PI4K2 β constrains non-canonical Wnt-PCP signalling and is localised by PAR-1 (MARK2/3) phosphorylation

S. M. Tsang¹, William Cheng², Jingjing Li³ and Jeremy B. A. Green*

Centre for Craniofacial and Regenerative Biology, King's College London, Guy's Tower Floor 27, London SE1 9RT, UK.

¹Present address: Breast Cancer Now Toby Robins Research Centre, Division of Breast Cancer Research, The Institute of Cancer Research, London, UK

²Present address: Pangenia Inc., Hong Kong

³Present address: AstraZeneca R&D, Milstein Building, Granta Park, Cambridge CB21 6GH.

*Corresponding author: jeremy.green@kcl.ac.uk

ABSTRACT

Canonical Wnt signalling is critically important in embryonic cell-type specification and cancer, while non-canonical Wnt signalling is primarily implicated in physical morphogenesis, especially planar cell polarity (PCP). Both are modulated by the polarity kinase PAR-1 (MARK2/3). PAR-1 phosphorylates the Wnt transducer Dishevelled, but there is evidence that it exerts control through other targets. Here we describe an *in vitro* screen for new targets of PAR-1 in which we identified phosphatidylinositol-4-kinase-2-beta (PI4K2 β) as a substrate. Perturbation phenotypes and reporter assays *in vivo* show that PI4K2 β inhibits both canonical and non-canonical Wnt pathways, in contrast to PI4K2 α , which promotes canonical but does not affect non-canonical signalling. We show that PI4K2 β acts in Wnt-responding tissue, not in Wnt production or secretion. Subcellularly, PI4K2 β is cortically enriched, unlike PI4K2 α , and is basolateral in polarised cells. Mutation of the PAR-1 phosphorylation site of PI4K2 β mis-localises it and the endogenous core PCP protein, Vangl2. Our results reveal that PAR-1 interacts with the vertebrate PCP signalling pathway via PI4K2 β .

INTRODUCTION

The polarity protein partitioning-defective 1 (PAR-1), also known as microtubule affinity regulating kinases (MARK2 or -3), is a highly conserved serine/threonine kinase with a number of polarity-associated functions ¹. PAR-1 plays essential roles in asymmetric cell division and the establishment of cell polarity from invertebrates to mammals ². PAR-1 interacts with multiple

signalling pathways including the canonical and non-canonical Wnt signalling pathways^{3, 4, 5, 6}. However, regulation and cross-talk between PAR-1 and Wnt signalling components are not well understood. Both canonical and non-canonical Wnt pathways are initiated by binding
 45 extracellular Wnt protein, which acts through a Frizzled (Fz) receptor and Dishevelled (Dvl) to activate downstream targets. Canonical Wnt pathway leads to stabilisation/translocation of β -catenin and transcriptional activation of target genes⁷, while non-canonical Wnt pathways diverge at the level of Dvl into the Planar Cell Polarity (PCP) pathway involving Rho/Rac small GTPase and Jun N-terminal kinase (JNK) or the Wnt/ Ca^{2+} pathway via protein kinase C (PKC)⁸.
 50 Functionally, the canonical pathway specifies dorsal structures during early *Xenopus* development⁹, while the non-canonical pathways regulate cell polarity including that required for convergent extension movements during zebrafish and *Xenopus* gastrulation^{10, 11}.

The PCP pathway promotes polarisation in the plane of the epithelial cells, perpendicular to their
 55 apicobasal axis¹², affecting cell morphology, at least in part independently of transcription¹³. A key feature of this pathway is the asymmetric distribution of their core PCP components such as transmembrane proteins Frizzled (Fz) and Van Gogh (Vang) and cytoplasmic regulators Dishevelled (Dvl) and Prickle (Pk) both within and between cells¹⁴. Disruption of the core PCP proteins is associated with developmental abnormalities such as defects in convergent extension
 60 and neural tube closure^{15, 16} and also implicated in cancer invasiveness¹⁷. Van Gogh-like (Vangl) is a mammalian homologue of the *Drosophila* PCP gene Van Gogh (Vang) (also known as Strabismus). Vang/Vangl is known to be involved in the non-canonical PCP pathway via the activation of JNK¹⁸. Studies have shown Vang/Vangl binds directly to Dvl¹⁸ and Prickle¹⁹ and is involved in their recruitment to the plasma membrane. The molecular networks that underlie its
 65 membrane targeting however, remain largely unknown.

Phosphatidylinositol (PI) can be phosphorylated at the 3-, 4- and 5-positions of the inositol ring by different lipid kinases to generate seven distinct phosphoinositides that mediate important signalling and trafficking functions^{20, 21}. Phosphatidylinositol 4-kinases (PI4Ks) generate
 70 phosphatidylinositol 4-phosphate (PI4P) and are structurally and biochemically classified into type II (PI4K2) and type III (PI4K3) families²⁰. Phosphatidylinositol 4-kinase type 2 (PI4K2) is the predominant PI4-kinase activity in cell extracts. Two structurally related isoforms, PI4K2 α and PI4K2 β , with similar enzymatic properties contribute to this activity.

75 Despite similarities between PI4K2 α and - β , accumulating evidence suggests that they have non-
identical biological functions. In cultured mammalian cells, PI4K2 α is catalytically active, tightly
membrane-associated, and predominantly located to intracellular membranes such as *trans*-
Golgi network ^{22, 23} and early and late endosomes ^{24, 25, 26}. PI4K2 α plays essential roles in
endocytosis ²², Golgi function ²⁴ and trafficking processes such as the delivery of AP-3-dependent
80 cargoes to lysosomal compartments ^{25, 27} and lysosomal degradation of epidermal growth factor
receptors ²⁶. Deletion of PI4K2 α gene in mice causes late onset neurodegeneration ²⁸. In addition,
PI4K2 α promotes LRP6 phosphorylation during canonical Wnt signalling in both mammalian cells
and *Xenopus* ^{29, 30}. Much less is known about PI4K2 β . Like PI4K2 α , PI4K2 β localises to membranes
of the Golgi and endosomal systems ^{24, 23} and lacks a transmembrane domain but associates
85 tightly with membranes through multiple palmitoylation sites ^{23, 31}. However, PI4K2 α and PI4K2 β
differ in their membrane recruitment: PI4K2 α is constitutively membrane associated, whereas
PI4K2 β is dynamically palmitoylated and recruited to the plasma membrane in response to
platelet-derived growth factor stimulation or Rac activation and in turn becomes activated ²³.
PI4K2 β therefore appears to be regulated by membrane recruitment. PI4K2 β does not activate
90 canonical Wnt signalling ²⁹ but has been associated with inhibition of invasiveness of tumour cells
³².

