Development 134, 503-513 (2007) doi:10.1242/dev.02739

Wnt11/β-catenin signaling in both oocytes and early embryos acts through LRP6-mediated regulation of axin

Matt Kofron[1], Bilge Birsoy[1], Douglas Houston[2], Qinghua Tao[1], Christopher Wylie[1] and Janet Heasman[1,*]

Current models of canonical Wnt signaling assume that a pathway is active if β-catenin becomes nuclearly localized and Wnt target genes are transcribed. We show that, in *Xenopus*, maternal LRP6 is essential in such a pathway, playing a pivotal role in causing expression of the organizer genes *siamois* and *Xnr3*, and in establishing the dorsal axis. We provide evidence that LRP6 acts by degrading axin protein during the early cleavage stage of development. In the full-grown oocyte, before maturation, we find that axin levels are also regulated by Wnt11 and LRP6. In the oocyte, Wnt11 and/or LRP6 regulates axin to maintain β-catenin at a low level, while in the embryo, asymmetrical Wnt11/LRP6 signaling stabilizes β-catenin and enriches it on the dorsal side. This suggests that canonical Wnt signaling may not exist in simple off or on states, but may also include a third, steady-state, modality.

KEY WORDS: *Xenopus*, β-catenin, Wnt11, LRP6, Axis formation

INTRODUCTION

The single-pass transmembrane protein, LDL receptor-related protein 6 (LRP6) was first identified as an essential co-receptor for mouse Wnt signal transduction (Pinson et al., 2000). LRP6 is required in the mouse for midbrain, axis and limb patterning (Kelly et al., 2004), and a mis-sense mutation in *LRP6* causes folate-sensitive neural tube defects in the *Crooked tail* mutant (Carter et al., 2005). LRP6 is upregulated in several human tumor cell lines (Li et al., 2004; Logan and Nusse, 2004).

Despite its importance in development and disease, the mechanism of LRP6 function is incompletely understood. LRP6 activity in Wnt signaling requires binding of the intracellular protein, axin, to its cytoplasmic domain through a phosphorylation-dependent process involving GSK-3β and CK1γ (Davidson et al., 2005; Tamai et al., 2000; Tamai et al., 2004; Wehrli et al., 2000; Zeng et al., 2005). Axin is an essential component of the canonical Wnt signaling pathway, and modulates the level of cytoplasmic β-catenin by targeting it for phosphorylation and degradation. When axin function is blocked by Wnt-pathway activation or by antisense depletion of *axin* mRNA, β-catenin is stable and enters nuclei to initiate the transcription of target genes (Kofron et al., 2001; Nusse, 2005; Willert et al., 1999a). However, the regulation of axin function by LRP proteins is not clear. LRP may bind to axin and sequester it from the β-catenin-destruction complex (Nusse, 2005), or may promote its degradation, thus inactivating the β-catenin-degradation complex (Mao et al., 2001; Tolwinski et al., 2003; Willert et al., 1999b). Alternatively, in the absence of Wnt signaling, axin may constitutively shuttle β-catenin out of the nucleus; Wnt signaling blocks shuttling by binding LRP to axin, allowing nuclear β-catenin to rise (Cong and Varmus, 2004; Wiechens et al., 2004). To determine which mechanism works in axis formation requires an analysis of endogenous axin in wild-type and LRP6-depleted embryos.

[1]Division of Developmental Biology, Cincinnati Children's Research Foundation, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA. [2]Department of Biological Sciences, The University of Iowa, 257 Biology Building, Iowa City, IA 52242-1324, USA.

*Author for correspondence (e-mail: heabq9@chmcc.org)

Accepted 14 November 2006

Here, we analyze the function of LRP6 in the dorsal axis-specifying pathway in early *Xenopus* embryos. In *Xenopus*, the most recent model of the mechanism of axis formation involves Wnt signaling during the cleavage stage (Tao et al., 2005). This model suggests that the axis-forming pathway is activated by Wnt11 because the protein is necessary and sufficient for dorsal-axis formation, is stored as a maternal transcript in the vegetal cortex of the oocyte, and is enriched in dorsal cells of the embryo at the 32-cell stage (Schroeder et al., 1999; Tao et al., 2005). However, the timing and mechanism of signal transduction is not known. The frizzled family member Xfz7, the EGF-CFC protein FRL1 and heparan sulphate proteoglycan activity are required downstream of Wnt11 (Sumanas et al., 2000; Tao et al., 2005). LRP6 has previously been implicated in this pathway because it is maternally expressed (Houston and Wylie, 2002) and because over-expression of LRP6 on the ventral side of the 4-cell-stage embryo causes the formation of a partial second axis (Tamai et al., 2000). However, when injected into early embryos, a dominant-negative form of LRP6, lacking the cytoplasmic domain, does not interfere with endogenous axis formation, suggesting either that this Wnt signaling pathway does not require LRP6, or that signaling occurs earlier in development and cannot be blocked by the expression of the dominant-negative construct (Tamai et al., 2000).

Axin is an essential negative regulator of canonical Wnt signaling pathways, acting by causing the degradation of β-catenin (Hamada et al., 1999; Lee et al., 2003; Salic et al., 2000; Willert et al., 1999a). In *Xenopus*, depletion of *axin* mRNA causes an increased nuclear localization of β-catenin, increased expression of Wnt target genes, including *siamois* and *Xnr3*, and radially dorsalized phenotypes (Kofron et al., 2001). In *Xenopus* egg extracts, axin is estimated to be present at low levels (picomolar quantities) compared with β-catenin, GSK3β and disheveled (nanomolar quantities), and β-catenin degradation is extremely sensitive to small changes in axin concentration (Lee et al., 2003; Salic et al., 2000). Injection of only 6 pg of *axin* mRNA is sufficient to rescue the dorsalized phenotype of axin-depleted *Xenopus* embryos (Kofron et al., 2001).

Here, we test the hypothesis that LRP6 is the necessary receptor for Wnt11 signal transduction and that it acts by degrading axin. We show first that LRP6 is essential for Wnt11-activated dorsal-axis formation in *Xenopus*. Second, we show that axin protein levels are

increased when LRP6 is depleted, indicating that LRP6 regulates the degradation of axin. Third, we identify a specific time in early development, the 8-cell stage, when axin levels are reduced on the dorsal side of the wild-type embryo, indicating the time of transduction of the Wnt signal. Finally, we present evidence that Wnt11 and LRP6 also act in oocytes to regulate axin, maintaining β-catenin in a low, steady-state level. This suggests that canonical Wnt signaling pathways may have three states (off, steady-state and transcriptional activation) rather than the generally accepted two (off and transcriptional activation).

MATERIALS AND METHODS

Oocytes and embryos

Full-grown oocytes obtained from adult-frog ovary were manually defolliculated and cultured in oocyte culture medium (OCM). Oocytes were injected with antisense oligo or mRNA in the doses described in the text and cultured for 72 hours at 18°C. Oocytes were matured by the addition of 2 μM progesterone in OCM, cultured for 12 hours after the addition of progesterone, labeled with vital dyes and fertilized using the host-transfer technique (Zuck et al., 1998).

