Development 134, 3339-3348 (2007) doi:10.1242/dev.02881

Porcupine-mediated lipid-modification regulates the activity and distribution of Wnt proteins in the chick neural tube

Lisa M. Galli[1], Tiffany L. Barnes[1,*], Stephanie S. Secrest[1,*], Tatsuhiko Kadowaki[2] and Laura W. Burrus[1,†]

A long-term goal of developmental biology is to understand how morphogens establish gradients that promote proper tissue patterning. A number of reports describe the formation of the Wg (Wnt1) gradient in *Drosophila* and have shown that Porcupine, a predicted membrane-bound O-acyl transferase, is required for the correct distribution of Wg protein. The discovery that Wnts are palmitoylated on a conserved cysteine residue suggests that porcupine activity and Wnt palmitoylation are important for the generation of Wnt gradients. To establish the role of porcupine in Wnt gradient formation in vertebrates, we tested the role of porcupine/Wnt palmitoylation in human embryonic kidney 293T cells and in the chick neural tube. Our results lead us to conclude that: (1) vertebrate Wnt1 and Wnt3a possess at least one additional site for porcupine-mediated lipid-modification; (2) porcupine-mediated lipid-modification of Wnt proteins promotes their activity in 293T cells and in the chick neural tube; and (3) porcupine-mediated lipid-modification reduces the range of activity of Wnt1 and Wnt3a in the chick neural tube. These findings highlight the importance of porcupine-mediated lipid modifications in the formation of vertebrate Wnt activity gradients.

KEY WORDS: Wnt1, Wnt3a, Porcupine, Palmitoylation, Chick, Neural tube, Gradient, Proliferation, BAT-gal

INTRODUCTION

Wnt proteins are potent modifiers of cell proliferation, differentiation and survival during both embryogenesis and carcinogenesis (Logan and Nusse, 2004; Moon et al., 2004). Wnts act as morphogens in a number of model systems (Johnston and Schubiger, 1996; Kiecker and Niehrs, 2001; Lawrence et al., 1996; Neumann and Cohen, 1997; Zecca et al., 1996); thus, the mechanism by which Wnt gradients are formed is a key question in developmental biology. One of the best-characterized Wnt activity gradients is in the developing chick neural tube, where dorsally expressed Wnt1 and Wnt3a create a dorsal-to-ventral proliferation gradient that drives the dorsal expansion of the neural tube (Megason and McMahon, 2002).

Activity gradients, like those observed for Wnt1 and Wnt3a in the neural tube, can reflect a protein activity gradient, which may be influenced by competence and inhibitory factors and/or a protein distribution gradient. The discovery that secreted signaling proteins like Wnts, hedgehogs and Spitz are palmitoylated has raised the possibility that palmitoylation participates in the formation of activity and distribution gradients (Miura et al., 2006; Pepinsky et al., 1998; Willert et al., 2003). A palmitoylation site for mouse Wnt3a has been mapped to cysteine 77 (Willert et al., 2003). Mutation of this cysteine to an alanine results in a significant loss of activity in both autocrine and paracrine assays (Willert et al., 2003). Likewise, mutation of the cognate residue in Wingless (Wg), the *Drosophila* ortholog of Wnt1, causes a loss-of-function *wg* phenotype (Couso and Martinez Arias, 1994; Willert et al., 2003) and a dramatic redistribution of the Wg protein in *Drosophila* embryos (Nusse, 2003). These studies suggest that palmitoylation regulates the activity and distribution of Wnt proteins.

[1]Department of Biology, San Francisco State University, 1600 Holloway Avenue, San Francisco, CA 94132, USA. [2]Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, 464-8601, Japan.

*These authors contributed equally to this work
†Author for correspondence (e-mail: LBurrus@sfsu.edu)

Accepted 29 June 2007

Porcupine is a ubiquitously expressed upstream regulator of Wnt/Wg glycosylation and lipid-modification that is localized to the endoplasmic reticulum (Fradkin et al., 2004; Kadowaki et al., 1996; Noordermeer et al., 1995; Nusse, 2003; Perrimon et al., 1989; Perrimon and Mahowald, 1987; Siegfried et al., 1994; Tanaka et al., 2002; Tanaka et al., 2000; van den Heuvel et al., 1993). Bioinformatic studies predict that Porcupine functions as a membrane bound O-acyl transferase (Hofmann, 2000). Porcupine is required for the lipid-modification of Wnt/Wg (Zhai et al., 2004), but it unknown whether Porcupine is a direct or indirect regulator of Wnt lipidation. Additionally, although Porcupine loss-of-function mutations phenocopy mutations in the palmitoylated cysteine of Wg and show a similar disruption of Wg secretion, it is unknown whether Porcupine directly regulates the modification of Wg (Kadowaki et al., 1996; Nusse, 2003; Siegfried et al., 1994; van den Heuvel et al., 1993; Zhai et al., 2004).

Here we test the hypothesis that porcupine-mediated lipid-modification of Wnt1 and Wnt3a proteins influences the generation of the proliferation gradient observed in the chick neural tube by regulating both the activity and distribution of Wnt proteins. Through a combination of in vitro and in vivo experiments, which include the use of newly developed monoclonal antibodies against chick Wnt1 and Wnt3a, we show that both Wnt1 and Wnt3a possess at least one previously unidentified site for porcupine-mediated lipid-modification. We further show that porcupine promotes Wnt activity and restricts the diffusion of Wnt proteins away from the site of synthesis. Our results suggest porcupine is a key regulator of the Wnt gradient in the developing vertebrate neural tube.

MATERIALS AND METHODS

DNAs and reagents

We thank Randy Moon for supplying pHippy (Kaykas and Moon, 2004), Super8XTopFlash and Super8XFopFlash (Veeman et al., 2003); Andy McMahon for pCIG (Megason and McMahon, 2002), chick (c) *Wnt1* partial cDNA (Hollyday et al., 1995), mouse (m) *Wnt1* (Fung et al., 1985) and *Wnt3a* cDNAs (Gavin et al., 1990); Elena Frolova for a partial *cWnt1* cDNA (Fokina and Frolova, 2006); Tsutomu Nohno for a partial *cWnt3a* cDNA (Kawakami et al., 2000); and Stefano Piccolo for BAT-gal (Maretto et al., 2003).

Other materials include: TX-114 (Fisher); fetal bovine serum (Hyclone); Hybridoma Cloning Factor (Origen); lipofectamine 2000 (Invitrogen); Dual Luciferase Reporter (Promega); Triton X-100; anti-phosphohistone H3 (ser10) polyclonal antibody (Upstate Technologies); anti-tubulin (Santa Cruz); anti-β-galactosidase J1E7 (Developmental Studies Hybridoma Bank); anti-NCAM (Chemicon); goat anti-mouse IgG(H+L)-AP, goat anti-mouse IgG(H+L)-Cy3 and goat-anti-rabbit IgG(H+L)-Cy5 (Jackson ImmunoResearch).

Plasmids

Full-length cDNAs for *cWnt1* and *cWnt3a* have not been isolated. Thus, we used a construct with the mWnt3a signal peptide fused to *cWnt3a* coding sequence (Galli et al., 2004). Because the mature protein contains only chick sequence, we refer to this chimeric protein as cWnt3a. We generated a similar fusion for the chick Wnt1 protein (cWnt1), piecing together amino acids 1-63 from mWnt1 (…EPSLQL) with amino acids 64-370 from cWnt1 (LSRKQ…).

We used pGEX-2T (Amersham) to generate GST:Wnt fusion proteins. For GST:cWnt1, the fusion protein corresponds to amino acids 73-112 of the full-length mouse-chick chimeric Wnt1 protein (RQNPG….QGPNI). For GST:cWnt3a, the fusion protein corresponds to amino acids 208-264 of the full-length mouse-chick chimeric Wnt3a protein (LSGSC….YNFFK). A construct encoding 6×his-tagged Wnt1 was generated by subcloning the same fragments into pET21(b) (Novagen).

We used sequence overlap extension to generate point mutations in cWnt1, cWnt3a and mPorcD (accession AB036749). For cWnt1 and cWnt3a, we changed C93 and C77, respectively, to either a serine or alanine. For mPorcD, we changed the H341 residue to either an aspartic acid or glutamine.

For uniformity of expression in cell culture studies, the following cDNAs were subcloned into pcDNA3.1(–)A (Invitrogen): *cWnt1*, *cWnt1*(C93S), *cWnt3a*, *cWnt3a*(C77S), *mPorcD* and *mPorcD*(H341Q) and *GFP*. The myc/his tag encoded by sequences in pcDNA was intentionally excluded in the resulting protein.

For electroporations, the sequences encoding cWnt1, cWnt1(C93S), cWnt3a, cWnt3a(C77S), mPorcD and mPorcD(H341Q) were subcloned into pCIG (Megason and McMahon, 2002).