Previously, we showed that different PAR-1 isoforms are involved in *Xenopus* development and
that each is associated with a different branch of Wnt signalling pathway ⁶. In most vertebrate
95 species examined, the isoforms are splice variants. Isoform PAR-1BY (MARK2 variant Y) controls
the localisation of one of its substrates, Dishevelled (Dvl), in the non-canonical Wnt planar cell
polarity (PCP) signalling pathway. In contrast, isoforms PAR-1A (MARK3) and PAR-1BX (MARK2
variant X) primarily act on canonical signalling to β -catenin. Interestingly, it was also found that
a PAR-1-non-phosphorylatable mutant of Dvl was able to induce a canonical Wnt signal just as
100 potently as wild type Dvl ⁶. This suggests that phosphorylation of Dvl may not be the only target
of PAR-1 in Wnt- β -catenin signal transduction.

Here we describe an unbiased screen for novel phosphorylation targets of PAR-1 in which we
identified PI4K2 β as a potential target. We then analysed the developmental role of PI4K2 β and
105 found that depletion produces phenotypes consistent with roles in inhibiting both canonical and
non-canonical Wnt signalling. These were confirmed with reporter assays, which were further
used to show that the effects on canonical signalling were in the responding rather than the Wnt-

secreting cells. We also observed that PI4K2 β is basolaterally localised in polarised epithelial cells in *Xenopus*, and that mutation of the PAR-1 phosphorylation site is sufficient to abrogate such localisation. Furthermore, we showed PI4K2 β co-localises with a key PCP protein Vangl2 at the basolateral domain and Vangl2 is mis-localised by expression of the PAR-1-unphosphorylatable PI4K2 β mutant protein. Our data suggests that PAR-1-dependent PI4K2 β basolateral localisation plays an important role in the regulation of the vertebrate PCP signalling pathway.

RESULTS

Phosphatidylinositol 4-kinase type II beta (PI4K2 β) is a novel phosphorylation target of PAR-1

We previously showed that a PAR-1-non-phosphorylatable mutant of Dvl was fully able to induce a canonical Wnt signal, and that PAR-1A could super-induce signalling triggered by this mutant⁶. This suggests that PAR-1 kinase could act by potentiating canonical signalling to Wnt- β -catenin via alternative (non-Dvl) target(s). We therefore carried out an unbiased screen for novel phosphorylation targets of PAR-1 to understand its mechanism of action in Wnt signalling branches. To screen for novel phosphorylation targets of PAR-1, pools of clones from a 7000-

Fig.1. Phosphatidylinositol 4-kinase type II beta (PI4K2 β) as a novel phosphorylation target of PAR-1. A) Schematic diagram of the *Xenopus* expression library screen. A 7000-clone arrayed normalized *Xenopus laevis* oocyte cDNA library was used. B) Western blot analysis showing that PI4K2 β *in vitro*-transcribed and translated from cloned DNA becomes phosphorylated after incubation with MARK3 wild-type (WT) kinase and migrated with an increased mobility upon gel electrophoresis as compared to the MARK3 kinase dead (KD)-treated protein. Purified GST-tagged human MARK3 wild type (WT) and kinase dead (KD) proteins were used. Numbers at the left are molecular mass in kDa. Arrow indicates the PI4K2 β protein becomes phosphorylated with a reduced mobility after kinase treatment.

clone arrayed normalized *Xenopus laevis* oocyte cDNA library³³ were first transcribed and translated *in vitro* in the presence of non-radioactively labelled amino acids. The resulting protein pools were then incubated with purified recombinant GST-tagged human MARK3 wild type (WT) or kinase dead (KD) proteins³⁴ and subsequently analyzed by SDS-PAGE and Western blotting (Fig.1A). Proteins phosphorylated after kinase treatment were identified by looking for alterations in their mobility in gel electrophoresis. “Band shifted” clones identified in the pool screen were further confirmed individually to verify the result. Using this approach, we identified 14 potential targets and 11 of them were known genes or close homologues (Fig.S1A). Among them, we selected phosphatidylinositol 4-kinase type II beta (PI4K2 β) for further investigation. PI4K2 β is an inositide kinase that participates in the phosphatidylinositol cycle. Western blot analysis in Fig.1B shows that PI4K2 β when tested individually becomes phosphorylated after incubation with MARK3 wild-type kinase and migrated with an increased mobility upon gel electrophoresis compared to the kinase dead-treated control. Although phosphorylation increases the molecular weight of a protein, an increase in mobility is not unprecedented (e.g. upon T-loop phosphorylation of CDK1³⁵) and presumably results from secondary structural changes, or phosphorylation-facilitated modifications in the cell free extract, that facilitate migration through the pores of the electrophoresis gel. It is known that both *Xenopus* PI4K2 α and - β contain the known PAR-1 phosphorylation consensus site KVGS at Ser-255 and 265 respectively (Fig.S1B) but neither has previously been identified as a PAR-1 substrate.

The *in vivo* function of PI4K2 β during *Xenopus* embryogenesis

PI4K2 α was previously implicated in Wnt signalling as an important cofactor in canonical signalling²⁹, but PI4K2 β has not previously been analysed *in vivo*. To evaluate the *in vivo* function of PI4K2 β during *Xenopus* development, we first examined whether *Xenopus* PI4k2 β is expressed during gastrulation. Analysis of the spatial expression of the PI4k2 β transcripts during *Xenopus* early embryogenesis by whole-mount *in situ* hybridization revealed ubiquitous expression of the transcripts (data not shown).

Next, we carried out the loss-of-function analysis by using translation-blocking morpholino oligonucleotides (Mos) against its mRNA at the 5'-UTR. Two non-overlapping Mos (designated PI4K2 β -Mo-1 and PI4K2 β -Mo-2) had the same effects as follows. Embryos injected with 15ng of either PI4K2 β -Mo into two dorsal blastomeres at 4-cell stage had no discernable effect up to gastrulation (data not shown). However, at tailbud and tadpole stages, more than 60% of the

PI4K2 β -depleted embryos (n=51) displayed severe defects in dorsoanterior structure (Fig.2A-F&M, Fig.S2A). The phenotype was variable but dose-dependent and an apparent combination
160 of two often distinct defects: reduced or missing head and a severely dorsally bent body axis. Absence of anterior structures if accompanied by loss of notochord can, in principle, be due to partial loss of the Spemann Organiser. Examination of the PI4K2 β -depleted embryos showed the presence of well-differentiated, albeit disorganised notochord tissue (Fig.2F). This implicates an

Fig.2. Both gain- and loss-of-function of PI4K2β cause severe defects in dorsoanterior structure during embryogenesis. (A-L) Representative tailbud stage embryos injected as indicated at 2-4 cell stage with control Mo 15 ng or GFP mRNA 2000 pg versus PI4K2β-Mo 15 ng or mRNA 2000 pg, show dorsally bent axes in PI4K2β-perturbed cases D, E, J, and K. Hematoxylin-Eosin-stained sagittal sections (C,F,I,L) show the presence of notochord in all cases (black arrows) but disorganised in PI4K2β-perturbed embryos (F,L). (M&N) Histograms quantifying frequencies of the phenotypes. Data summarise at least three independent experiments with at least 20 embryos in each condition for each. (N -R) Rescue experiments by co-injecting Mo with GFP- or full length PI4K2β mRNA. 500pg of PI4K2β mRNA, but not GFP alone, partially rescues the convergent extension defect to give a shorter but straight axis (N,Q-R).

excess of late posteriorising canonical Wnt signalling which has previously been associated with loss of anterior structures with retention of notochord³⁶. Notochord disorganisation suggested a defects in the control of convergent extension movements, which are regulated by non-canonical Wnt-planar cell polarity (PCP) signalling^{37, 38}. Neither ventral injections (not shown) nor control Mo (Fig.2A-C) had any overt effects.