Collagenase treatment

Oocytes were washed in Ca^{2+}-free 1×MMR (100 mM NaCl, 2 mM KCl, 15 mM HEPES) and nutated in 0.02 mg/ml collagenase (Sigma Type 1) in Ca^{2+}-free MMR +1.6 mM $CaCl_2$. Denuded oocytes were washed in 1×MMR and then in OCM.

Inhibition of proteasomal-mediated protein degradation

Oocytes were treated with MG132 (Sigma) as described by Dupont et al. (Dupont et al., 2005). A concentration of 1:100 MG132 was made in sterile water from a stock of 10 mM MG132 in DMSO and 10 nl was injected into oocytes. Injected oocytes were cultured in OCM containing 10 μM MG132 for 2-3 days. Oocytes were harvested for western blots in lysis buffer containing 10 μM MG132.

Oligos and mRNAs

Antisense oligo for *LRP6* 5′-T*C*G*AGGCTGATCCAG*C*T*C-3′; for *Wnt11* 5′-G*T*C*GGAGCCATTGGT*A*C*T-3′; and for *Axin* 5′-T*T*C*CTCGCCAGGAAC*T*G*G-3′, where phosphorothioate-modified residues are indicated by an asterisk (*).

Oligos were resuspended in sterile distilled water and injected in doses of 4-5 ng per oocyte. Mouse *LRP6* was subcloned into pRN3, linearized with *Sfi*I and transcribed with T3 message machine (Ambion). *Xenopus* pCS2+ Myc-β-catenin plasmid was linearized with *Not*I, and pSP64T-Wnt11 with *Xba*I, and transcribed with SP6 message machine kit (Ambion). Analysis of gene expression using real-time RT-PCR was as described by Tao et al. (Tao et al., 2005). The β-catenin constructs used for Fig. 7F,G,H were either wild-type or stabilized β-catenin in pCS2-MT (Yost et al.1996). The stabilized β-catenin lacks the four N-terminal Gsk3β phosphorylation sites because of their mutation from serine to alanine.

Western blotting

In total, five embryos or oocytes were homogenized in 200 μl of ice-cold PBS plus protease inhibitors (PIC P8340 Sigma 1:100, PMSF 10 μg/ml) and spun for 5 minutes at 700 ***g***, 4°C. Supernatants were precipitated with acetone for 20 minutes on ice. Samples were spun for 5 minutes at 3000 ***g***, 4°C. Pellets were resuspended in 50 μl 2×SDS sample buffer and boiled for 5 minutes. A total of 18 μl was loaded onto 7.5% Tris-glycine ready gel (Bio-Rad) and electrophoresed for 2.5 hours at 75 V. Protein was transferred to nitrocellulose membranes and blocked overnight in PBS-Tween 0.1%, 5% dry milk at 4°C. Antibody conditions were anti-axin 1067 AP 1:400 (gift from Peter Klein, University of Pennsylvania, Philadelphia, PA), anti-β-catenin: Sigma C2206 1:2000, Roche anti-c-myc clone 9E10 1:2000. Membranes incubated with HRP-conjugated antibodies were incubated with ECL developing solution (Amersham) and exposed to X-ray film at variable times to avoid the saturation of bands (Hyperfilm, Amersham). Quantification was performed using IPlab densitometry software, normalized to α-tubulin levels and values expressed as the percentage change relative to controls. Blots were re-probed with a FITC-conjugated anti-α–tubulin antibody (Sigma F2168) at 1:4000 for loading control, and imaged on a Typhoon variable mode imaging system.

Co-immunoprecipitation

Animal cells of embryos were injected with 500 pg *Wnt11-HA* mRNA and 1.5 ng *LRP6-N-myc* RNA (lacking the carboxyl intracellular domain (Tamai et al., 2000) at the 4- to 8-cell stage. Adjacent blastomeres were injected such that only secreted protein could interact in order to avoid non-specific interactions between the two proteins in the ER/golgi complex. Embryos were harvested at stage 10.5 and centrifuged to remove yolk protein. Co-immunoprecipitation was carried out as described previously (Tao et al., 2005). The anti-HA high-affinity rat antibody (3F10, Roche) was used to pull-down Wnt11-HA protein. Lrp6-N-myc protein was detected using the anti-c-myc antibody (9e10, Roche). Detection of proteins in western blots used HRP-conjugated anti-rat or -mouse antibodies and ECL plus (GE healthcare, RPN2132). Blots were visualized with ECL fluorescence on a Molecular Dynamics typhoon phosphor imager.

Luciferase assays

TOPflash DNA (60 pg) containing three copies of the TCF-binding site upstream of a minimal TK promoter and luciferase open reading frame, together with 20 pg pRLTK DNA (Renilla reniformis luciferase used as an internal control), was injected into two dorsal cells at the 4-cell stage of control and LRP6-depleted embryos. Three replicate samples, each of four embryos, were frozen at the late blastula stage and luciferase assays were carried out using the Promega luciferase assay system.

RESULTS

LRP6 is necessary and sufficient for axis formation

To study the role of maternal LRP6 in Wnt signaling in the early *Xenopus* embryo, we first determined whether *LRP6* mRNA was localized. Real-time RT-PCR of oocytes cut into animal and vegetal halves, or into animal, equatorial and vegetal thirds, showed that, compared with vegetally localized *Wnt11* mRNA, *LRP6* mRNA was distributed throughout the oocyte (Fig. 1A). Next, we showed that an antisense oligonucleotide caused a dose-dependent depletion of *LRP6* mRNA from full-grown oocytes, without significantly affecting the levels of expression of the related family member *LRP5* (Fig. 1B).

When LRP6-depleted oocytes were fertilized, they developed normally until gastrulation, but then phenocopied maternal β-catenin- or Wnt11-depleted embryos, displaying delayed gastrulation (data not shown) and the loss of axial structures at the tailbud stage – the so called ‘ventralized’ phenotype (Fig. 1C,D; 121/132 cases [92%] had this appearance in ten experiments). Over-expression of mouse *LRP6* mRNA in the oocyte had the opposite effect, causing dorsalization (Fig. 1C). The effect of antisense depletion was specific, because ventralization was rescued by the re-introduction of *LRP6* mRNA into LRP6-depleted oocytes (Fig. 1D; 80/83 embryos [96%] rescued in this way in five experiments). The expression of the early zygotic Wnt11 target genes *siamois*, *Xnr3*, *chordin* and *goosecoid* was severely reduced in LRP6-depleted embryos at the early gastrula stage (Fig. 1E), and remained so throughout gastrulation (Fig. 1F, Fig. 2). Wnt target gene expression was rescued by the injection of *LRP6* mRNA before fertilization, showing that the effect was specific (Fig. 1E). The expression of the endoderm marker *XSox17* and the ventral marker X*wnt8* was delayed, but reached wild-type levels (Fig. 1F). Expression of the mesodermal marker *Xbra* was also reduced in LRP6-depleted embryos (Fig. 1E). Although embryos and molecular markers were not completely rescued to the wild-type state, Wnt target gene expression and dorsal-axis formation were consistently recovered by the injection of mouse *LRP6* mRNA (Fig. 1G), showing the requirement for LRP6 in this process.