For RNAi studies, the Whitehead siRNA selection algorithm identified sequences directed against human (h) PORC (Caricasole et al., 2002; Tanaka et al., 2000; Yuan et al., 2004). The first sequence ('a'), which corresponds to nucleotide 434 of *hPORCD*, is 5′-aaaaAAGGCAGTGTCTCTGGGCTT-CGA-3′, whereas the 'b' sequence corresponds to nucleotide 946 and is 5′-aaaaAAGCTGGAACCTGCCCATGTCTT-3′ (lower case letters represent nucleotides added for subcloning). BLAST analysis verified no significant cross-reactivity with other genes. Double-stranded oligonucleotides encoding these two regions were subcloned into pHippy. A control RNAi construct against *GFP* was generated as described (Kaykas and Moon, 2004).

To test the efficacy of our RNAi constructs, we fused sequences encoding HA-tagged mPorcD (Tanaka et al., 2000) to *hPORC* sequences that include target sequences for the RNAi constructs (Caricasole et al., 2002). This insert was subcloned into pcDNA3.1 for transfections. For transfections, 0.1-1 μg of pcDNA.HA-Porc was transfected alone or with 1.5-18 μg of the GFP or hPORC RNAi constructs.

Generation of monoclonal antibodies against cWnt1 and cWnt3a

GST-Wnt fusion proteins were produced in *E. coli* and purified over a glutathione-agarose affinity column (Burrus and McMahon, 1995). Concentrated pooled fractions (1-8 mg/ml) were sent to Covance for mouse immunizations and fusions. We identified positive clones by ELISA and western blot analysis. Clones 5F1-G11-D1 (Wnt1; IgG1) and 3E9-1B11-H3 (Wnt3a; IgG2b) were used exclusively in this manuscript. These antibodies do not detect mWnt1 or mWnt3a (data not shown).

TX-114 phase-separation assay

Human embryonic kidney 293T cells were plated on 12-well plates the day prior to transfection at a density that would yield 90% confluence on the day of transfection. Transfections were performed with lipofectamine with minor modifications from the manufacturer's instructions. Cells were transfected with 1.6 μg of each vector per well and incubated overnight at 37°C in 5% CO_2. Cells were washed once with PBS (pH 7.4). Then, 0.5 ml 100 mM Hepes (pH 7.4) containing 100 mM NaCl was added to each well. After scraping, cells were triturated with 18G and 25G needles and transferred to microcentrifuge tubes. The TX-114 extraction was performed essentially as described previously (Willert et al., 2003). Samples were precipitated, resuspended, and boiled in 1×SDS-PAGE sample buffer prior to electrophoresis and electroblotting. Western blots were probed with anti-Wnt1, anti-Wnt3a and anti-tubulin. Alkaline phosphatase-conjugated secondary antibodies were used for detection. Each experiment was performed at least three times.

TCF reporter assay

293T cells were plated onto 24-well plates as described above. DNA quantities used in transfections are as follows: pcDNA.GFP, pcDNA.Wnt1, pcDNA.Wnt1(C93S or C93A), pcDNA.Wnt3a, pcDNA.Wnt3a(C77S or C77A), pcDNA.mPorcD, pcDNA.mPorcD(H341D or H341Q) at 0.25 μg/well; pHippy.GFP, pHippy.hPorc(a), pHippy.hPorc(b) at a total of 125 ng/well; Super8XTopFlash or Super8XFopFlash at 0.1 μg/well; RL-CMV at 0.1 ng/well. Luciferase measurements were carried out in a TD-20/20 luminometer. For each assay, five independent replicates were performed. The assay was performed at least three times.

Electroporations

Hamburger and Hamilton (HH) stage-11 to -14 chick embryos (Hamburger and Hamilton, 1951) were electroporated in the neural tube as previously described (Galli et al., 2004). Embryos were embedded and sectioned (16-30 μm) on a Leica cryostat (Galli et al., 2004).

Immunohistochemistry and in situ hybridization of tissue sections

Tissue sections were immunostained with anti-Wnt1, anti-Wnt3a, anti-NCAM, anti-phosphohistone H3, or anti-β-galactosidase and imaged by confocal microscopy as previously described (Galli et al., 2006). In situ hybridizations were performed according to standard protocols.

Quantitation and statistical analysis

Quantification of the data in Fig. 7 by two independent investigators yielded identical results. Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test.

RESULTS

Endogenous Wnt1 protein is localized close to the site of synthesis in the chick neural tube

Previous reports show that a dorsal-to-ventral gradient of Wnt1 and Wnt3a-mediated proliferative activity exists in the developing chick neural tube (Megason and McMahon, 2002). Because regulators of both protein activity and distribution play important roles in the formation of activity gradients, we analyzed the distribution of Wnt1 and Wnt3a proteins in the chick neural tube. To do this, we generated monoclonal antibodies against chick (c) Wnt1 and Wnt3a. Both antibodies are capable of specifically recognizing cWnt1 and cWnt3a on western blots (Fig. 1A) and via immunohistochemistry when overexpressed in cells (Fig. 1B) and tissues (Fig. 2A-F). Only the cWnt1 antibody, however, was able to detect endogenous Wnt protein (Fig. 2G-O). In the western blot (Fig. 1A), the presence of multiple bands migrating at approximately 40 kDa is consistent with previous reports of differential glycosylation of Wnt proteins (Brown et al., 1987; Burrus and McMahon, 1995; Papkoff et al., 1987; Smolich et al., 1993). Likewise, the distribution of cWnt1 and cWnt3a overexpressed in COS7 cells (Fig. 1B) is indistinguishable from that of mouse (m) Wnt1 and Wnt3a, which are localized to the endoplasmic reticulum (Burrus and McMahon, 1995).

We then determined the localization of Wnt proteins expressed in the chick neural tube. The vast majority of ectopic cWnt1 and cWnt3a was distributed at or very near the site(s) of synthesis (Fig. 2A-F). The perinuclear staining observed in the GFP-positive cells

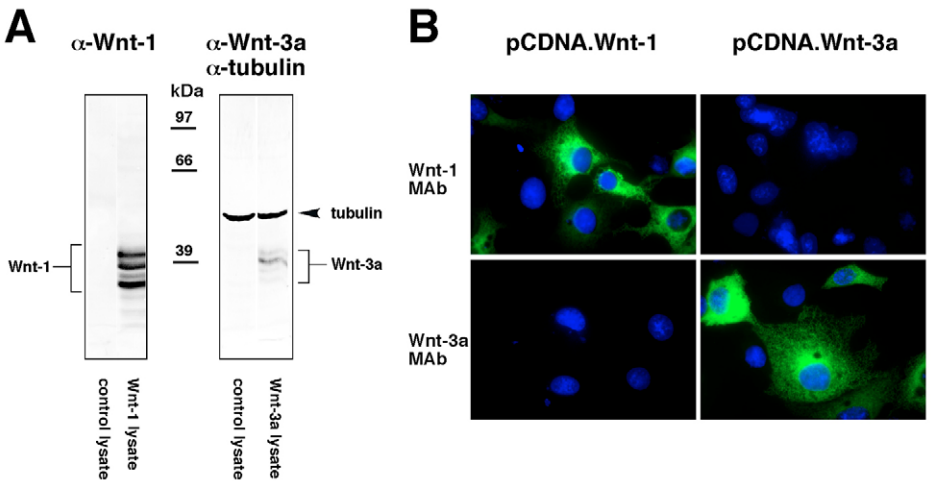

Fig. 1. Antibodies generated against chick Wnt1 and Wnt3a can be utilized for western blots and immunohistochemistry. (**A**) Western blot analysis of 293T cells transfected with control, Wnt1 and Wnt3a shows that antibodies specifically immunoreact with their cognate Wnt protein. A cluster of Wnt proteins migrates at approximately 40 kDa. The Wnt3a blot was also probed with anti-tubulin antibodies as a loading control. (**B**) Immunohistochemical analysis of COS cells transfected with Wnt1 or Wnt3a. Wnt1/Wnt3a were visualized by the addition of Cy3-labeled secondary antibody (green); nuclei were visualized with DAPI (blue).

was reminiscent of that observed in COS cells (Fig. 1B, Fig. 2A-F). We also occasionally observed immunopositive punctae away from the site(s) of synthesis (Fig. 2D).

We then used our Wnt1 antibodies to investigate the distribution of endogenous Wnt1 in sections from wild-type chick embryos. Wnt1 protein was detected in the dorsal neural tube of HH stage-18 chick embryos (Fig. 2G-O). To determine how far endogenous Wnt1 can diffuse from the site of synthesis, we compared adjacent sections that were subjected to immunostaining and in situ hybridization, respectively (Fig. 2J-L). Like ectopic Wnt, endogenous Wnt1 protein was only detectable very close to the site of synthesis. To further delineate the subcellular localization of Wnt1, we double-labeled sections with anti-NCAM (also known as Ncam1), which marks the plasma membrane (Fig. 2M-O). We observed Wnt1 protein adjacent to and overlapping with NCAM, suggesting that Wnt1 is localized within the cell (presumably in the secretory pathway) as well as on the plasma membrane. To our knowledge, this is the first visualization of any Wnt protein in a vertebrate embryo.