For gain-of-function analysis, we injected PI4K2 β mRNA into two dorsal blastomeres at 4-cell stage. The embryos injected with up to 2000pg PI4K2 β mRNA also resulted in a shorter and dorsally bent axis (Fig.2J-L). As shown in Fig.2M, more than 50% of the PI4K2 β mRNA-injected embryos (n=50) have the similar “super bent” phenotype to that of embryos injected with PI4K2 β -Mo. H&E stained sections of the PI4K2 β mRNA-injected embryos also showed the presence of well-differentiated but disorganised notochord as compared to GFP mRNA-injected embryos (Fig.2I&L). Thus, both gain- and loss-of-function of PI4K2 β has the effect of disorganising the notochord, similar to other proteins previously implicated in Wnt-PCP pathway e.g. Vangl and Dishevelled^{12, 39}.

As an additional control for the specificity of the phenotypes, we performed rescue experiments by co-injecting morpholinos with full length PI4K2 β mRNA. Fig.2Q-R show 500pg of PI4K2 β mRNA but not GFP alone (Fig.2O-P) is able to reduce the severity of the observed phenotypes, resulting in a greater percentage of normal and weakly affected embryos i.e. shorter with straight axis. The amount of PI4K2 β mRNA for the rescue experiment was limited to 500pg, as higher amounts also resulted in dorsally bent axes (Fig.2J-L). It is likely that this limitation might have prevented the complete rescue of the PI4K2 β -depleted embryos.

We also examined effects of depleting embryos of the PI4K2 α , both as another control for specificity, and also to compare side-by-side the published phenotypes²⁹ with those for PI4K2 β in the same experiment. In agreement with the published data, and in contrast to effects of PI4K2 β , embryos injected with 15ng of PI4K2 α -Mo into the two dorsal blastomeres at 4-cell stage led to anteriorisation with a shorter axis and an enlarged cement gland (Fig.S2B). Our data is consistent with Pan *et al* 2008 and anteriorisation is thought to be due to suppression of late, posteriorising canonical Wnt signalling. Taken together, our data suggest that PI4K2 β normally inhibits or restricts both the canonical Wnt and non-canonical PCP/Wnt pathways.

PI4K2 β differentially affects Wnt signalling pathways

To test molecularly whether PI4K2 α and - β were indeed affecting Wnt pathways, we quantified Wnt canonical and non-canonical outputs in embryos depleted for, or overexpressing each PI4K2 isoform using luciferase reporter assays. For canonical Wnt/ β -catenin signalling, early embryos were co-injected with Wnt8 mRNA, TOPFLASH β -catenin reporter and CMV-Renilla reporter together with PI4K2 α or - β -Mo and/or the corresponding mRNA and assayed at gastrula stage.

Fig.3A shows that TOPFLASH-Luciferase reporter activated by Wnt8 was further potentiated by co-injection with PI4K2 β -Mo. In contrast, PI4K2 α -Mo inhibited Wnt8-induced TOPFLASH-Luciferase reporter activity (Fig.3B), in line with the reported canonical Wnt inhibitory effect²⁹. To control for the specificity, we performed rescue experiments (Fig.3A). Unlike the GFP negative control, full length GFP-PI4K2 β mRNA was able to provide substantial luciferase rescue with augmented TOPFLASH activity. Overexpression of PI4K2 β by injection of up to 2000pg mRNA did not have significant effect on the Wnt8-induced TOPFLASH-Luciferase reporter activity (Fig.3A) and higher doses were toxic.

Fig.3. PI4K2 β is involved in both the canonical and non-canonical Wnt signalling. (A-D) TOPFLASH/ATF-2-luciferase reporter assays in *Xenopus* embryos show potentiation of Wnt signals by PI4K2 β -Mo and rescue by PI4K2 β mRNA in contrast to effects of PI4K2 α . Embryos were injected at the animal region of two dorsal blastomeres at 4-cell stage with reporter DNAs, morpholinos (Mos) and/or mRNAs as indicated and assayed at stage 11 in pools of 3. Histograms represent three separate samples from each of three independent egg batches for each condition. Values are given as mean $\pm$ SEM (Student's *t*-test without ANOVA⁵⁸, $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***).

To determine effects of perturbations on the non-canonical PCP/Wnt signalling, embryos were co-injected with Wnt11 mRNA, ATF-luciferase reporter⁴⁰ and CMV-Renilla reporter together with PI4K2 α or - β -Mo and/or the corresponding mRNA. Fig.3C shows that ATF-luciferase reporter activated by 600pg of wnt11 was further potentiated by co-injection with PI4K2 β -Mo, but not

PI4K2 α -Mo (Fig.3D). To control for the specificity, we performed luciferase rescue experiments. Fig.3C shows that GFP alone did not repress the ATF-luciferase reporter activity, while a substantial luciferase rescue with augmented ATF-luciferase activity using 2000g of full length PI4K2 β mRNA. Overexpression of PI4K2 β by injection of up to 2000pg mRNA did not have significant effect on the ATF-luciferase reporter activity. It is noted that PI4K2 β cannot activate either reporter on its own (data not shown). Taken together, the reporter gene assays confirm that PI4K2 β indeed acts on the Wnt8-induced canonical and Wnt11-induced non-canonical PCP signalling pathways.