As further confirmation that LRP6 was required to activate the canonical Wnt signaling pathway, we used the TOPflash reporter to examine the effects of the loss of LRP6 activity on Tcf3- and/or LEF-mediated transcription. Fig. 1H shows that LRP6 depletion, at doses that cause ventralization, abolished the activity of this reporter in embryos at the late blastula stage.

LRP6 acts downstream of Wnt11 and upstream of β-catenin in the axis-forming pathway

LRP6-depleted embryos resemble Wnt11-depleted embryos (Fig. 1) (Tao et al., 2005). To analyze this further, we compared the expression of Wnt target genes in sibling Wnt11- and LRP6-depleted embryos derived from the same batch of oocytes. Fig. 2

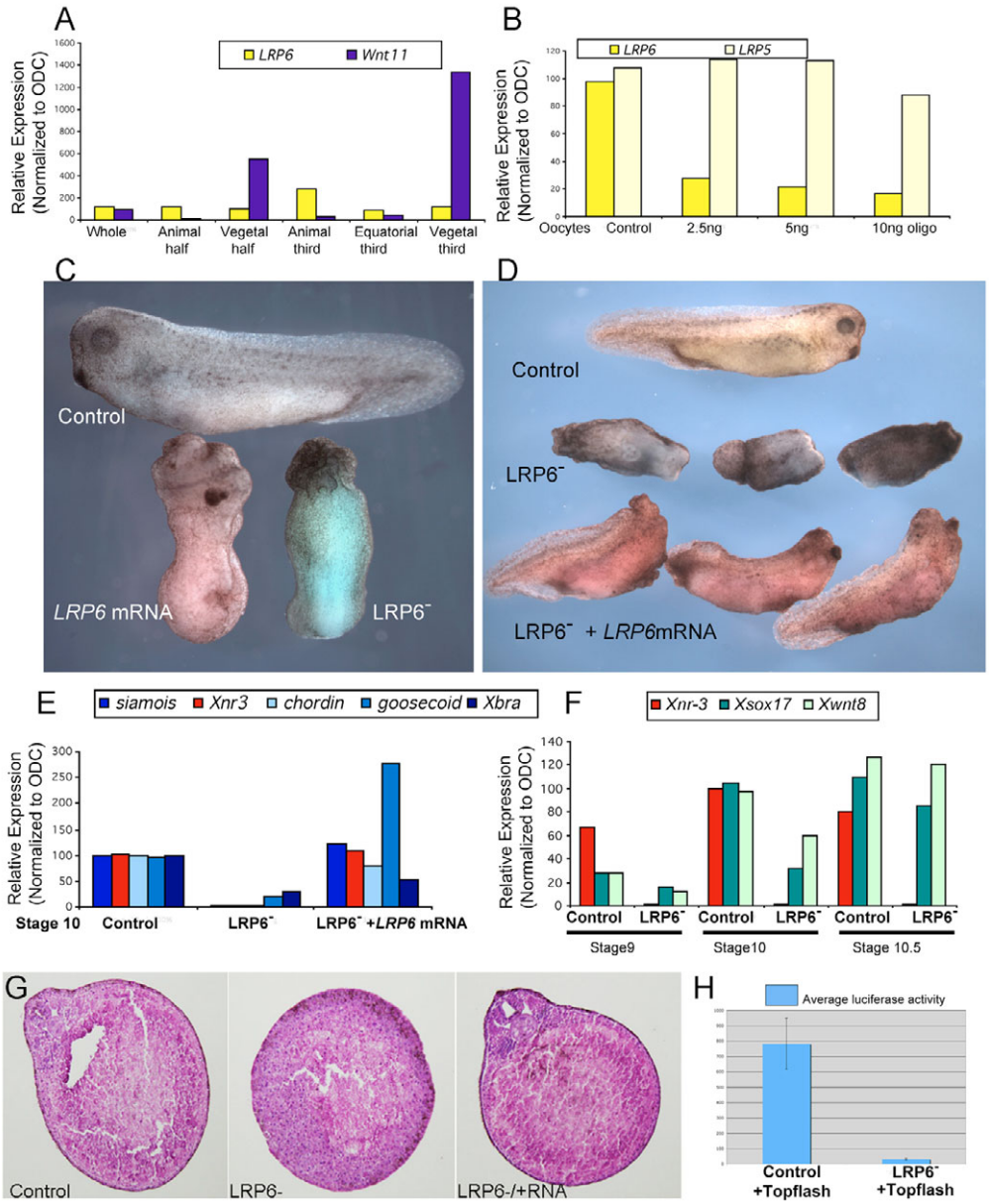

Fig. 1. LRP6 is necessary for dorsal axis formation. (**A**) Two wild-type whole oocytes, and four animal and vegetal halves, or six animal, equatorial and vegetal thirds, were frozen and assayed by real-time RT-PCR for the expression of *LRP6* and *Wnt11* mRNAs. *LRP6* mRNA was expressed throughout the oocytes. (**B**) Groups of two control and LRP6 antisense oligo-injected oocytes (2.5, 5 and 10 ng oligo) were incubated for 24 hours, and assayed for the expression of *LRP6* and the related *LRP5* mRNA. *LRP6*, but not *LRP5*, mRNA levels were reduced by the antisense oligo. (**C**) The phenotype of tailbud-stage embryos derived from oocytes injected with 500 pg mouse *LRP6* mRNA (dorsalized) or 5 ng LRP6 antisense oligo (LRP6^{-}; ventralized). (**D**) The LRP6-depleted ventralized phenotype at tailbud stage caused by the injection of LRP6 antisense oligo (middle row; 3 ng antisense oligo injected) was rescued by the injection of 100 pg mouse *LRP6* mRNA (bottom row). *LRP6* mRNA was injected 48 hours after oligo injection and 24 hours before oocyte maturation. (**E**) The expression of Wnt target genes (*siamois*, *Xnr3*, *chordin* and *goosecoid*) assayed by real-time RT-PCR at the early gastrula stage in sibling embryos to those in D. (**F**) The expression of endoderm marker *Xsox17α* and ventral mesodermal marker *Xwnt8* was delayed in LRP6-depleted embryos at the late blastula stage, but reached wild-type levels of expression by the early gastrula stage in LRP6-depleted embryos. By comparison, *Xnr3* expression remained off in LRP6-depleted embryos. (**G**) Transverse sections of tailbud-stage embryos derived from a control, LRP6-depleted and LRP6^{-} + *LRP6*-mRNA-injected oocytes. LRP6 depletion resulted in a lack of dorsal structures, which was rescued by *LRP6*-mRNA injection. (**H**) TOPflash reporter activation in control and LRP6-depleted late blastulae after injection into two dorsal cells at the 4-cell stage. Error bars indicate the standard deviation from the mean (s.e.m.).

shows that, in both depletions, the expression of *siamois* and *Xnr3* was severely reduced. Because ventralized phenotypes occur because of either reduced dorsal gene expression, or the upregulation of ventral gene expression, we examined the expression of the BMP target genes *Xvent1* and *Xvent2*, as well as of *BMP4*. These markers were not upregulated at stage 9.5-10.25, confirming that LRP6 depletion causes ventralization by preventing dorsal gene activation.