Porcupine promotes lipid-modification of Wnt1/Wnt3a

Although Porcupine is an upstream regulator of Wnt activity and distribution in invertebrates, porcupine has not been directly linked to the lipid-modification of vertebrate Wnt proteins. To test whether porcupine might have a role in the lipid-modification of vertebrate Wnt proteins, we transiently expressed wild-type cWnt1 or cWnt3a along with either mPorcD or GFP (control) in 293T cells (Figs 3, 4). We then used a TX-114 phase-separation assay to compare the hydrophobicity of Wnt protein expressed in the presence of either mPorcD or GFP. Four splice variants of porcupine have been identified in the mouse (designated A-D); we chose to use the 'D' variant for these studies because it is the predominant variant expressed in neural tissue (Tanaka et al., 2000). Upon co-expression of cWnt1 or cWnt3a with GFP, we observed that Wnt protein was present in both the aqueous and detergent phases (Figs 3, 4). The presence of some protein in the detergent phase was not unexpected, as porcupine expression is ubiquitous and we had determined via RT-PCR that 293T cells express endogenous porcupine (data not shown). The protein in the aqueous phase exhibited more heterogeneity in apparent molecular mass than that in the detergent phase. These results are consistent with reports that Wnts are differentially glycosylated (Brown et al., 1987; Burrus and McMahon, 1995; Papkoff et al., 1987; Smolich et al., 1993) and that ectopic porcupine promotes processing into more fully glycosylated forms, presumably by an indirect mechanism (Tanaka et al., 2002). It is possible that a different subset of isoforms would have been observed had we assayed Wnt proteins in conditioned medium rather than in cell lysates, as was done here. Upon co-expression of cWnt1 or cWnt3a with mPorcD, we observed a dramatic shift of Wnt protein into the detergent phase. This shift was accompanied by an increase in the total amount of Wnt protein in the aqueous and detergent phases. Tubulin levels remained constant in the presence or absence of porcupine. Thus, in addition to promoting an increase in the hydrophobicity of Wnt, ectopic porcupine also stimulated an overall accumulation of cell-associated Wnt protein. These data are consistent with a role for porcupine as an upstream regulator of the lipid-modification of Wnt proteins.

Bioinformatic analysis coupled with functional analysis of other MBOAT-family members suggested that the H341 residue is required for the acyl transferase activity of mPorcD (Guo et al., 2005; Hofmann, 2000; Lin et al., 2003). To test whether the predicted catalytic domain is required to shift Wnt protein into the hydrophobic phase of a TX-114 extraction, we carried out parallel experiments using a porcupine construct with an aspartic acid (D) or a glutamine (Q) residue substitution of H341 [designated mPorcD(H341D) and mPorcD(H341Q), respectively]. Upon co-transfection of cells with Wnt3a and either mPorcD(H341D) or mPorcD(H341Q), the relative proportion of Wnt3a protein in the aqueous/detergent fractions was not detectably changed as compared with co-transfection of Wnt3a and GFP (Fig. 3). Furthermore, co-transfection of the mutant porcupine proteins did not increase the level of cell-associated Wnt protein (Fig. 3). In fact, we consistently observed a reduction in Wnt protein accumulation in cells co-transfected with mutant porcupine. Thus, the histidine in the predicted acyl transferase active site of porcupine is required for the hydrophobic shift observed for Wnt protein in the TX-114 phase-separation assay.

Wnt1 and Wnt3a possess at least one additional site for porcupine-mediated lipid-modification

Although a single palmitoylated cysteine residue has been identified in Wnt proteins, the existence of additional modification sites was not ruled out (Willert et al., 2003). To determine whether additional modification sites exist, we tested the ability of mPorcD to stimulate the hydrophobic modification of Wnt1 and Wnt3a, in which the previously identified cysteine residue was changed to a serine residue [designated Wnt1(C93S) and Wnt3a(C77S)]. As

before, mPorcD dramatically stimulated the shift of mutant cWnt1 and cWnt3a into the detergent phase as compared with co-transfection with GFP (Fig. 4). Because oxyester-linked palmitate occasionally occurs on serines (Bernstein et al., 2004), we also tested the ability of porcupine to modify cWnt1(C93A) and cWnt3a(C77A) proteins. Our results show that porcupine is still able to promote the lipid-modification of these mutant proteins (see Fig. S1 in the supplementary material), demonstrating that the previously identified cysteine residue is not the only site modified by porcupine.

Porcupine and palmitoylation are required for activity in 293T cells

Because porcupine and palmitoylation might regulate the activity as well as the distribution of Wnt proteins, we investigated the role of porcupine and palmitoylation on Wnt activity. To assay for Wnt activity, we transiently transfected 293T cells with Wnt and porcupine constructs and TCF reporter constructs. TopFlash, a construct containing eight TCF-binding sites upstream of a minimal TA viral promoter that drives expression of firefly luciferase, was used to measure Wnt activity; FopFlash, a similar construct with mutated TCF sites, was used as a negative control (Veeman et al., 2003). All cells were co-transfected with *Renilla* luciferase (pRL-CMV) to control for differences in cell lysis. It is important to note that this assay does not distinguish between autocrine and paracrine Wnt activity.

We first tested whether overexpression of porcupine could augment the activity of cWnt3a in 293T cells. Co-transfection of porcupine and cWnt3a caused a 1.2-fold increase in reporter activity as compared with co-transfection of GFP and cWnt3a (Fig. 5, $P<0.05$). Thus, overexpression of porcupine promotes Wnt activity.