PI4K2 β is required for Wnt signalling in the receiving tissue

Both PI4K2 α / β have been implicated in Golgi function and protein secretion²³ and hence, we wanted to test if PI4K2 α / β -Mo acts on secretion of Wnt or on the response to Wnt. We carried out the animal cap explant sandwich assay, an established method in *Xenopus* for examining cell-autonomous versus non-autonomous effects on signalling^{41, 42}. As described in Materials and Methods and schematized in Fig.4A-C, two groups of embryos (i.e. “sending” versus “receiving”) were injected separately at 2-4-cell stage with Wnt8 mRNA and reporter DNA respectively (Fig.4A). PI4K2 α / β -Morpholinos were co-injected in some embryos with the Wnt8 mRNA to test for effects of PI4K2 α / β depletion on the production or secretion of Wnt, and in some embryos injected with the TOPFLASH reporter to test effects on the response to Wnt. Animal caps were subsequently cut out at stage 8 - 10 and the caps of the sending embryos were conjugated with caps from receiving embryos to form “sandwiches” in which only one contained Mo (Fig. 4B,C). The explants were cultured until they reached stage 12 and harvested for luciferase assay. Fig. 4D,E shows reporter activity induced by Wnt8 was potentiated by co-injection with PI4K2 β -Mo and inhibited by PI4K2 α -Mo respectively in the whole embryo control or when co-injected in the same half of the conjugate as the reporter (Fig.4D). No significant difference was observed when PI4K2 β -Mo was co-injected in the Wnt-overexpressing tissue. Similarly, PI4K2 α -Mo in the same experiments inhibited canonical Wnt signal response and had no effect in the Wnt overexpressing tissue (Fig.4E), consistent with its published role in Wnt signal transduction²⁹. These results indicate that both PI4K2 α / β act on the response to a Wnt signal rather than Wnt production or secretion. Unlike PI4K2 α , PI4K2 β depletion potentiates the canonical Wnt signal response, suggesting that its normal function is inhibitory for this pathway.

Fig.4. Both PI4K2 α /- β -Mo act on signal reception/transduction of Wnt. TOPFLASH luciferase reporter assay in *Xenopus* embryos and explants. A) Schematic diagram of the experimental procedure. See text (Materials & Methods and Results) for details. D&E) Both PI4K2 α /- β -Mos potentiated Wnt8-induced TOPFLASH-Luciferase reporter activity when co-injected in the same half of the conjugate as the reporter. Histograms represent luciferase assays from three independent experiments (total of at least 3 pools of 5 sandwiches each per condition per experiment). Values are shown as mean $\pm$ SEM

Phosphorylation of serine-265 by PAR-1 regulates the subcellular localisation of PI4K2 β *in vivo*

The primary sequence of PI4K2 β contains a PAR-1 phosphorylation consensus site KVG^S⁴³, but it has not previously been identified as a PAR-1 substrate. To test whether this site is phosphorylated by PAR-1, we mutated serine (Ser265) to alanine to block PAR-1 phosphorylation. The PI4K2 β wild type and the mutated (PI4K2 β SA) proteins were transcribed and translated *in vitro* and then incubated with purified GST-tagged human MARK3 wild type (WT) or kinase dead (KD) proteins and analyzed by SDS-PAGE and Western blotting. Fig.5A shows the Ser265Ala mutation abolishes the appearance of a down-shifted band seen with wild type protein confirming Ser265 as a phosphorylation site *in vitro*.

Fig.5. The consensus PAR-1 (Mark2/3) target site on PI4K2 β is phosphorylated *in vitro* and used *in vivo* to regulate its apicobasal localisation. (A) Western blot showing *in vitro*-synthesised PI4K2 β shows PAR-1 kinase activity-dependent downshift (strong lower band of doublet) which is lost upon mutation of Ser-265 to Ala. (B) Western blot of GFP-tagged and probed PI4K2 β wild type or SA mutant showing altered mobility of the latter. (C-F'') Confocal images of cryosections of stage 10.5-11 embryonic ectoderm stained using anti-mCherry/GFP primary antibodies to detect mCherry- or GFP-tagged PI4K2 α / β wild type or -SA mutant from injected mRNA encoding the indicated proteins (F' and F'' are boxed details of panel F). mCherry-PI4K2 β wild type has a more cell cortical localisation, predominantly in the basolateral cortex of superficial (apicobasally polarised) cells, while the mCherry-PI4K2 β SA mutant is seen at both apical and basolateral cell cortex domains. (G-L) Confocal images of sections from mCherry-PI4K2 β wild type or -SA mutant mRNA-injected embryos as indicated co-stained using antibodies to endogenous Vangl2 protein. Both PI4K2 β wild type and SA mutant partially co-localise with endogenous Vangl2 in the superficial ectoderm, ectopically apical in the SA mutant (white arrowhead in panel L). At least 5 embryos per experiment were examined and (C-P) are representative sections of at least three independent experiments.

To test whether this site is also phosphorylated *in vivo*, mRNA encoding GFP-PI4K2 β wild type or SA mutant were injected at the animal region of two dorsal blastomeres at 4-cell stage and the mobility of their protein products compared by Western blot analyses. Fig.5B shows that PI4K2 β wild type migrated with a reduced, rather than increased, mobility upon gel electrophoresis as compared to PI4K2 β SA mutant. These results suggest that serine residue serine-265 within the PAR-1 consensus phosphorylation site is a target for *in vivo* modification, very likely phosphorylation by PAR-1.

To investigate the significance of this site, we studied the subcellular distribution of mCherry-PI4K2 β wild type or SA mutant protein and its mCherry-PI4K2 α counterparts in *Xenopus* embryonic ectodermal cells and determine if such distribution is affected by PAR-1 phosphorylation (Fig.5C-F). Fig.5C shows overexpression of mCherry alone resulted in a diffuse pattern in the cytoplasm. Similarly, mCherry-PI4K2 α wild type and -SA mutant were localised relatively diffusely (Fig.S4A-B). In contrast, mCherry-PI4K2 β wild type (Fig.5D) has a striking enrichment in the basolateral cortex of superficial ectoderm cells, and appears to be excluded from the apical domain. It should be noted that this domain in *Xenopus* embryos contains a number of proteins associated with adherens junctions in more mature mammalian epithelia e.g. occludin⁴⁴. The mCherry-PI4K2 β SA mutant (Fig.5E) is localised to both apical and basolateral membranes and appears somewhat less restricted to the cortex compared to the deeper cytoplasm (allowing for the fact that this is also the location of large, impermeable yolk platelets). Co-injection of the two constructs interfered with expression of both, a phenomenon we have observed with other proteins⁴⁵. However, injection of GFP-PI4K2 β wild type and mCherry-PI4K2 β SA mutant into nearby parts of same embryo highlighted that GFP-PI4K2 β wild type is more basolaterally localised than the mCherry-PI4K2 β SA mutant (Fig.5F-F"). Our data suggest that phosphorylation of Serine-265 of PI4K2 β by PAR-1 regulates the subcellular localisation of PI4K2 β , which is consistent with PAR-1's larger role in apicobasal polarization in epithelia.