To confirm that LRP6 acts in the maternal Wnt11 signaling pathway, we tested whether *LRP6* mRNA could rescue Wnt11-depleted embryos. Oocytes were depleted of Wnt11 using an antisense oligo (Tao et al., 2005), cultured for 2 days and injected with mouse *LRP6* mRNA before fertilization. Mouse *LRP6* mRNA (75 pg) rescued Wnt11-depleted embryos in producing neural fold formation, although the size of the neural folds and head formation were abnormal, whereas higher doses caused embryos to develop with a dorsalized phenotype (Fig. 3A). This abnormal morphology may be explained by the fact that Wnt11 depletion causes a loss of a localized asymmetric signal, which is not replaced by LRP6, so that the rescued axis is not normal. *LRP6* mRNA also rescued the expression of *siamois* and *Xnr3* in Wnt11-depleted embryos at the gastrula stage (Fig. 3B).

By contrast, the effects of maternal LRP6 depletion were not rescued by the injection of 100-300 pg *Wnt11* mRNA (Fig. 3C,D), although these doses of *Wnt11* RNA caused the dorsalization of wild-type siblings (data not shown). Thus, LRP6 lies downstream of Wnt11 in the signaling pathway.

To place β-catenin relative to LRP6 in the pathway, we asked whether LRP6-depleted embryos could be rescued by the injection of *β-catenin* mRNA. *β-catenin* mRNA (50 pg) was injected into one dorsal cell in LRP6-depleted embryos at the 4-cell stage, and rescued the formation of axial structures as well as *siamois* and *Xnr3* expression (Fig. 3E,F). Thus, LRP6 is upstream of β-catenin in the axis-forming pathway.

To determine whether Wnt11 physically interacts with LRP6, HA-tagged Wnt11 (500 pg) and LRP6 lacking the C-terminus (Tamai et al., 2000) (LRP6-N-myc 1.5 ng) were injected into adjacent cells at the 4- to 8-cell stage, and were frozen at the early gastrula stage for co-immunoprecipitation analysis. Fig. 3G shows that LRP6-N-myc interacts with Wnt11-HA.

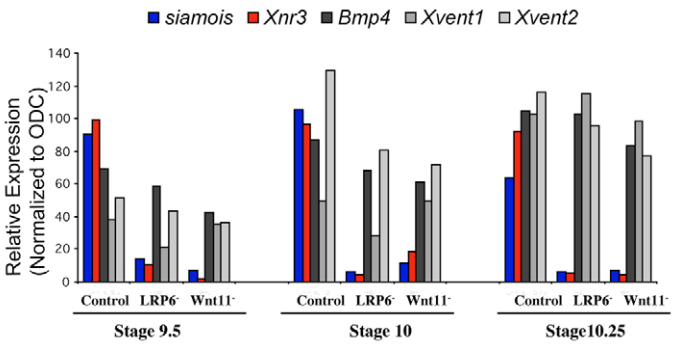

Fig. 2. LRP6 and Wnt11 depletion cause similar effects on dorsal gene expression. The expression of Wnt target genes (*siamois*, *Xnr3*) assayed by real-time RT-PCR at stage E9.5-E10.25 in sibling embryos depleted of Wnt11 or LRP6. Expression of Wnt target genes was reduced in both cases, whereas that of *Xvent1*, *Xvent2* and *BMP4* were not.

LRP6 depletion causes increased axin levels in early embryos

The above data raise the question of the mechanism of LRP6 function. Current models suggest that LRP6 either sequesters or degrades axin protein, or shuttles it continuously out of the nucleus. To determine how LRP6 acts in the early *Xenopus* embryo, we compared the amounts of endogenous axin in western blots of wild-type and LRP6-depleted embryos using an affinity purified anti-*Xenopus* axin antibody in embryos at the 8-cell and early blastula stages. Remaining sibling embryos were cultured to the tailbud stage to confirm that they were ventralized. Fig. 4A shows that LRP6 depletion resulted in increased levels of endogenous axin in whole-embryo lysates. In three experiments, increases ranged from 25-47% greater than control levels. Although these changes were not extreme, axin is known to be extremely active and present at limiting amounts in wild-type embryos (Lee et al., 2003; Salic et al., 2000), such that small changes can dramatically alter Wnt signal transduction. This finding supports the model that LRP6 transduces Wnt signals by causing axin degradation.

Because axin is known to cause β-catenin degradation, such increased axin levels would be predicted to result in reduced β-catenin protein in LRP6-depleted embryos. Blots were stripped and re-probed using a β-catenin-specific antibody, and this prediction was confirmed; the trend for β-catenin levels was opposite to that for axin, particularly at the early blastula stage (Fig. 4A).

Fig. 3A shows that Wnt11 is upstream of LRP6 in the axis-forming pathway. Therefore, we asked whether Wnt11 depletion also affects axin levels in the early embryo. Wnt11 depletion enhances axin levels in the 8-cell-stage embryo (Fig. 4B), with increases ranging from 19-46% in three experiments.

The most recent model of Wnt11 function in the axis-forming pathway proposes that sperm activates the cortical rotation movements of the first cell cycle, which leads to a dorsal asymmetry of *Wnt11* mRNA and protein, causing increased Wnt11 signaling dorsally during the cleavage to blastula stages (Heasman, 2006; Larabell et al., 1997; Schneider et al., 1996; Tao et al., 2005). The work here suggests the following model (Fig. 4): by the 8-cell stage, Wnt11 protein is secreted, mostly by dorsal vegetal cells, and binds to LRP6 (Fig. 4Cii). Association of Wnt11 with the extracellular domain of LRP6 causes LRP6 phosphorylation (Davidson et al., 2005; Tamai et al., 2000; Tamai et al., 2004; Wehrli et al., 2000; Zeng et al., 2005) and axin degradation. Because less axin is present to degrade β-catenin, β-catenin is stabilized, binds to *Xenopus* Tcf3 and enters nuclei on the dorsal side. On the ventral side, low levels of Wnt11 signaling occur, maintaining a level of axin that degrades sufficient β-catenin to reduce nuclear accumulation. In LRP6-depleted embryos, Wnt11 molecules are unable to activate the pathway, so axin increases in concentration and β-catenin is degraded on the dorsal as well as the ventral side (Fig. 4Ciii). Next, we tested four predictions of this model, as follows:

(1) *LRP6* is not a dorsally localized mRNA

To determine whether *LRP6* mRNA is asymmetrically localized in embryos, we examined *LRP6* mRNA levels in dorsal versus ventral half-embryos at the 32-cell stage (Fig. 5A). Fig. 5A shows that *LRP6* mRNA is not enriched dorsally.

