg each) alone or in combination with PAPCc (800 pg, *PAPCcflag*) or full-length PAPC (1,000 pg, *fl-papc-HA*). (–) RT, (negative) reverse transcription; AC, animal cap; CK2, casein kinase 2; d, dorsal; dorsal, dorsal halves of early gastrula embryo; HA, haemagglutinin; PAPC, *Xenopus* paraxial protocadherin; we, whole embryo; v, ventral; ventral, ventral halves; wt, wild-type, uninjected embryos; *xnr3*, *nodal-related 3*.

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 CK2 α and CK2 β in the ventral mesoderm and analysed the induction of the Wnt target genes *xnr3* and *siamois* (*xsia*) by quantitative reverse transcription–polymerase chain reaction (qRT–PCR) in ventral halves of bisected gastrula embryos. CK2 α / 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 CK2 β 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

(*ck2 α K1*) (Dominguez et al, 2005). In line with our results in animal caps, overexpression of PAPCc 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 CK2 β .

During gastrulation, *papc* 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 *papc* 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

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_cflag* (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 *ck2 α KI*). Real-time PCR was performed for *xnr3* on hemi-sectioned gastrula embryos (wt, wild-type, uninjected 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 uninjected 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 CK α / β levels were raised. Low doses of PAPC-MO and synthetic mRNA for CK α / β were injected either alone or in combination into dorsal blastomeres of *Xenopus* embryos. PAPC-MO or CK α / β mRNA alone elevated *xnr3* mRNA levels only moderately and had no effect on *siamois* expression. Co-injection of PAPC-MO and CK α / 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 CK2 β 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,

Fig 4 | *Xenopus* paraxial protocadherin (PAPC) is a negative regulator of the Wnt/ β -catenin signalling pathway. (A) Synergy of PAPC morpholino antisense oligonucleotide (PAPC-MO) and casein kinase 2 (CK2) on the dorsal side of early gastrula. Embryos were injected in dorsal blastomeres with synthetic mRNA of CK2 α/β (500 pg each) or with 14 ng PAPC-MO1 and 2 or a combination of both. Embryos were hemi-sectioned at early gastrula stage, and real-time PCR was performed for *xnr3* and *siamois*. wt, wild-type, uninjected embryos; dorsal, dorsal halves of early gastrula embryo; ventral, ventral halves. (B) Model of negative regulation of Wnt/ β -catenin signalling by PAPC. In the absence of PAPC (upper diagram), CK2 β is free to form the tetrameric complex, which positively regulates Wnt/ β -catenin signalling. When PAPC is present (lower diagram) it binds CK2 β and sequesters the regulatory subunit of the CK2 holoenzyme. Dsh, Dishevelled; TCF, T-cell factor.

protein, it can recruit CK2 β 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 CK2 β . 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 PAPCc, or pNLX3-xPCNSc and pACT2-CK2 β 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 $1 \times$ 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 μ g/ml penicillin). The embryos and animal caps were cultured in $0.1 \times$ MBSH and $0.5 \times$ MBSH, respectively. Embryos were staged according to Nieuwkoop & Faber (1994).

Co-immunoprecipitation. *Xenopus* embryos were co-injected with synthetic mRNA of Myc-CK2 β (600 pg) in combination with *papc*_{flag} or *papc* Δ 899_{flag} (600 pg of each) at the four-cell stage and cultured until late gastrula. Protein was extracted in Nonidet P40 lysis buffer and incubated with 2 μ g rabbit anti-Flag (OctA-probe: Santa Cruz) or 2 μ g rabbit anti-IgG antibody (Dianova). To the supernatant of 30 embryos, 20 μ l 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'-TCCATTCGCTCTCCTGAGCAC-3', 5'-GTCAATGATGGAGTGTATGGATC-3', 57 °C for 24 cycles; *xnr3* primers: 5'-TGAATCCACTTGTGCAGTTC-3', 5'-GACAGTCTGTGTTACATGTCC-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'-TGCACATGTCAAGCCAGTTC-3', 5'-GCCCCATCACAGTTGGTC-3'; *xnr3* primers: 5'-CCAAAGCTTCATCGCTAAAG-3', 5'-AAAAGAAGGGAGGCAAATACG-3'; *siamois* primers: 5'-TCTGGTAGAACTTTACTCTGTTTGG-3', 5'-AACTTCATGGTTTGCTGACC-3'.

Statistics. The figures show the mean of $n \geq 3$ 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 *pdcx-nr3* (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 *xwnt8* mRNA. Triplicates of five embryos were lysed according to the manufacturer's protocol (Promega), and 20 μ l 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.

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