Finally, we asked whether PAR-1-phosphorylation-dependent localisation of PI4K2 β might begin to explain its interaction with Wnt-PCP signalling, since the latter has long been known to occur exclusively at or near the apical domain (mostly peri-apically near adherens junctions) and depends on that localisation⁴⁶. We examined the effect of expression of mCherry-PI4K2 β wild type or SA mutant on the localisation of a core PCP protein, Vangl2 (Fig.5G-P). We found that,

Vangl2 was co-localised with PI4K2 β , being basolateral in wild-type tissue (Fig.5G-I), but apically mislocalised in PI4K2 β SA mutant-injected embryos (Fig.5J-P).

DISCUSSION

In this paper, we have described an *in vitro* screen for novel targets of PAR-1 in which we identified phosphatidylinositol 4-kinase 2-beta (PI4K2 β) as one such target (Fig.1). We have further shown that depletion of PI4K2 β in *Xenopus* embryos leads to phenotypes characteristic of perturbation of Wnt pathways, particularly the non-canonical PCP pathway required for normal convergent extension (Fig.2). We go on to demonstrate that these Wnt pathways are indeed perturbed by depletion of PI4K2 β and rescued by re-expression (Fig.3). It is surprising that both a gain- and loss-of-function of PI4K2 β cause similar dose-dependent defects in dorsoanterior structure associated with Wnt signalling – a severely reduced head and a dorsally bent axis. Severe reduction of heads can be due to early loss of canonical Wnt signalling, which in turn leads to reduced organiser size or activity and such “ventralisation” usually has a strong effect in reducing or even abolishing notochord tissue⁴⁷. This is however not the case since our results show that PI4K2 β -depleted embryos have normally differentiated but disorganised notochords. Head reduction can also be due to excess canonical Wnt signalling after midblastula stages or later in development⁴⁸. Our reporter assays show that PI4K2 β is indeed involved in the Wnt8-induced canonical pathway, with depletion producing a modest increase. This suggests an inhibiting role for the endogenous protein and a late excess of canonical Wnt signalling as the cause of the head reduction phenotype. Although PI4K2 β has previously been identified as a promoter of canonical Wnt signalling in zebrafish⁴⁹, it is possible that the regulation of Wnt signalling by PI4K2 β is context-dependent as we have yet to demonstrate that xPI4K2 β itself is able to induce double axis formation in *Xenopus* embryos (internal communication), a positive functional readout for Wnt activity^{50, 51}.

Dorsally bent axes suggest a defect in the control of convergent extension movement, which is regulated by non-canonical Wnt-planar cell polarity³⁸ or Wnt-calcium signalling pathways^{8, 10, 52}. It is well established that the bent-axis phenotype in *Xenopus* is caused by both gain- and loss-of-function of PCP components^{12, 39}. Further link between PI4K2 β and non-canonical Wnt pathway is demonstrated by the thickened and disorganised notochord of embryos depleted for,

or overexpressing with PI4K2 β , suggesting the PCP pathway is indeed disrupted. This is consistent with our reporter assays which show that PI4K2 β , in contrast to PI4K2 α , inhibits the Wnt11-induced non-canonical Wnt pathway. In addition, our experiments using the animal cap “sandwich” assay show that this inhibitory activity is in Wnt-responding tissue, not in Wnt production or secretion (Fig.4). Taken together, our data suggest that PI4K2 β is a functional inhibitor in both canonical and non-canonical Wnt pathways in early *Xenopus* development.

Par1 phosphorylation excludes PI4K2 β from the apical cortex

It is known that PI4K2 β contain a PAR-1 phosphorylation consensus site KVGS, but it has yet been identified as a PAR-1 substrate. We mutated serine (Ser-265) of PI4K2 β to alanine and the electrophoretic mobility shift assay showed that the Ser-265Ala mutation abolishes the appearance of a down-shifted band seen with wild type protein, confirming that this site is critical for PAR-1 phosphorylation in *in vitro* system (Fig.5). We showed that PI4K2 β wild type migrated with a reduced mobility upon gel electrophoresis as compared to PI4K2 β SA mutant, suggesting that serine residue 265 is a target for *in vivo* PAR-1 phosphorylation. In addition, PI4K2 β wild type and its non-phosphorylatable SA mutant have a more prominent plasma membrane localisation as compared to PI4K2 α . We show that PI4K2 β wild type is found predominantly in the basolateral cortex of superficial ectoderm cells, while mutation of the PAR-1 phosphorylation site is sufficient to abrogate this localisation. PAR-1 is known to organise other downstream polarity proteins via mutual phosphorylation reactions to exclude each other from their respective regions of the cortex⁵³. For instance, Par1 directly phosphorylates Par3 and exclude it from the apical cortex⁵⁴. The same mechanism may apply to exclude PI4K2 β from cortical regions that contain Par1. We believe that segregation of PI4K2 β into basolateral domain of the cell cortex by PAR-1 phosphorylation is essential to its biological functions.

PAR-1, PI4K2 β and non-canonical Wnt signalling

Previously, we showed that different PAR-1 isoforms control different branches of the Wnt signalling pathways⁶ i.e. Par1A and Par1BX control the canonical branch while PAR-1BY controls the non-canonical Wnt planar cell polarity (PCP) signalling branch. Here, we show how the polarity information of the cell could affect the non-canonical Wnt-PCP pathway via PI4K2 β . A key feature of the non-canonical Wnt-PCP pathway is the asymmetric distribution of their core PCP components such as Dishevelled (Dvl)³⁰, Lethal Giant Larvae (Lgl)⁵⁵ and Van Gogh-like (Vangl)⁵. Specifically, we showed PI4K2 β colocalised with the Vangl2 at the basolateral domain

of apicobasally polarised superficial cells in *Xenopus* ectoderm (Fig.5). Vangl has been shown to be involved in the non-canonical PCP pathway via the activation of the Jun N-terminal kinase (JNK) cascade¹⁸. Furthermore, dorsal injection of PI4K2 β disturbs convergent extension movements and the resulting phenotypes strongly resemble those produced by perturbation of Vangl¹⁸, suggesting that these genes may act in a common pathway.

We speculate that the correct localisation of PI4K2 β is a prerequisite for the proper function of PCP proteins. This is consistent with the data of Hammond, who reported the function of plasma membrane PI4P is required to contribute to the pool of polyanionic lipids for membrane targeting of proteins⁵⁶. We believe that the co-localisation of PI4K2 β and Vangl2 may involve Dishevelled (Dvl) since Dvl was found to interact with both PI4K2 β ³⁰ and Vangl in mammalian cells⁵⁷.

In conclusion, using gain-of-function and loss-of-function approaches, we demonstrate that PI4K2 β regulates the vertebrate Wnt-PCP signalling pathway. Our results provide compelling evidence that the novel interaction between PI4K2 β and the core PCP component Vang2 links it to the apicobasal polarity regulator PAR-1. These findings represent an important advancement in our understanding of the molecular mechanisms that integrate the establishment of cell polarity with Wnt signalling pathways.