(2) Axin protein is less abundant dorsally than ventrally in wild-type 8-cell-stage embryos

We examined total axin protein in western blots of dorsal and ventral wild-type half embryos at the 8-cell stage. Axin levels were reduced dorsally compared with ventral levels in three repeats of the

experiment (Fig. 5B; 32% and 59% less than the ventral levels of expression). Blots were stripped and re-probed for β-catenin levels, and β-catenin expression was found to be increased on the dorsal side compared with ventral levels (Fig. 5B; 14% and 20% more than the ventral side).

To determine whether low levels of axin were maintained dorsally, we examined a series of embryos from the 8- to the 64-cell stage. Only at the 8-cell stage was axin at a low level dorsally compared to ventrally. β-catenin was enriched dorsally compared to ventrally throughout the 16- to 64-cell stages (Fig. 5C). This correlates with the reported time of nuclear localization of β-catenin protein in dorsal cells (Larabell et al., 1997).

(3) The dorsal enrichment of β-catenin is lost in LRP6-depleted embryos

To test whether the result of LRP6 depletion was a reduction in the dorsal enrichment of β-catenin, we compared the amounts of endogenous β-catenin in western blots of dorsal and ventral half-

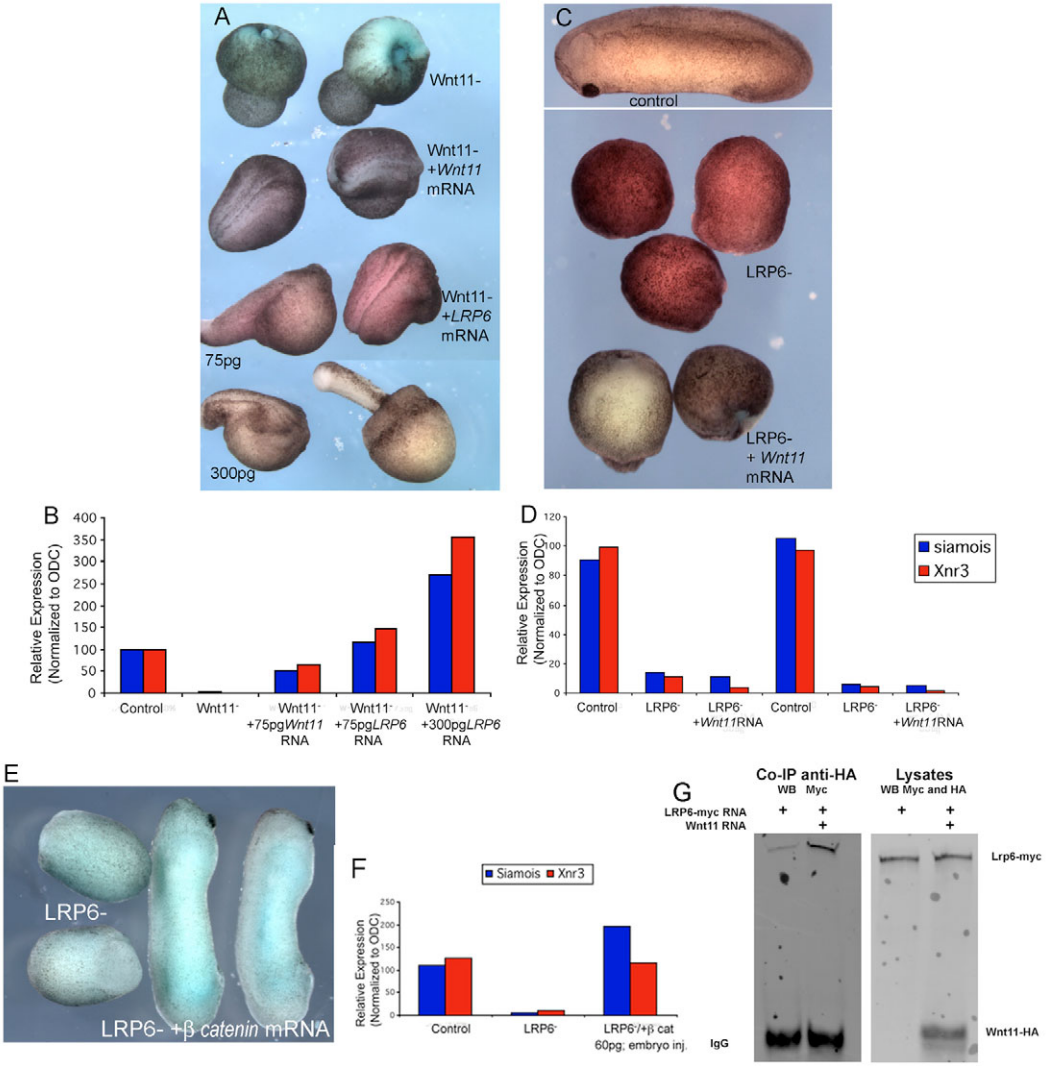

Fig. 3. LRP6 is downstream of Wnt11 and upstream of β-catenin in the axis-forming pathway. (**A**) The ventralized Wnt11-depletion phenotype at late neurula stage (top row; 9/9 cases ventralized), was rescued by 75 pg *Wnt11* mRNA (second row; 0/8 cases ventralized), and by 75 pg *LRP6* mRNA (third row; 0/12 cases ventralized), injected 24 hours before oocyte maturation. Additionally, 300 pg of *LRP6* mRNA also rescued Wnt11-depleted embryos, but caused a dorsalized phenotype (fourth row; 0/8 cases ventralized). (**B**) The expression of Wnt target genes at the early gastrula stage (stage 10) in sibling embryos to those shown in A, assayed by real-time RT-PCR. *LRP6* mRNA rescued the expression of *siamois* and *Xnr3* in Wnt11-depleted embryos. (**C**) The ventralized LRP6-depletion phenotype at tailbud stage (red embryos; 9/9 cases ventralized) was not rescued by the injection of 100 pg *Wnt11* mRNA 24 hours prior to maturation (bottom row; 6/6 cases ventralized). (**D**) The expression of Wnt target genes at the late blastula stage (stage 9.5) and early gastrula stages (stage 10) in embryos that were siblings to those shown in C, assayed by real-time RT-PCR. Wnt11 was not able to rescue the expression of Wnt target genes in LRP6-depleted embryos. (**E**) The ventralized LRP6-depletion phenotype (7/7 cases ventralized) shown at tailbud stage was rescued by the injection of 50 pg *β-catenin* mRNA into one dorsal cell at the 4-cell stage (6/20 cases ventralized). (**F**) The expression of Wnt target genes at the early gastrula stages (stage 10) in embryos that were siblings to those shown in C, assayed by real-time RT-PCR. β-catenin rescued the expression of Wnt target genes in LRP6-depleted embryos. (**G**) Lrp6-N-Myc interacts with Wnt11-HA in co-immunoprecipitation assays. Lrp6-N-Myc (1.5 ng) and Wnt11-HA (500 pg) were injected into two different blastomeres at the 4- to 8-cell stage. Embryos were harvested at stage 10.5. Panel on the right is a western blot of the embryo lysates, blotted with both anti-myc and anti-HA antibody. Panel on the left shows the result of the lysates immunoprecipitated with anti-HA antibody and blotted with anti-myc antibody. IgG band is indicated on the bottom of the left panel.