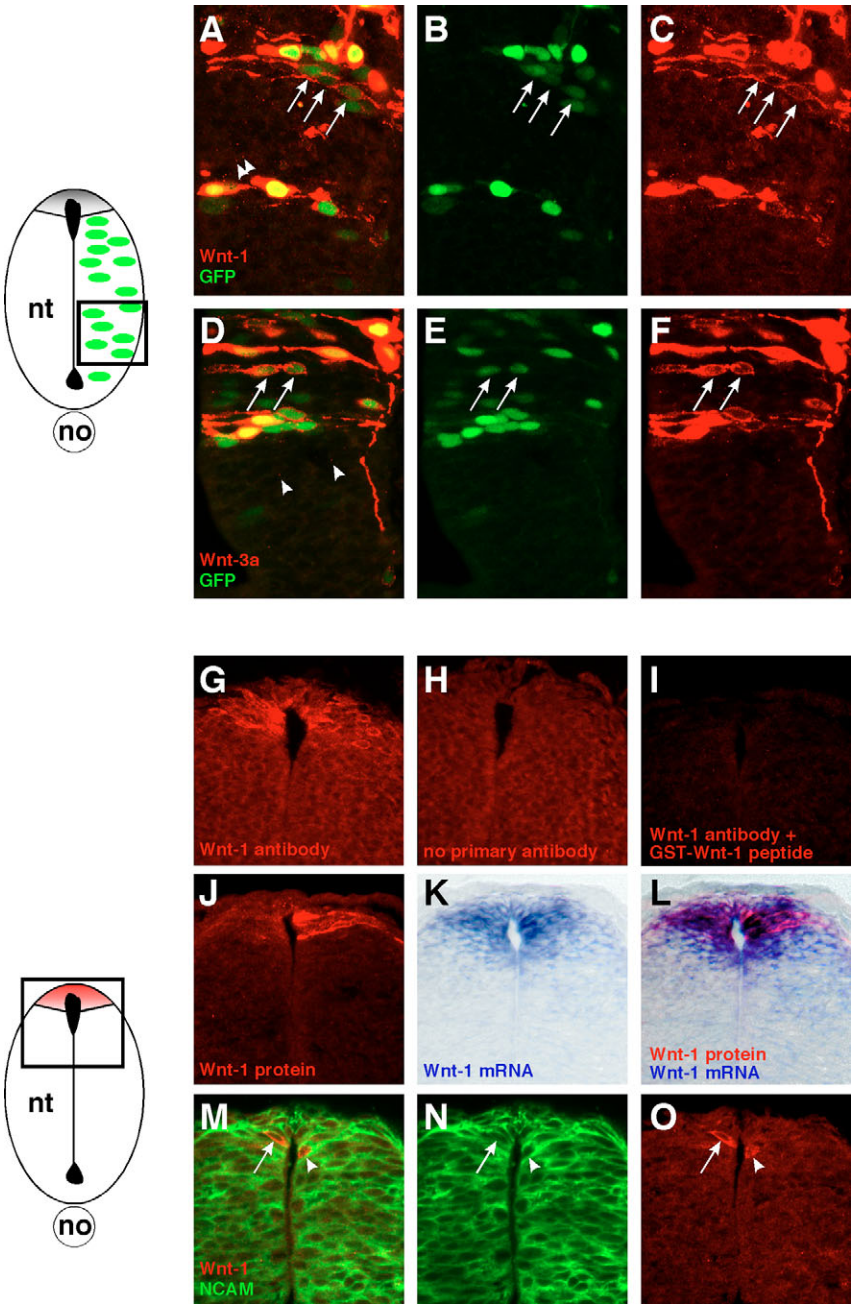

Fig. 2. Localization of Wnt proteins in the chick neural tube. Wnt1 and GFP (**A-C**) or Wnt3a and GFP (**D-F**) were expressed in the neural tube following electroporation. Diagram to left depicts GFP (green) expression in an electroporated neural tube; the boxed region is shown in A-F. GFP-positive embryos were harvested 24 hours post-electroporation (HH stage 18/19), fixed and cryosectioned prior to immunostaining with anti-Wnt1/Wnt3a antibodies. Goat anti-mouse-Cy3 secondary antibodies were used to visualize Wnt proteins (red). All images were collected by confocal microscopy. White arrows point to perinuclear staining; arrowheads indicate immunopositive punctae. No punctae were observed when primary antibody was omitted (data not shown). (**G-O**) A wild-type chick embryo (HH stage 18) was cryosectioned and stained with Wnt1 primary antibody and a Cy3-labeled secondary antibody (red). Diagram to left shows endogenous Wnt1 expression (red) in the neural tube; the boxed region is shown in G-O. G and H are adjacent sections, as are I-L. In H, primary antibody was omitted. In I, excess GST-Wnt1 blocking peptide was added. In J-L, we compare the localization of Wnt1 protein (J) with that of *Wnt1* transcript (K). In M-O, we compare the localization of Wnt1 (red) with NCAM (green) in a single optical confocal layer. In M and O, white arrows point to staining on or just interior to the plasma membrane, whereas the white arrowheads indicate staining that is inside the cell. nt, neural tube; no, notochord.

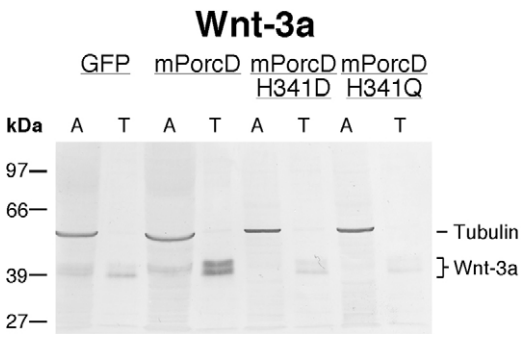

Fig. 3. The conserved histidine residue in the predicted porcupine active site is required for the lipid-modification of Wnt3a. 293T cells were transfected with chick (c) Wnt3a in the presence of either GFP (control), mouse (m) PorcD, mPorcD(H341D) or mPorcD(H341Q). Cells were lysed 24 hours post-transfection and extracted with TX-114 before analysis by SDS-PAGE and western blot. The blots were probed with Wnt3a and tubulin (control) antibodies. Monomeric Wnt3a proteins migrate at ~40 kDa, whereas tubulin migrates at 50 kDa. In the presence of GFP alone, Wnt3a is in both aqueous (A) and detergent (T) phases. Whereas the presence of ectopic porcupine promotes the partitioning of Wnt3a into the hydrophobic phase, ectopic mPorcD(H341D) and mPorcD(H341Q) failed to show a similar effect.

To determine the role of the H341 residue of porcupine, we tested the ability of mPorcD(H341D) and mPorcD(H341Q) to promote Wnt activity. Interestingly, co-transfection with either mutant resulted in diminished reporter activity as compared with controls (Fig. 5, $P<0.05$). These results suggest that the catalytically inactive porcupine is able to act as an inhibitor of endogenous porcupine, possibly via direct competition for substrate.

Although the ability of the porcupine mutants to disrupt Wnt signaling suggests porcupine is required for vertebrate Wnt signaling, we sought to further test this using RNAi specifically targeted against endogenous human (h) PORC in the 293T cells. Double-stranded oligonucleotides containing hPORC target sequences were subcloned into pHippy, which uses opposing and convergent H1 and U6 promoters to drive the expression of both strands (Kaykas and Moon, 2004). As a negative control, we used an RNAi construct designed to knockdown the expression of GFP.

Because no antibodies for hPORC are currently available, we overexpressed HA-tagged hPORC to confirm the ability of hPORC RNAi constructs to knockdown *hPORC* expression (see Fig. S2 in the supplementary material). Because the levels of endogenous *hPORC* transcripts are very low compared with levels of ectopic HA-hPORC in this control experiment (data not shown), we expect even greater levels of knockdown in experiments in which endogenous *hPORC* is the sole target of our RNAi constructs.

Co-transfection of either hPORC RNAi construct along with cWnt3a caused a dramatic reduction of cWnt3a activity as compared with co-transfection with GFP RNAi (Fig. 5). Cells transfected with both hPORC RNAi constructs exhibited a less dramatic loss of cWnt3a-induced TopFlash activity (Fig. 5). Co-transfection of ectopic mPorcD partially rescued the effects of the hPORC RNAi constructs (data not shown). Similar data were obtained for cWnt1 (data not shown). Together, these results show that porcupine is required for Wnt activity in 293T cells ($P<0.01$ for all RNAi constructs).

We then tested whether mutation of C93/C77 in Wnt1/Wnt3a would yield similar results as knocking down endogenous porcupine. cWnt1(C93S) and cWnt3a(C77S) exhibited reduced activity as compared with their wild-type counterparts (Fig. 5). Thus, this cysteine residue is important for the biological activity of Wnt1 and Wnt3a.

Because our TX-114 assays indicated that porcupine can promote the lipid-modification of at least one site in addition to C93/C77, we also tested whether additional sites were likely to play a role in the regulation of Wnt activity by porcupine. To do this, we co-transfected cWnt3a(C77S) with a mixture of the two hPORC RNAi constructs. Cells transfected with these constructs exhibited less TopFlash activity than cells transfected with cWnt3a(C77S) and GFP RNAi (Fig. 5, $P<0.01$). Cumulatively, these results provide strong evidence for the existence of at least one additional site for porcupine modification.

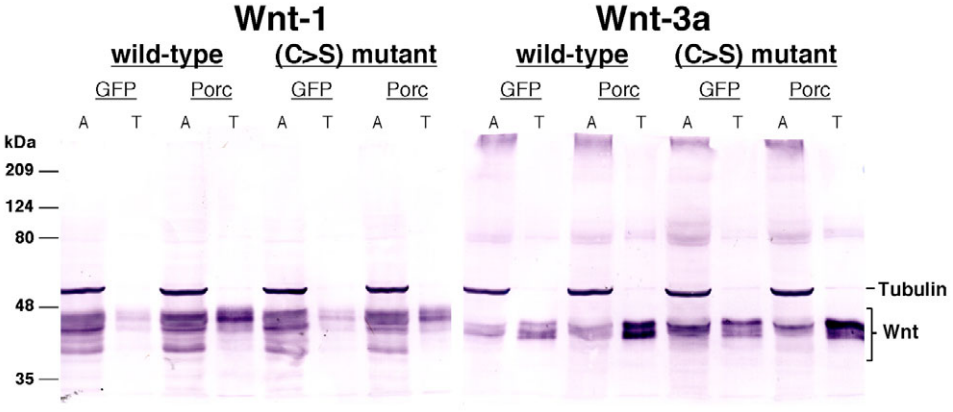

Fig. 4. Porcupine promotes the lipid-modification of both wild-type Wnt1/Wnt3a and mutant Wnt1/Wnt3a, which possess C-to-S point mutations in the cysteine that is known to be palmitoylated. 293T cells were transfected with Wnt1, Wnt3a, Wnt1(C93S) or Wnt3a(C77S) in the presence of GFP (control) or mPorcD. Cells were lysed 24 hours post-transfection and extracted with TX-114 before analysis by SDS-PAGE and western blot. The blots were probed with Wnt1 (left), Wnt3a (right) and tubulin antibodies. Monomeric Wnt1/Wnt3a proteins migrate at ~40 kDa, whereas tubulin migrates at 50 kDa. In the absence of ectopic porcupine, Wnt1 and Wnt3a are found in both aqueous (A) and detergent (T) phases. The presence of ectopic porcupine promotes the partitioning of Wnt1, Wnt3a, Wnt1(C93S) and Wnt3a(C77S) into the hydrophobic phase.