METHODS

Screen for novel phosphorylation targets of PAR-1

The full-length, normalized *Xenopus laevis* oocyte cDNA library used in this study was gifted by Vincenzo Costanzo (CRUK, now at IFOM, Milan). An expression library screening method described³³ was adapted with modifications. FluoroTect™ Green Lys *in vitro* Translation Labelling System (Promega) was used for protein labelling in place of ³⁵S-methionine in the original protocol. GST-tagged human MARK3 wild type (WT) and kinase dead (KD) proteins were expressed from cDNA clones in pGEX6P-2 vector (gift of Dario Alessi, MRC Protein Phosphorylation Unit, Dundee, UK) and purified as published³⁴. *In vitro*-translated protein pools were subjected to *in vitro* phosphorylation reaction and separated by SDS-PAGE. Gels were rinsed with water and scanned by Typhoon 9210 Imager (GE Healthcare, Amersham, UK) using the green laser (532 nm), emission filter “526 SP Fluorescein, AlexaFluor488”, sensitivity “High” and pixel size at 100 μ m. Image files were analysed using ImageJ (<http://rsbweb.nih.gov/ij/index.html>). For further analysis, full-length *Xenopus laevis*

phosphatidylinositol 4-kinase type 2- α and - β (PI4K2 α / β) clones in pExpress-1 vector
 395 were purchased from Source Bioscience and were sub-cloned into pCS2plus with eGFP/mCherry
 at the N-terminus, respectively.

Ethical Approval

Animal work was approved by the King's College Institutional Ethics Committee and authorised
 under a UK Home Office Project Licence to J.B.A.G.

Preparation of synthetic RNA and microinjection of embryos

Plasmids were linearised using *Not1* and capped mRNAs were synthesized by *in vitro*
 transcription using the mMessage mMachine SP6 transcription kit (Life Technologies). The
 quality of synthesised mRNA was checked by 1% agarose gel electrophoresis. Embryos were
 obtained by artificial fertilisation, dejellied in 2% cysteine hydrochloride (pH 7.8-8.0) and cultured
 405 in 1/10 NAM. Microinjections were carried out in 3% Ficoll at 2- to 4-cell stage in a total volume
 of 10nl. Amounts injected indicated in the text are per embryo.

TOPFLASH/ATF Luciferase reporter assay

Embryos were injected at the animal region of the two dorsal blastomeres at 4-cell stage. 50pg
 TOPFLASH (Upstate), 25pg CMV-Renilla-Luciferase reporter and 6pg Wnt8 mRNA were used for
 410 the canonical pathway. 100pg ATF-luciferase reporter⁴⁰, 0.5pg CMV-Renilla-Luciferase reporter
 and 600pg Wnt11 mRNA were used for the non-canonical pathway. The reporter plasmids were
 injected alone or in combination with 15ng PI4K2 α / β -Mo or 500-1500pg PI4K2 α / β mRNAs.
 Embryos were collected at stage 11 in pools of 3 embryos and assayed for luciferase activity in
 triplicate (total of >27 injected embryos). Luciferase activity was measured in accordance with
 415 the manufacturer's recommendations (Promega) and 25 μ l of cell lysate was used for Luciferase
 detection. The measured luciferase activity was normalised against the measured Renilla
 reporter reading.

Animal cap explant conjugation assay

Embryos were injected at the animal region of the two dorsal blastomeres at 4-cell stage with
 420 either 6pg Wnt8 mRNA or 50 pg TOPFLASH reporter plus 25 pg Renilla reporter DNAs. To test
 effects on the response to Wnt, 15 ng PI4K2 α or - β -Morpholinos (Mo) were co-injected with the
 reporter, while to test effects on the production/secretion of Wnt, the Mo was co-injected with
 the Wnt8 mRNA. Animal caps were excised at mid-blastula (stage 8-9) and Wnt8-injected caps
 placed against reporter DNA-injected caps for culturing overnight in 3/4NAM at 14°C to early
 425 neurula (stage 14) when extracts were made from pools of 5 conjugates and luciferase assays
 conducted as above.

Immunofluorescence and hematoxylin staining

Embryos were fixed at stage 10.75 to 12 in MEMFA for 2 hours at room temperature or Dent's fixative⁶ for 6 hours at -20°C (rehydrate with gradual change of methanol/PBS) and embedded in fish gelatin (16.7ml of 40% gelatin (Sigma-Aldrich) and 7.5g sucrose in 50ml ddH₂O) at 4°C overnight. Embedded samples were sectioned using a Cryostat and serial 15µm frozen transverse sections of the entire embryos were collected. For hematoxylin staining, slides were stained with 50% hematoxylin solution (Sigma-Aldrich), washed with tap water, and differentiated with 0.1% HCl. For immunofluorescence, sections were permeabilised and blocked in PBS containing 0.01% Triton X-100 and 5% Goat/Donkey serum at room temperature for 10 minutes. Mouse monoclonal to GFP (clone 3E6, Molecular Probes) 1:500, rabbit/mouse polyclonal to mCherry (Abcam) 1:500, goat polyclonal to Vang2 (N-13, Santa Cruz) 1:250 were used overnight at 4°C. This is followed by Alexi Fluor-488 and/or -568 (Molecular Probes) 1:500 for 1 hour and DAPI 1:10 000 for 5 minutes at room temperature. Samples were mounted with glycerol and analysed with confocal microscope. Confocal images were taken using either a 20x, 40x oil or 63x glycerol objective. For phenotypic studies, embryos were grown to stage 33 to 35 and fixed with 4% PFA. Embryos were scored as normal (white), shorter (light gray), bent (dark gray) and super bent (black) as shown in the Fig.S3.

Western Blot

Two animal blastomeres of four-cell stage were injected with 500pg eGFP-PI4K2α/-β mRNA. Five injected embryos per sample were lysed at stages 10.5–11.5 in 50ul lysis buffer (1% Triton X-100, 50 mM Tris [pH 7.6], 50 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 10 mM NaF, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, Protease Inhibitor Cocktail Complete [Roche]), and the supernatants were centrifuged at 13,000 × g for 10 min. One-third to one embryo equivalents were loaded at per lane on 4%-12% or 8% Bolt® Bis-Tris (Invitrogen). GFP Rabbit IgG Polyclonal Antibody (Molecular Probes) 1:500 and HRP-conjugated goat anti-rabbit IgG (Thermo Scientific) 1:3000 were used sequentially in blocking buffer (1×PBS, 0.1% Tween, 5% BSA).

Morpholino antisense oligonucleotide injection

Morpholinos (Gene Tools) were re-suspended in water to a concentration of 1mM and stored at -20°C. The following gene specific morpholinos were used in this study: PI4K2α-Mo(ATACTAACGGGCTGGTCTCATCCAT)²⁹, PI4K2β-Mo-1 (GCTCAGTCGTTTGCTTCTGCTCCAT) and PI4K2β-Mo-2 (CTCCCTT CTATTCCCAATGAAAGCA). 10-20ng per embryo was used.