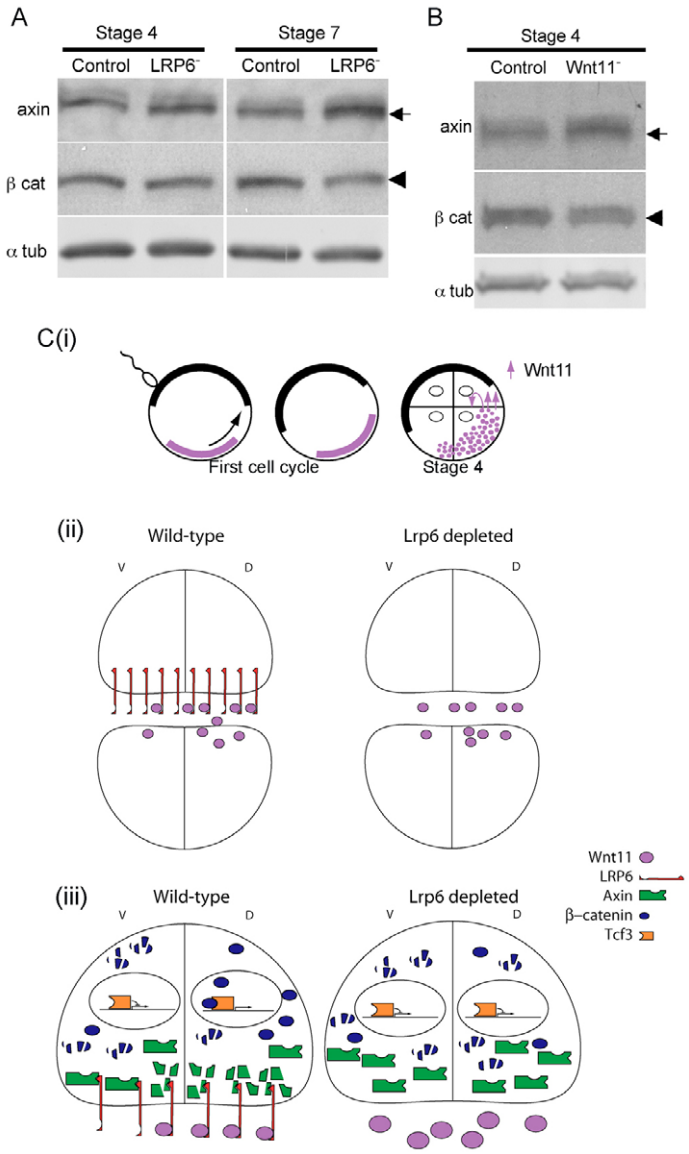

Fig. 4. Wnt11-LRP6 signaling regulates axin levels in the early embryo. (**A**) Western blot of embryos derived from wild-type and LRP6-depleted oocytes (LRP6^{-}) that were frozen at the 8-cell (stage 4) and early blastula (stage 7) stages and probed with an affinity purified axin polyclonal antibody. Blots were stripped and re-probed for β-catenin, and α-tubulin antibody was used as a loading control. Axin levels were increased in LRP6-depleted embryos (arrow; increased by 47% compared with control). β-catenin levels were decreased by LRP6 depletion (arrowhead; decreased by 17% compared with controls). (**B**) Western blot of embryos derived from wild-type and Wnt11-depleted oocytes (Wnt11^{-}) that were frozen at stage 4 and probed with an affinity purified axin polyclonal antibody. Blots were stripped and re-probed for β-catenin. Axin levels were increased (arrow; 46% compared to control) in Wnt11-depleted embryos and β-catenin levels were decreased (arrowhead; 23% compared with control). (**C**) A model of LRP6 function in the axis-forming pathway. (**Ci**) Sperm activates the cortical rotation movements of the first cell cycle, leading to a dorsal asymmetry of *Wnt11* mRNA and protein, which causes increased Wnt11 signaling dorsally (purple arrows). (**Cii**) By the 8-cell stage, Wnt11 protein is secreted, mostly by dorsal vegetal cells, and binds to LRP6. Signaling is shown between a dorsal vegetal and animal cell, although LRP6 is likely to be present in both cells, and autocrine signaling may occur. In LRP6-depleted embryos, Wnt11 molecules are secreted dorsally but are unable to activate the pathway. (**Ciii**) An enlargement of the animal cells shown in Fig. 3Cii at the 8-cell stage. Association of Wnt11 with the extracellular domain of LRP6 causes the degradation of axin. β-catenin enters nuclei and binds to Xenopus Tcf3 on the dorsal side. In LRP6-depleted embryos, Wnt11 molecules do not bind LRP6, so axin increases in concentration and β-catenin is degraded to the same extent on dorsal and ventral sides, and does not enter nuclei.

embryos bisected at the 32-cell stage. The dorsal enrichment of β-catenin was lost in dorsal compared to ventral halves of LRP6-depleted embryos (Fig. 4D; repeated in three experiments).

(4) Embryos depleted of LRP6 and axin are less dorsalized than axin-depleted embryos

The model suggests a causative relationship between LRP6 and its modulation of axin in regulating β-catenin levels, in turn affecting the transcription of Wnt target genes. To explore this further, we tested the effect of depleting both *axin* and *LRP6* mRNA. We have shown previously that maternal axin depletion causes the upregulation of *siamois* and *Xnr3* (Kofron et al., 2001), whereas LRP6 depletion causes their downregulation (Fig. 1E). In embryos depleted of both LRP6 and axin, the loss of LRP6 would be predicted to allow remaining axin to be stabilized, thus attenuating the up-regulation of *siamois* and *Xnr3*. Fig. 5E shows that this was the case. Depletion of both axin and LRP6 partially rescued the over-expression of *siamois* and *Xnr3* caused by axin depletion. However, sibling LRP6- and axin-depleted embryos did not develop normal axes, most probably due to the lack of asymmetry of dorsal gene expression (data not shown).

Together, these results are consistent with the model shown in Fig. 4C, in which LRP6 transduces Wnt11 signals dorsally by causing axin degradation at the 8-cell stage. When this process is blocked by LRP6 depletion, axin levels rise, leading to increased β-catenin degradation and reduced expression of Wnt target genes.

LRP6 regulates axin levels in stage-6 oocytes

The Wnt pathway is assumed to be inactive in full-grown oocytes, because transcription is repressed, and chromatin is methylated and hypo-acetylated (Bird and Wolffe, 1999; Landsberger and Wolffe, 1995). However, because axin protein levels act as an early indicator of LRP6 function, we examined the effects of LRP6 depletion on endogenous axin protein levels in oocytes. Unexpectedly, we found that endogenous axin protein levels were increased in LRP6-depleted oocytes compared to controls, suggesting that maternal LRP6 has a role in maintaining oocyte axin at a steady-state level (Fig. 6A; repeated in three experiments).