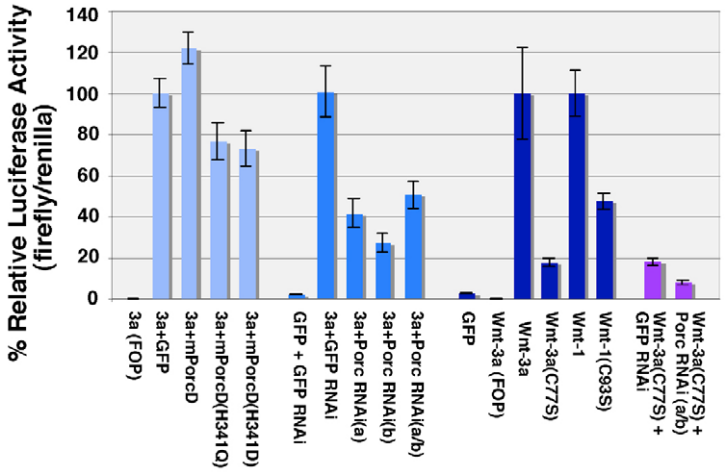

Fig. 5. Porcupine/palmitoylation regulates the activity of Wnt1 and Wnt3a in 293T cells. 293T cells were transfected with Top/FopFlash, *Renilla* luciferase and the indicated pHIPPY (siRNA) and pcDNA expression constructs (Kaykas and Moon, 2004). DNA concentrations were held constant within each series of experiments. The effects of mPorcD and mPorcD mutants on Wnt3a activity are shown in light blue. The effects of co-transfection of hPORC RNAi constructs with Wnt3a are shown in medium blue. The activity of Wnt3a(C77S) and Wnt1(C93S) mutants is shown in dark blue. Finally, TopFlash activity in cells co-transfected with Wnt3a(C77S) and human PORC RNAi constructs is shown in purple. 'FOP' indicates samples in which FopFlash was transfected in place of TopFlash. Error bars indicate standard error. Each experiment was repeated a minimum of three times with five independent replicates each time.

Porcupine and palmitoylation also regulate Wnt activity in the chick neural tube

To test whether the observed roles for porcupine and Wnt palmitoylation in 293T cells are conserved in the chick neural tube, we electroporated porcupine and Wnt constructs into the neural tube and assessed their effect on proliferation. All cDNAs were subcloned into pCIG, a vector that uses the β-actin promoter to drive the expression of a bi-cistronic mRNA, which is then translated into two independent proteins: the experimental protein and a nuclear-localized variant of GFP (Megason and McMahon, 2002). Electroporations were conducted with the anode on the left side of the embryo and the cathode on the right, resulting in transfection of the right side of the neural tube. GFP-positive embryos were harvested 24 hours post-electroporation and immunostained with anti-phosphohistone H3, a marker for cells in late G2/M phase (Hendzel et al., 1997; Wei et al., 1999). In the developing neural tube, mitotic cells are positioned adjacent to the lumen in the ventricular zone. To quantify proliferation in electroporated embryos, we counted the total number of H3-positive cells on the control (left) and experimental (right) sides of the neural tube. To normalize the number of mitotic cells back to the total number of cells, we divided the number of mitotic cells by the area of the control and experimental sides of the neural tube to generate a 'proliferation index'. This means of normalization is valid because we have previously determined that cell size remains constant (Galli et al., 2006). Because the proliferation index is dependent on the stage of the embryo and the axial level analyzed, we further normalized the data by dividing the experimental proliferation index by the control proliferation index. Thus, we expect that ectopic expression of inert constructs would produce a ratio close to 1, whereas those that induce proliferation would yield a ratio of greater than 1, and those that reduce proliferation would generate a ratio of less than 1.

With only a single exception, our results measuring proliferative activity in the neural tube (Fig. 6) mirrored the results obtained in the TopFlash assay in 293T cells (Fig. 5). As in 293T cells, overexpression of either cWnt1, cWnt3a or mPorcD in the neural tube caused a statistically significant increase in the proliferation

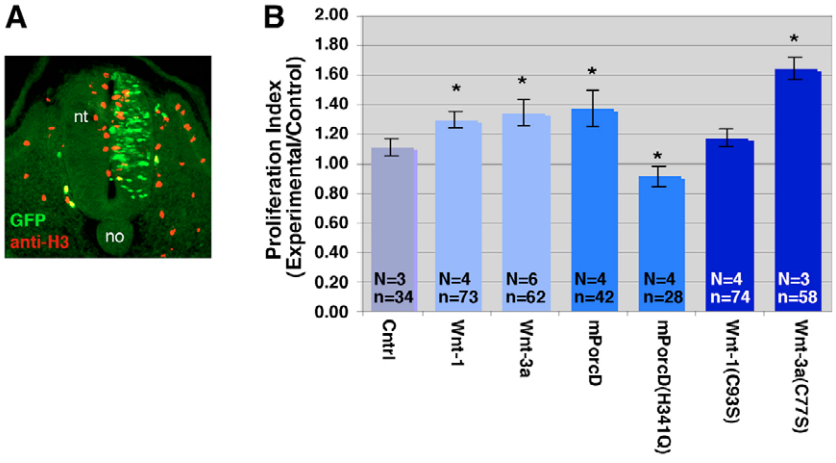

Fig. 6. Porcupine/palmitoylation regulates Wnt-induced proliferation in the chick neural tube. Embryos were electroporated in the neural tube as described in Materials and methods. GFP-positive embryos were harvested for analysis 24 hours post-electroporation. Embryos were fixed, cryosectioned and immunostained with anti-phosphohistone H3 (red), which marks cells in late G2/M phase. (**A**) A section from a representative embryo electroporated with mPorcD. The right side of the neural tube has been electroporated (experimental), whereas the contralateral side serves as a control. (**B**) The ratio of experimental/control proliferation indices for embryos electroporated with Wnt1, Wnt3a, mPorcD, mPorcD(H341Q), Wnt1(C93S) and Wnt3a(C77S). Error bars show the standard error. N, number of embryos analyzed for each data point; n, number of sections analyzed. *, Significantly different from the control (P<0.05). nt, neural tube; no, notochord.

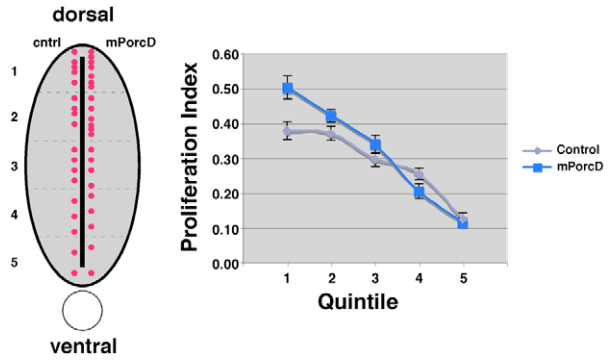

Fig. 7. Ectopic expression of porcupine in the chick neural tube steepens the Wnt1/Wnt3a proliferation gradient. mPorcD was transfected into the neural tube by electroporation. GFP-positive embryos were harvested for analysis 48 hours post-electroporation. Embryos were fixed, cryosectioned and immunostained with anti-phosphohistone H3 (red), which marks cells in late G2/M phase. The neural tube was divided into quintiles for quantitation of proliferation, as illustrated in schematic to left. The red dots represent mitotic cells in each quintile. The proliferation index for the control and experimental sides of the neural tube was determined and graphed for each quintile. A total of 43 sections derived from three different embryos were analyzed. Error bars indicate standard error.

ratio compared with electroporation of GFP alone (P<0.05). Also consistent with results in 293T cells, electroporation of mPorcD(H341Q) caused a reduction of Wnt activity. Likewise, cWnt1(C93S) showed diminished proliferation as compared with wild-type cWnt1. Surprisingly, cWnt3a(C77S) was more active than wild-type cWnt3a.

Ectopic porcupine steepens the Wnt1 and Wnt3a-induced proliferation gradient in the chick neural tube

To assess the effect of porcupine on the Wnt gradient, we subdivided the neural tube into five equally sized quintiles along the dorsal-ventral axis and quantified the effect of porcupine. The proliferation index of the ventral-most three quintiles on the experimental side of the neural tube, as compared with the control side, revealed no significant differences in proliferation (Fig. 7). By contrast, there was a striking increase in proliferation in the dorsal-most two quintiles (Fig. 7; P<0.05). These data are consistent with either increased affinity/avidity of Wnt proteins or increased concentrations of Wnt protein in the dorsal neural tube. Increased Wnt concentration could be achieved by a number of mechanisms, including increased stability, increased secretion or a restricted range of diffusion.

Ectopic porcupine also shortens the BAT-gal activity gradient in the chick neural tube

To further assess the effect of porcupine on the gradient of Wnt activity, we co-electroporated embryos with BAT-gal, a Wnt reporter construct (Maretto et al., 2003), and either a GFP-expressing control vector (pCIG) or pCIG containing Wnt1, Wnt3a or porcupine inserts. We then divided the length of the Wnt activity gradient (along the dorsal-ventral axis), as indicated by immunostaining with antibodies against β-galactosidase, by the length of the transfected area as indicated by GFP (Fig. 8). It should be noted that these measurements do not provide a measure of the level of Wnt activity, which would require measurement of the intensity of the β-galactosidase immunostaining.