REFERENCES

- 460 1. Matenia D, Mandelkow EM. The tau of MARK: a polarized view of the cytoskeleton. *Trends in biochemical sciences* **34**, 332-342 (2009).
2. Huynh JR, St Johnston D. The origin of asymmetry: early polarisation of the Drosophila germline cyst and oocyte. *Current biology : CB* **14**, R438-449 (2004).
- 465 3. Kusakabe M, Nishida E. The polarity-inducing kinase Par-1 controls Xenopus gastrulation in cooperation with 14-3-3 and aPKC. *The EMBO journal* **23**, 4190-4201 (2004).
4. Sun TQ, *et al.* PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. *Nature cell biology* **3**, 628-636 (2001).
- 470 5. Ossipova O, Chu CW, Fillatre J, Brott BK, Itoh K, Sokol SY. The involvement of PCP proteins in radial cell intercalations during Xenopus embryonic development. *Developmental biology* **408**, 316-327 (2015).
- 475 6. Ossipova O, Dhawan S, Sokol S, Green JB. Distinct PAR-1 proteins function in different branches of Wnt signaling during vertebrate development. *Developmental cell* **8**, 829-841 (2005).
- 480 7. Saito-Diaz K, *et al.* The way Wnt works: components and mechanism. *Growth factors (Chur, Switzerland)* **31**, 1-31 (2013).
8. Kuhl M. Non-canonical Wnt signaling in Xenopus: regulation of axis formation and gastrulation. *Seminars in cell & developmental biology* **13**, 243-249 (2002).
- 485 9. Miller JR, Rowning BA, Larabell CA, Yang-Snyder JA, Bates RL, Moon RT. Establishment of the dorsal-ventral axis in Xenopus embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. *The Journal of cell biology* **146**, 427-437 (1999).
- 490 10. Wallingford JB, Ewald AJ, Harland RM, Fraser SE. Calcium signaling during convergent extension in Xenopus. *Current biology : CB* **11**, 652-661 (2001).
- 495 11. Tada M, Smith JC. Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development (Cambridge, England)* **127**, 2227-2238 (2000).
12. Wallingford JB, Fraser SE, Harland RM. Convergent extension: the molecular control of polarized cell movement during embryonic development. *Developmental cell* **2**, 695-706 (2002).
- 500 13. Komiya Y, Habas R. Wnt signal transduction pathways. *Organogenesis* **4**, 68-75 (2008).
14. Gray RS, Roszko I, Solnica-Krezel L. Planar cell polarity: coordinating morphogenetic cell behaviors with embryonic polarity. *Developmental cell* **21**, 120-133 (2011).
- 505 15. Goto T, Keller R. The planar cell polarity gene strabismus regulates convergence and extension and neural fold closure in Xenopus. *Developmental biology* **247**, 165-181 (2002).

16. Wallingford JB, Goto T, Keller R, Harland RM. Cloning and expression of *Xenopus* Prickle, an orthologue of a *Drosophila* planar cell polarity gene. *Mechanisms of development* **116**, 183-186 (2002).
17. Luga V, *et al.* Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* **151**, 1542-1556 (2012).
18. Park M, Moon RT. The planar cell-polarity gene *stbm* regulates cell behaviour and cell fate in vertebrate embryos. *Nature cell biology* **4**, 20-25 (2002).
19. Bastock R, Strutt H, Strutt D. Strabismus is asymmetrically localised and binds to Prickle and Dishevelled during *Drosophila* planar polarity patterning. *Development (Cambridge, England)* **130**, 3007-3014 (2003).
20. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**, 651-657 (2006).
21. Balla A, Balla T. Phosphatidylinositol 4-kinases: old enzymes with emerging functions. *Trends Cell Biol* **16**, 351-361 (2006).
22. Wang YJ, *et al.* Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell* **114**, 299-310 (2003).
23. Wei YJ, *et al.* Type II phosphatidylinositol 4-kinase beta is a cytosolic and peripheral membrane protein that is recruited to the plasma membrane and activated by Rac-GTP. *The Journal of biological chemistry* **277**, 46586-46593 (2002).
24. Balla A, Tuymetova G, Barshishat M, Geiszt M, Balla T. Characterization of type II phosphatidylinositol 4-kinase isoforms reveals association of the enzymes with endosomal vesicular compartments. *The Journal of biological chemistry* **277**, 20041-20050 (2002).
25. Craige B, Salazar G, Faundez V. Phosphatidylinositol-4-kinase type II alpha contains an AP-3-sorting motif and a kinase domain that are both required for endosome traffic. *Molecular biology of the cell* **19**, 1415-1426 (2008).
26. Minogue S, Waugh MG, De Matteis MA, Stephens DJ, Berdichevski F, Hsuan JJ. Phosphatidylinositol 4-kinase is required for endosomal trafficking and degradation of the EGF receptor. *Journal of cell science* **119**, 571-581 (2006).
27. Salazar G, Craige B, Wainer BH, Guo J, De Camilli P, Faundez V. Phosphatidylinositol-4-kinase type II alpha is a component of adaptor protein-3-derived vesicles. *Molecular biology of the cell* **16**, 3692-3704 (2005).
28. Simons JP, *et al.* Loss of phosphatidylinositol 4-kinase 2alpha activity causes late onset degeneration of spinal cord axons. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 11535-11539 (2009).
29. Pan W, *et al.* Wnt3a-mediated formation of phosphatidylinositol 4,5-bisphosphate regulates LRP6 phosphorylation. *Science (New York, NY)* **321**, 1350-1353 (2008).