This raises the query as to whether axin levels in the oocyte are controlled by Wnt signaling. Because the manually defolliculated oocytes used in these experiments retain some follicle cells, these cells could secrete a Wnt signal. We removed all remaining follicle

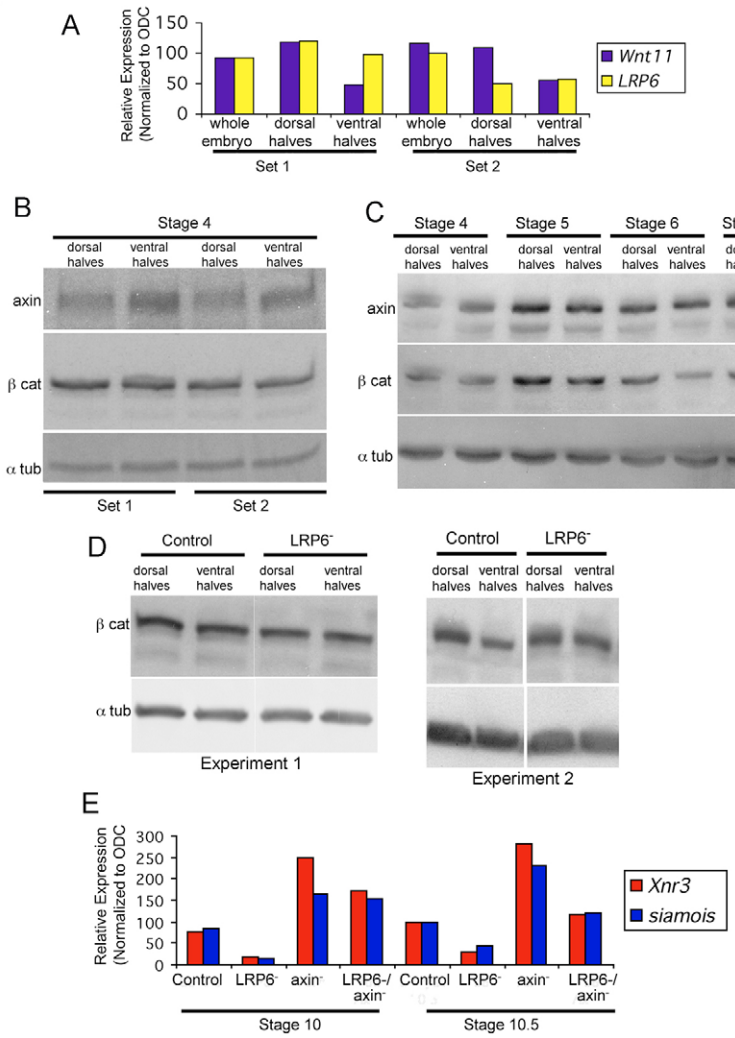

Fig. 5. Testing the predictions of the axin-degradation model of axis formation. (**A**) *LRP6* is not a dorsally localized mRNA. Real-time RT-PCR analysis of groups of two wild-type embryos, and four dorsal- or ventral-half embryos at the 32-cell stage shows that endogenous *LRP6* mRNA was not enriched dorsally, in comparison with *Wnt11* mRNA. Data from two separate sets of embryos is shown. (**B**,**C**) Axin protein is less abundant dorsally than ventrally in wild-type 8-cell-stage embryos. (B) Total axin protein in western blots of ten dorsal- and ten ventral-half embryos at stage 4. Axin levels were reduced dorsally compared with ventrally. Blots were stripped and re-probed for β-catenin levels. (C) Total axin protein levels in a series of sibling embryos from the 8- to the 64-cell stage. Only at the 8-cell stage was axin at a low level dorsally compared with ventrally. β-catenin was enriched dorsally compared with ventrally throughout the 16- to 64-cell stage. (**D**) The dorsal enrichment of β-catenin is lost in LRP6-depleted embryos. Two experiments showing western blots of ten dorsal or ten ventral halves of wild-type and LRP6-depleted embryos hemisected and frozen at the 32-cell stage, and probed with a β-catenin polyclonal antibody. β-catenin levels were enriched dorsally in control dorsal halves compared to ventral halves (32% more in experiment 2), and this difference was lost in LRP6-depleted embryos (dorsal halves have 8% less β-catenin in experiment 2). (**E**) Embryos depleted of LRP6 and axin are less dorsalized than axin-depleted embryos. Real-time RT PCR analysis of the expression of Wnt target genes at the early gastrula stages in wild-type control embryos and embryos depleted of LRP6, axin or both LRP6 and axin. Axin depletion causes the upregulation, which is partially rescued by the depletion of LRP6, of *Xnr3* and *siamois*.

cells using collagenase treatment, and compared endogenous axin levels in control and denuded oocytes after culturing for 3 days (Fig. 6B). No differences were seen between collagenase-treated oocytes compared to control oocytes, suggesting that follicle cells do not play a role in regulating oocyte axin levels.

An alternative possibility is that Wnt11 acts in an autocrine fashion. Thus, we compared the effects on axin levels in oocytes depleted of maternal *Wnt11* mRNA with that of controls. Fig. 6C shows that Wnt11-depleted oocytes had increased levels of axin compared with controls, indicating that Wnt11 normally stimulates LRP6-mediated degradation of axin in full-grown oocytes.

We then investigated whether this mechanism regulates oocyte β-catenin levels by examining endogenous β-catenin levels in wild-type and LRP6-depleted oocytes. Levels were also compared 8 hours after progesterone-stimulated oocyte maturation. Fig. 6D shows that β-catenin levels were low in oocytes compared with matured oocytes, and that new β-catenin synthesis occurred during oocyte maturation. β-catenin protein in matured oocytes decreased as a result of LRP6 depletion (by 23% compared with controls).

Because β-catenin levels in non-matured oocytes are low, we next asked whether maternal axin is required to maintain this state. We depleted *axin* mRNA using an antisense oligo (Kofron et al., 2001), and cultured the oocytes for 3 days to reduce axin protein levels. Fig. 6E shows that axin-depletion caused an increase in β-catenin levels in non-matured oocytes compared with controls. Axin-depleted oocytes did not transcribe *siamois* and *Xnr3* even with these increased β-catenin levels (data not shown). These results suggest a role for maternal axin in maintaining β-catenin at low levels in the oocyte, and suggest that Wnt11 signals in an autocrine fashion through LRP6 to degrade sufficient axin to maintain a steady-state level (Fig. 6F).

One prediction of the model that maternal axin regulates endogenous β-catenin in oocytes is that exogenously introduced β-catenin should also be degraded. To test this, we injected myc-tagged *β-catenin* mRNA into oocytes, which were then cultured in the presence or absence of the proteasomal inhibitor MG132. The amount of myc-tagged β-catenin was enhanced when proteasomal degradation was prevented, suggesting that exogenous protein is degraded (Fig. 7A). To test the role of LRP6, we co-injected *LRP6* mRNA together with *β-catenin-myc* mRNA and cultured oocytes for 3 days. Fig. 7B shows a substantial increase in the amount of myc-tagged β-catenin protein in oocytes when *LRP6* mRNA was co-injected with *β-catenin-myc* mRNA, showing that LRP6 enhances the stability of the exogenous β-catenin-myc protein.