When co-electroporated with pCIG (GFP) alone, the BAT-gal gradient extended throughout most of the dorsal half of the neural tube (Fig. 8). Consistent with previous reports, ectopic expression of cWnt1 and cWnt3a extended the Wnt activity range ventrally as compared with expression of pCIG (P<0.001; Fig. 8). If the function of porcupine is to simply increase the affinity/avidity of Wnt, we expect porcupine overexpression to lengthen the Wnt gradient. This is because low concentrations of Wnt protein at the distal end of the Wnt gradient would have effectively greater activity, permitting activation of BAT-gal in more-ventral domains. Similarly, if porcupine promotes the stability or secretion of Wnt protein, we expect overexpression of porcupine to lengthen the gradient. However, if porcupine restricts the range of Wnt distribution, we expect a shortened gradient in the presence of porcupine. Indeed, we observed that porcupine expression restricted the Wnt activity range compared with control embryos (P<0.01; Fig. 8). These data suggest that the range of Wnt distribution is restricted by the presence of porcupine. Predicted outcomes for the mPorcD(H341Q) mutant are less straightforward. On the one hand, Porcupine is required for Wg/Wnt activity and Wg secretion (Kadowaki et al., 1996; Nusse, 2003; Siegfried et al., 1994; van den Heuvel et al., 1993; Zhai et al., 2004). On the other hand, ectopic porcupine appears to restrict the range of diffusion. Sections with fewer than five β-galactosidase-positive cells were eliminated from the analysis as we felt there was not adequate data to suggest a distal limit for the range of activity. Because effects on secretion are expected to supersede effects on the range of diffusion, we predicted that expression of the slightly inhibitory mutant porcupine [mPorcD(H341Q)] construct would result in a decrease in the range of Wnt activity. Though not statistically significant (P=0.14), this trend was observed (Fig. 8).

DISCUSSION

What is the role of palmitoylation of secreted signaling proteins such as Wnts, hedgehogs and Spitz in regulating the activity and distribution of these proteins? Although Hedgehog and Spitz are palmitoylated by the same member of the MBOAT superfamily (Amanai and Jiang, 2001; Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002; Miura et al., 2006), this modification has differential effects on the two ligands. Whereas palmitoylation of sonic hedgehog (Shh) is required for long-range signaling (Chen et al., 2004), palmitoylation of Spitz restricts its range of activity (Miura et al., 2006). We show here that porcupine restricts the range of Wnt activity.

To reach this conclusion, we analyzed the role of porcupine in regulating the lipid-modification, activity and distribution of Wnt proteins in vertebrates. First, antibodies against Wnt1 were used to assess distribution of ectopic and endogenous Wnt1 in the developing chick neural tube. When we overexpressed either cWnt1 or cWnt3a protein in the neural tube, the vast majority of the staining was very close to the site of synthesis. Although we observed immunopositive punctae resembling *Drosophila* vesicular/argosome/lipoprotein-type structures (Gonzalez et al., 1991; Greco et al., 2001; Panakova et al., 2005; Strigini and Cohen, 2000; van den Heuvel et al., 1993), we cannot rule out the possibility that these represent aggregates of improperly folded Wnt protein. Immunostaining for endogenous Wnt1 showed that the protein localized very close to the site of synthesis. We were unable to reliably detect a Wnt protein gradient extending as far ventrally as the expression of BAT-gal in control embryos. We suspect this difference is due to the ability to detect cell-associated, but not secreted, Wnt protein. For instance, tissue fixation precludes the detection of secreted Wg in *Drosophila* (Strigini and Cohen, 2000).

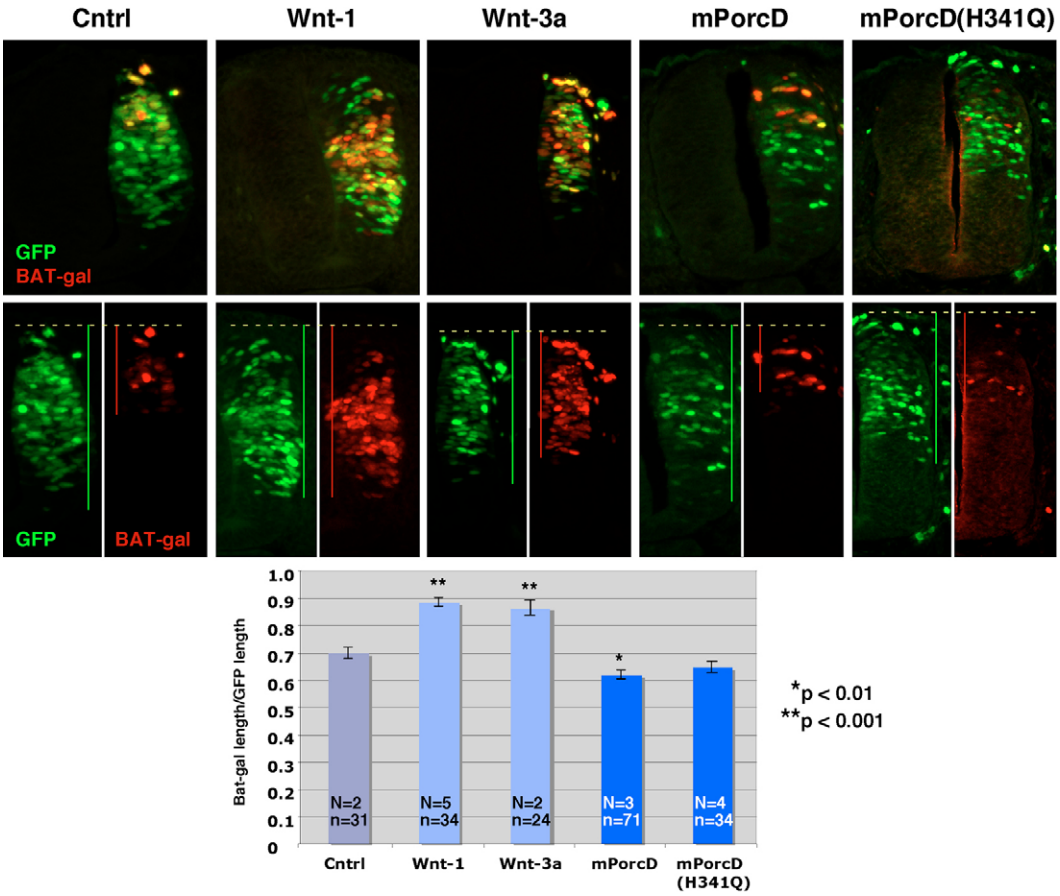

Fig. 8. Ectopic expression of porcupine in the chick neural tube shortens the BAT-gal reporter gradient. The BAT-gal reporter construct was co-electroporated into chick neural tubes along with either pCIG (control), Wnt1, Wnt3a, mPorcD or mPorcD(H341Q) constructs. GFP-positive embryos were harvested for analysis 24 hours post-electroporation. Embryos were fixed, cryosectioned and immunostained with antibodies against β-galactosidase. Sections were imaged with confocal microscopy. Merged images of the GFP and β-galactosidase expression are shown above, separate channels below. The vertical green line demarcates the distance of GFP expression from the dorsal-most extent of the neural tube; a red line demarcates the distance of BAT-gal expression from the dorsal neural tube. A graphic representation of the ratio of the BAT-gal distance divided by the GFP distance is shown. N, number of embryos analyzed; n, number of sections analyzed. Error bars indicate standard error.

We observe similar results with fixed or fresh frozen sections, even though our Wnt1 antibody recognizes native Wnt1 protein (data not shown). The exceptional intractability of Wnt immunostaining is further highlighted by the fact that our immunostaining protocol yields robust staining of the Shh gradient emanating from the floor plate/notochord (data not shown).

Data from the TX-114 phase-separation assay suggest that ectopic expression of porcupine promotes the lipid-modification of Wnt proteins and that Wnt1/Wnt3a possess at least one additional site for modification by porcupine. These data are consistent with recent reports demonstrating that Wnts are lipid-modified on at least two residues, including C93/C77/C104 of Wnt1/Wnt3a/Wnt5a, respectively (Kurayoshi et al., 2007; Willert et al., 2003), and S209 of Wnt3a (Takada et al., 2006). This observation is significant because membrane interactions generally require two separate lipid modifications in order meet the energetic requirements of the association between protein and membrane (Arni et al., 1998; Hancock et al., 1990; Resh, 1994). Because these two sites exhibit little sequence similarity, we wondered whether porcupine regulates the modification of both of these sites. As porcupine is able to promote lipid-modification of the C93/C77 mutants, it is likely that porcupine regulates lipid-modification of the S224/S209 site of Wnt1 and Wnt3a. Our observation that porcupine can also promote the modification of Wnt1 when S224 is mutated (data not shown), further suggests that porcupine regulates the modification of C93. We do not know whether the modification of these sites represents a direct or indirect activity of porcupine. Data showing that the histidine in the acyl transferase active site of porcupine is required for Wnt modification is consistent with the hypothesis that porcupine is the enzyme that directly modifies Wnt proteins. However, the only way to unequivocally test this is to determine whether purified porcupine can mediate lipid-modification of purified Wnt proteins.