30. Qin Y, Li L, Pan W, Wu D. Regulation of phosphatidylinositol kinases and metabolism by Wnt3a and Dvl. *The Journal of biological chemistry* **284**, 22544-22548 (2009).
31. Jung G, *et al.* Molecular determinants of activation and membrane targeting of phosphoinositol 4-kinase IIbeta. *The Biochemical journal* **409**, 501-509 (2008).
32. Alli-Balogun GO, Gewinner CA, Jacobs R, Kriston-Vizi J, Waugh MG, Minogue S. Phosphatidylinositol 4-kinase IIbeta negatively regulates invadopodia formation and suppresses an invasive cellular phenotype. *Molecular biology of the cell* **27**, 4033-4042 (2016).
33. Smith E, *et al.* An ATM- and ATR-dependent checkpoint inactivates spindle assembly by targeting CEP63. *Nature cell biology* **11**, 278-285 (2009).
34. Lizcano JM, *et al.* LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *The EMBO journal* **23**, 833-843 (2004).
35. Solomon MJ, Lee T, Kirschner MW. Role of phosphorylation in p34cdc2 activation: identification of an activating kinase. *Molecular biology of the cell* **3**, 13-27 (1992).
36. Niehrs C, Kazanskaya O, Wu W, Glinka A. Dickkopf1 and the Spemann-Mangold head organizer. *The International journal of developmental biology* **45**, 237-240 (2001).
37. Heisenberg CP, *et al.* Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature* **405**, 76-81 (2000).
38. Axelrod JD, McNeill H. Coupling planar cell polarity signaling to morphogenesis. *TheScientificWorldJournal* **2**, 434-454 (2002).
39. Darken RS, Scola AM, Rakeman AS, Das G, Mlodzik M, Wilson PA. The planar polarity gene strabismus regulates convergent extension movements in Xenopus. *The EMBO journal* **21**, 976-985 (2002).
40. Ohkawara B, Niehrs C. An ATF2-based luciferase reporter to monitor non-canonical Wnt signaling in Xenopus embryos. *Developmental dynamics : an official publication of the American Association of Anatomists* **240**, 188-194 (2011).
41. Darras S, Marikawa Y, Elinson RP, Lemaire P. Animal and vegetal pole cells of early Xenopus embryos respond differently to maternal dorsal determinants: implications for the patterning of the organiser. *Development (Cambridge, England)* **124**, 4275-4286 (1997).
42. Nieuwkoop PD. The organization center of the amphibian embryo: its origin, spatial organization, and morphogenetic action. *Advances in morphogenesis* **10**, 1-39 (1973).
43. Drewes G, Ebner A, Preuss U, Mandelkow EM, Mandelkow E. MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell* **89**, 297-308 (1997).

- 605 44. Chalmers AD, Strauss B, Papalopulu N. Oriented cell divisions asymmetrically segregate aPKC and generate cell fate diversity in the early *Xenopus* embryo. *Development (Cambridge, England)* **130**, 2657-2668 (2003).
45. Panousopoulou E, Tyson RA, Bretschneider T, Green JB. The distribution of Dishevelled in convergently extending mesoderm. *Developmental biology* **382**, 496-503 (2013).
- 610 46. Wasserscheid I, Thomas U, Knust E. Isoform-specific interaction of Flamingo/Starry Night with excess Bazooka affects planar cell polarity in the *Drosophila* wing. *Developmental dynamics : an official publication of the American Association of Anatomists* **236**, 1064-1071 (2007).
- 615 47. Kao KR, Elinson RP. The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Developmental biology* **127**, 64-77 (1988).
- 620 48. Yao J, Kessler DS. Goosecoid promotes head organizer activity by direct repression of *Xwnt8* in Spemann's organizer. *Development (Cambridge, England)* **128**, 2975-2987 (2001).
49. Wieffer M, *et al.* PI4K2beta/AP-1-based TGN-endosomal sorting regulates Wnt signaling. *Current biology : CB* **23**, 2185-2190 (2013).
- 625 50. Kuhl M, Pandur P. Dorsal axis duplication as a functional readout for Wnt activity. *Methods in molecular biology (Clifton, NJ)* **469**, 467-476 (2008).
51. McMahon AP, Moon RT. Ectopic expression of the proto-oncogene *int-1* in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* **58**, 1075-1084 (1989).
- 630 52. Kuhl M, Geis K, Sheldahl LC, Pukrop T, Moon RT, Wedlich D. Antagonistic regulation of convergent extension movements in *Xenopus* by Wnt/beta-catenin and Wnt/Ca²⁺ signaling. *Mechanisms of development* **106**, 61-76 (2001).
- 635 53. McCaffrey LM, Macara IG. Signaling pathways in cell polarity. *Cold Spring Harbor perspectives in biology* **4**, (2012).
54. Benton R, St Johnston D. *Drosophila* PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. *Cell* **115**, 691-704 (2003).
- 640 55. Dollar GL, Weber U, Mlodzik M, Sokol SY. Regulation of Lethal giant larvae by Dishevelled. *Nature* **437**, 1376-1380 (2005).
- 645 56. Hammond GR, *et al.* PI4P and PI(4,5)P₂ are essential but independent lipid determinants of membrane identity. *Science (New York, NY)* **337**, 727-730 (2012).
57. Torban E, Wang HJ, Groulx N, Gros P. Independent mutations in mouse Vangl2 that cause neural tube defects in looptail mice impair interaction with members of the Dishevelled family. *The Journal of biological chemistry* **279**, 52703-52713 (2004).
- 650 58. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology* **1**, 43-46 (1990).

655

ACKNOWLEDGMENTS

We thank H. Steinbeisser and P. Angel (DKFZ-ZMBH Alliance) for ATF-2 luciferase reporter, Vincenzo Costanzo (CRUK, now at IFOM, Milan) for the *Xenopus laevis* oocyte cDNA library and
660 Shane Minogue (UCL) for critically reading the manuscript. This work was supported by a BBSRC research grant BB/J015075/1 to J.B.A. Green.

Author contributions statement:

S.M.T, W.C and J.B.A.G designed the research and analysed data; S.M.T, W.C and J.L performed
665 the experiments; S.M.T and J.B.A.G wrote the manuscript. All authors reviewed the manuscript. The authors declare no conflict of interest.

Supplementary Figures

Fig.S1. Novel phosphorylation targets of PAR-1. A) The screen identified 14 potential targets with MARK3 phosphorylation and 11 of them were known genes/ homologues. B) Sequence comparison of PAR-1 phosphorylation consensus site KVGSR with Xenopus PI4K2 α (top) / PI4K2 β (bottom). The red box highlights PAR-1 phosphorylation consensus site KVGSR.

Fig.S2. PI4K2 α and - β perturbations cause severe but different defects in dorsoanterior structure during embryogenesis. Representative phenotypes at tailbud and tadpole stages of embryos injected with A) PI4K2 β -Mo-2 at the doses shown; B) PI4K2 α or PI4K2 β -Mo-1. Embryos were injected with PI4K2 β -Mo-1/2 resulting in distinct dose-dependent defects, whereas PI4K2 α -Mo led to anteriorisation with a shorter axis and an enlarged cement gland.

Fig.S3. Embryos injected with PI4K2 β -Mo cause severe defects in dorsoanterior structure. Representative phenotypes of embryos injected with control Mo 15ng or PI4K2 β -Mo 15ng. Embryos were scored at tailbud and tadpole stages as normal (white), shorter (light gray), bent (dark gray) and super bent (black) as shown.

Fig.S4. Localisation of PI4K2 α wild type or SA mutant in the superficial ectoderm. Embryos were injected with 100pg mCherry-PI4K2 α wild type or SA mutant mRNA Cryosections of stage 10.5-11 embryonic ectoderm were stained with anti-mCherry. Both mCherry-PI4K2 α wild and SA mutant were localised relatively diffusely.