We then tested the role of porcupine/lipid-modification in mediating the activity of Wnt1 and Wnt3a. Consistent with previous studies in *Drosophila* (Kadowaki et al., 1996; Nusse, 2003; Siegfried et al., 1994; Zhai et al., 2004), porcupine loss-of-function studies in 293T cells and in chick neural tube show a decrease in Wnt activity. Likewise, mutation of C93/C77 and S224/S209 show no detectable activity in 293T cells (data not shown). In the neural tube, Wnt1(C93S) shows decreased activity, whereas Wnt3a(C77S) shows increased activity. Although we do not know the reason the singly mutated Wnt3a is hypermorphic, we suspect that mutation of the second lipid-modification site in Wnt3a

will result in a loss of activity. As our studies do not differentiate between the autocrine and paracrine activities of Wnt1 and Wnt3a, we cannot definitively distinguish whether we are observing a decrease in the affinity/avidity of Wnt1/Wnt3a or a decrease in secretion.

We also found that expression of ectopic mPorcD promotes the activity of cWnt1 and cWnt3a in 293T cells and in the chick dorsal neural tube. As per above, this might reflect an increase in affinity/avidity or an increase in secretion. Our results from the TX-114 assays showing that porcupine increases the amount of cell-associated Wnt protein, suggest that ectopic porcupine does not simply promote secretion. Furthermore, whereas porcupine loss-of-function experiments cause improper secretion and localization of Wnt proteins (Nusse, 2003; Takada et al., 2006; van den Heuvel et al., 1993; Zhai et al., 2004), gain-of-function experiments show little effect on secretion (Kadowaki et al., 1996). Thus, it is unlikely that the ability of ectopic porcupine to promote Wnt activity can be solely attributed to increased secretion. We predict that lipid-modification of Wnts promotes their affinity/avidity for cell surface receptors. This prediction is consistent with recently published data showing that binding of Wnt5a to frizzled 5 requires palmitoylation of C104 (Kurayoshi et al., 2007).

Because porcupine is an upstream regulator of glycosylation and lipid-modification of vertebrate Wnt proteins (Kurayoshi et al., 2007; Takada et al., 2006; Tanaka et al., 2002), differences in Wnt activity observed upon porcupine overexpression might stem from changes in glycosylation and not lipidation. Although both glycosylation and lipid-modification are required for the secretion of Wnts, the removal of the two known lipid-modification sites causes a more dramatic loss of activity than the mutation of all asparagine-linked glycosylation sites (data not shown) (Komekado et al., 2007; Kurayoshi et al., 2007; Mason et al., 1992; Takada et al., 2006; Willert et al., 2003; Zhai et al., 2004). As these effects of removing the two lipid-modification sites more closely mimic the effects of knocking down porcupine (shown here) (Kurayoshi et al., 2007; Takada et al., 2006; Willert et al., 2003), it seems likely that the primary role of porcupine with respect to Wnt proteins is the regulation of lipid-modification.

The most important findings of our work are derived from our use of the chick model system to assess the effect of porcupine on the Wnt1/Wnt3a gradient in the developing neural tube. Loss-of-function studies identify a requirement for porcupine in the secretion of vertebrate Wnt proteins (Takada et al., 2006); however, the early requirement for porcupine in secretion precluded determination of the role of porcupine/lipid-modification in regulating the distribution of Wnt protein after secretion. Our gain-of-function studies show that porcupine steepens the Wnt-mediated proliferation gradient in the neural tube and shortens the Wnt range of activity as measured by BAT-gal activity. Although the ability of porcupine to steepen the proliferation gradient could be attributed to the ability of porcupine to augment Wnt activity, these results strongly suggest that porcupine limits the distribution of Wnt protein. Thus, we conclude that porcupine plays a role in regulating both the activity and distribution of Wnt proteins. Our interpretation is that lipid-modification by porcupine results in Wnts being tethered closer to their site of synthesis, thus raising their local concentration. Interestingly, heparan sulfate proteoglycans (HSPGs) are also able to promote such an increase in local concentration (Baeg et al., 2001; Giraldez et al., 2002; Han et al., 2005; Kirkpatrick et al., 2004). Our data from the TX-114 extractions showing that porcupine overexpression promotes increased cell-associated Wnt protein is consistent with this idea.

Other proteins involved in Wnt secretion and/or the establishment of Wnt gradients have been identified (Banziger et al., 2006; Bartscherer et al., 2006; Coudreuse and Korswagen, 2007; Coudreuse et al., 2006; Hausmann et al., 2007; Prasad and Clark, 2006). It will be interesting to determine how these proteins coordinate with porcupine to regulate Wnt secretion and gradient formation.

We are very grateful to Dr Shinji Takada for sharing data prior to publication; Dr Diana Chu for her invaluable help with editing; Rowena Suriben for her assistance with section in situs; and Dr Annette Chan of SFSU CMIC for assistance with imaging. This work was funded by NIH-MBRS (SO6 GM52588) and MDA grants to L.W.B. and a NIH-RIMI (P20 MD000262) grant to SFSU.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/18/3339/DC1

References

Amanai, K. and Jiang, J. (2001). Distinct roles of Central missing and Dispatched in sending the Hedgehog signal. *Development* **128**, 5119-5127.

Arni, S., Keilbaugh, S. A., Ostermeyer, A. G. and Brown, D. A. (1998). Association of GAP-43 with detergent-resistant membranes requires two palmitoylated cysteine residues. *J. Biol. Chem.* **273**, 28478-28485.

Baeg, G. H., Lin, X., Khare, N., Baumgartner, S. and Perrimon, N. (2001). Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. *Development* **128**, 87-94.

Banziger, C., Soldini, D., Schutt, C., Zipperlen, P., Hausmann, G. and Basler, K. (2006). Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. *Cell* **125**, 509-522.

Bartscherer, K., Pelte, N., Ingelfinger, D. and Boutros, M. (2006). Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. *Cell* **125**, 523-533.

Bernstein, L. S., Linder, M. E. and Hepler, J. R. (2004). Analysis of RGS protein palmitoylation. *Methods Mol. Biol.* **237**, 195-204.

Brown, A. M., Papkoff, J., Fung, Y. K., Shackleford, G. M. and Varmus, H. E. (1987). Identification of protein products encoded by the proto-oncogene int-1. *Mol. Cell. Biol.* **7**, 3971-3977.

Burrus, L. W. and McMahon, A. P. (1995). Biochemical analysis of murine Wnt proteins reveals both shared and distinct properties. *Exp. Cell Res.* **220**, 363-373.

Caricasole, A., Ferraro, T., Rimland, J. M. and Terstappen, G. C. (2002). Molecular cloning and initial characterization of the MG61/PORC gene, the human homologue of the Drosophila segment polarity gene Porcupine. *Gene* **288**, 147-157.

Chamoun, Z., Mann, R. K., Nellen, D., von Kessler, D. P., Bellotto, M., Beachy, P. A. and Basler, K. (2001). Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. *Science* **293**, 2080-2084.

Chen, M. H., Li, Y. J., Kawakami, T., Xu, S. M. and Chuang, P. T. (2004). Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. *Genes Dev.* **18**, 641-659.

Coudreuse, D. and Korswagen, H. C. (2007). The making of Wnt: new insights into Wnt maturation, sorting and secretion. *Development* **134**, 3-12.

Coudreuse, D. Y., Roel, G., Betist, M. C., Destree, O. and Korswagen, H. C. (2006). Wnt gradient formation requires retromer function in Wnt-producing cells. *Science* **312**, 921-924.

Couso, J. P. and Martinez Arias, A. (1994). Notch is required for wingless signaling in the epidermis of Drosophila. *Cell* **79**, 259-272.

Fokina, V. M. and Frolova, E. I. (2006). Expression patterns of Wnt genes during development of an anterior part of the chicken eye. *Dev. Dyn.* **235**, 496-505.

Fradkin, L. G., van Schie, M., Wouda, R. R., de Jong, A., Kamphorst, J. T., Radjkoemar-Bansraj, M. and Noordermeer, J. N. (2004). The Drosophila Wnt5 protein mediates selective axon fasciculation in the embryonic central nervous system. *Dev. Biol.* **272**, 362-375.

Fung, Y. K., Shackleford, G. M., Brown, A. M., Sanders, G. S. and Varmus, H. E. (1985). Nucleotide sequence and expression in vitro of cDNA derived from mRNA of int-1, a provirally activated mouse mammary oncogene. *Mol. Cell. Biol.* **5**, 3337-3344.

Galli, L. M., Willert, K., Nusse, R., Yablonka-Reuveni, Z., Nohno, T., Denetclaw, W. and Burrus, L. W. (2004). A proliferative role for Wnt-3a in chick somites. *Dev. Biol.* **269**, 489-504.

Galli, L. M., Barnes, T., Cheng, T., Acosta, L., Anglade, A., Willert, K., Nusse, R. and Burrus, L. W. (2006). Differential inhibition of Wnt-3a by Sfrp-1, Sfrp-2, and Sfrp-3. *Dev. Dyn.* **235**, 681-690.

Gavin, B. J., McMahon, J. A. and McMahon, A. P. (1990). Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. *Genes Dev.* **4**, 2319-2332.

Giraldez, A. J., Copley, R. R. and Cohen, S. M. (2002). HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Dev. Cell* **2**, 667-676.
Gonzalez, F., Swales, L., Bejsovec, A., Skaer, H. and Martinez Arias, A. (1991). Secretion and movement of wingless protein in the epidermis of the Drosophila embryo. *Mech. Dev.* **35**, 43-54.
Greco, V., Hannus, M. and Eaton, S. (2001). Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell* **106**, 633-645.
Guo, Z.-Y., Lin, S., Heinen, J. A., Chang, C. C. Y. and Chang, T.-Y. (2005). The active site His-460 of human acyl-coenzyme A:cholesterol acyltransferase 1 resides in a hitherto undisclosed transmembrane domain. *J. Biol. Chem.* **280**, 37814-37826.
Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.
Han, C., Yan, D., Belenkaya, T. Y. and Lin, X. (2005). Drosophila glypicans Dally and Dally-like shape the extracellular Wingless morphogen gradient in the wing disc. *Development* **132**, 667-679.
Hancock, J. F., Paterson, H. and Marshall, C. J. (1990). A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* **63**, 133-139.
Hausmann, G., Banziger, C. and Basler, K. (2007). Helping Wingless take flight: how WNT proteins are secreted. *Nat. Rev. Mol. Cell Biol.* **8**, 331-336.
Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P. and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-360.
Hofmann, K. (2000). A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling. *Trends Biochem. Sci.* **25**, 111-112.
Hollyday, M., McMahon, J. A. and McMahon, A. P. (1995). Wnt expression patterns in chick embryo nervous system. *Mech. Dev.* **52**, 9-25.
Johnston, L. A. and Schubiger, G. (1996). Ectopic expression of wingless in imaginal discs interferes with decapentaplegic expression and alters cell determination. *Development* **122**, 3519-3529.
Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K. and Perrimon, N. (1996). The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes Dev.* **10**, 3116-3128.
Kawakami, Y., Wada, N., Nishimatsu, S. and Nohno, T. (2000). Involvement of frizzled-10 in Wnt-7a signaling during chick limb development. *Dev. Growth Differ.* **42**, 561-569.
Kaykas, A. and Moon, R. T. (2004). A plasmid-based system for expressing small interfering RNA libraries in mammalian cells. *BMC Cell Biol.* **5**, 16.
Kiecker, C. and Niehrs, C. (2001). A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. *Development* **128**, 4189-4201.
Kirkpatrick, C. A., Dimitroff, B. D., Rawson, J. M. and Selleck, S. B. (2004). Spatial regulation of Wingless morphogen distribution and signaling by Dally-like protein. *Dev. Cell* **7**, 513-523.
Komekado, H., Yamamoto, H., Chiba, T. and Kikuchi, A. (2007). Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes Cells* **12**, 521-534.
Kurayoshi, M., Yamamoto, H., Izumi, S. and Kikuchi, A. (2007). Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *Biochem. J.* **402**, 515-523.
Lawrence, P. A., Sanson, B. and Vincent, J. P. (1996). Compartments, wingless and engrailed: patterning the ventral epidermis of Drosophila embryos. *Development* **122**, 4095-4103.
Lee, J. D. and Treisman, J. E. (2001). Sightless has homology to transmembrane acyltransferases and is required to generate active Hedgehog protein. *Curr. Biol.* **11**, 1147-1152.
Lin, S., Lu, X., Chang, C. C. Y. and Chang, T.-Y. (2003). Human acyl-coenzyme A:cholesterol acyltransferase expressed in Chinese hamster ovary cells: membrane topology and active site location. *Mol. Biol. Cell* **14**, 2447-2460.
Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.
Maretto, S., Cordenonsi, M., Dupont, S., Braghetta, P., Broccoli, V., Hassan, A. B., Volpin, D., Bressan, G. M. and Piccolo, S. (2003). Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. *Proc. Natl. Acad. Sci. USA* **100**, 3299-3304.
Mason, J. O., Kitajewski, J. and Varmus, H. E. (1992). Mutational analysis of mouse Wnt-1 identifies two temperature-sensitive alleles and attributes of Wnt-1 protein essential for transformation of a mammary cell line. *Mol. Biol. Cell* **3**, 521-533.
Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-2098.
Micchelli, C. A., The, I., Selva, E., Mogila, V. and Perrimon, N. (2002). Rasp, a putative transmembrane acyltransferase, is required for Hedgehog signaling. *Development* **129**, 843-851.
Miura, G. I., Buglino, J., Alvarado, D., Lemmon, M. A., Resh, M. D. and Treisman, J. E. (2006). Palmitoylation of the EGFR ligand Spitz by Rasp increases Spitz activity by restricting its diffusion. *Dev. Cell* **10**, 167-176.
Moon, R. T., Kohn, A. D., De Ferrari, G. V. and Kaykas, A. (2004). WNT and beta-catenin signalling: diseases and therapies. *Nat. Rev. Genet.* **5**, 691-701.
Neumann, C. J. and Cohen, S. M. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. *Development* **124**, 871-880.
Noordermeer, J., Klingensmith, J. and Nusse, R. (1995). Differential requirements for segment polarity genes in wingless signaling. *Mech. Dev.* **51**, 145-155.
Nusse, R. (2003). Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface. *Development* **130**, 5297-5305.
Panakova, D., Sprong, H., Marois, E., Thiele, C. and Eaton, S. (2005). Lipoprotein particles are required for Hedgehog and Wingless signalling. *Nature* **435**, 58-65.
Papkoff, J., Brown, A. M. and Varmus, H. E. (1987). The int-1 proto-oncogene products are glycoproteins that appear to enter the secretory pathway. *Mol. Cell. Biol.* **7**, 3978-3984.
Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K. et al. (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. *J. Biol. Chem.* **273**, 14037-14045.
Perrimon, N. and Mahowald, A. P. (1987). Multiple functions of segment polarity genes in Drosophila. *Dev. Biol.* **119**, 587-600.
Perrimon, N., Engstrom, L. and Mahowald, A. P. (1989). Zygotic lethals with specific maternal effect phenotypes in Drosophila melanogaster. I. Loci on the X chromosome. *Genetics* **121**, 333-352.
Prasad, B. C. and Clark, S. G. (2006). Wnt signaling establishes anteroposterior neuronal polarity and requires retromer in C. elegans. *Development* **133**, 1757-1766.
Resh, M. D. (1994). Myristylation and palmitylation of Src family members: the fats of the matter. *Cell* **76**, 411-413.
Siegfried, E., Wilder, E. L. and Perrimon, N. (1994). Components of wingless signalling in Drosophila. *Nature* **367**, 76-80.
Smolich, B. D., McMahon, J. A., McMahon, A. P. and Papkoff, J. (1993). Wnt family proteins are secreted and associated with the cell surface. *Mol. Biol. Cell* **4**, 1267-1275.
Strigini, M. and Cohen, S. M. (2000). Wingless gradient formation in the Drosophila wing. *Curr. Biol.* **10**, 293-300.
Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T. and Takada, S. (2006). Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Dev. Cell* **11**, 791-801.
Tanaka, K., Okabayashi, K., Asashima, M., Perrimon, N. and Kadowaki, T. (2000). The evolutionarily conserved porcupine gene family is involved in the processing of the Wnt family. *Eur. J. Biochem.* **267**, 4300-4311.
Tanaka, K., Kitagawa, Y. and Kadowaki, T. (2002). Drosophila segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the endoplasmic reticulum. *J. Biol. Chem.* **277**, 12816-12823.
van den Heuvel, M., Harryman-Samos, C., Klingensmith, J., Perrimon, N. and Nusse, R. (1993). Mutations in the segment polarity genes wingless and porcupine impair secretion of the wingless protein. *EMBO J.* **12**, 5293-5302.
Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H. and Moon, R. T. (2003). Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr. Biol.* **13**, 680-685.
Wei, Y., Yu, L., Bowen, J., Gorovsky, M. A. and Allis, C. D. (1999). Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. *Cell* **97**, 99-109.
Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., 3rd and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448-452.
Yuan, B., Latek, R., Hossbach, M., Tuschl, T. and Lewitter, F. (2004). siRNA Selection Server: an automated siRNA oligonucleotide prediction server. *Nucl. Acids Res.* **32**, W130-W134.
Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. *Cell* **87**, 833-844.
Zhai, L., Chaturvedi, D. and Cumberledge, S. (2004). Drosophila Wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts; a process that requires porcupine. *J. Biol. Chem.* **279**, 33220-33227.