A second test of whether β-catenin is degraded in oocytes compared to embryos is to compare the ability of *β-catenin* mRNA to rescue LRP6-depleted oocytes in which the mRNA was injected either before or after fertilization. We used a dose of *β-catenin* mRNA (50 pg) that caused axis-duplication when injected into the ventral side of wild-type embryos at the 4-cell stage (data not

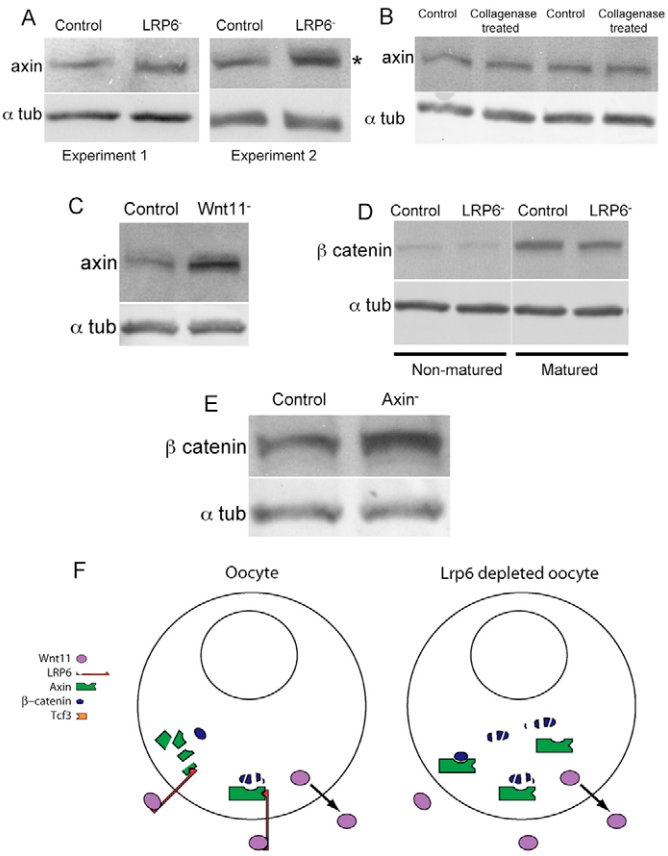

Fig. 6. Wnt11-LRP6 signaling regulates axin levels in the stage-6 oocyte. (**A**) Western blots of wild-type control and LRP6-depleted (LRP6$^-$) stage-6 oocytes frozen after 3 days in culture and probed with an affinity purified axin polyclonal antibody. Axin levels were increased in LRP6-depleted oocytes compared with controls (*36% and 46% increases in experiment 1 and 2, respectively). (**B**) Western blots of wild-type, manually defolliculated oocytes (Control) and collagenase-treated oocytes frozen after 3 days in culture and probed with an axin antibody. Axin levels were unchanged as a result of culture without the follicle cell layer. Two repeats of the experiment are shown. (**C**) Western blots of wild-type and Wnt11-depleted (Wnt11$^-$) stage-6 oocytes frozen after 3 days in culture and probed with an axin antibody. Axin levels were increased in Wnt11-depleted oocytes compared with controls (increased by 128%; range 10-128% in three experiments). (**D**) Western blots of wild-type control and LRP6-depleted (LRP6$^-$) stage-6 oocytes that were frozen after 3 days in culture and probed with a β-catenin antibody. Oocytes were non-matured or stimulated with progesterone and cultured for 8 hours before freezing. β-catenin was expressed at low levels in oocytes before maturation, compared with the levels after maturation. LRP6 depletion reduced the amount of β-catenin protein in matured oocytes (23% less β-catenin compared with controls). (**E**) Western blots of wild-type and axin-depleted (Axin$^-$) stage-6 oocytes that were frozen after 3 days in culture and probed with a β-catenin antibody. β-catenin levels were increased in axin-depleted oocytes compared with controls (99% increase compared with controls). (**F**) A model for steady-state canonical Wnt signaling. Axin is continually synthesized in full-grown, non-matured oocytes and degrades cytoplasmic β-catenin. Wnt11 is also continually synthesized and secreted, binds to LRP6, and degrades some axin protein. Thus, a steady-state level of axin is maintained by Wnt signaling, which, in turn, keeps β-catenin at a low level in the cytoplasm. In LRP6-depleted oocytes, Wnt11 is secreted, but signals cannot be transduced. Thus, axin levels increase, and β-catenin is further reduced.

shown). Fig. 3E and Fig. 7C,D show that *β-catenin* mRNA rescued axis formation and dorsal marker expression when injected at the 4-cell stage, but the same dose did not rescue LRP6-depleted embryos when *β-catenin* mRNA was injected into oocytes before maturation. This experiment was repeated three times with the same result. One trivial explanation of this difference might be that *β-catenin* mRNA was broken down when injected into oocytes but not embryos. We confirmed that *β-catenin* mRNA was not degraded when injected into oocytes (data not shown). Finally, to confirm that more degradation of β-catenin protein occurs in oocytes than in embryos, we examined the levels of β-catenin in western blots of siblings of the embryos shown in Fig. 7C, at the 64-cell stage (Fig. 7E). Embryos injected as oocytes with *β-catenin* mRNA had less total β-catenin protein at the 64-cell stage than those injected at the 4-cell stage. These findings are consistent with the hypothesis that, in the oocyte, axin degrades β-catenin synthesized from the injected mRNA.

To confirm that the failure of β-catenin to rescue the LRP6-depletion phenotype was due to the degradation of β-catenin in the oocyte through an axin- and/or GSK-dependent mechanism, we repeated the rescue experiment comparing the rescuing ability of equivalent doses of wild-type β-catenin-myc mRNA and stabilized β-catenin-myc lacking the 4 N-terminal Gsk3β phosphorylation sites (Yost et al., 1996). Fig. 7G shows that the stabilized form of β-catenin was indeed protected from degradation in LRP6-depleted oocytes, and Fig. 7F,H shows that it rescued the expression of *siamois* and *Xnr3*, and caused a dorsalized phenotype. Embryos derived from LRP6-depleted oocytes injected with stabilized β-catenin before fertilization were radially dorsalized, unlike those injected with β-catenin after fertilization (Fig. 3E). This is consistent with the fact that RNA injected into oocytes is able to diffuse widely, whereas that injected into a single site at the 4-cell stage does not diffuse and allows a dorsal-ventral asymmetry to be established.

Finally, to determine whether, acting in parallel to LRP6, the co-receptor Xfz7 was able to activate the axis-forming pathway, we injected *Xfz7* mRNA into LRP6-depleted oocytes before fertilization. LRP6-depleted embryos were not rescued by the injection of *Xfz7* mRNA either phenotypically (not shown) or by the expression of *siamois* and *Xnr3* (Fig. 7H).

Taken together, these data provide insight into a novel steady-state role for Wnt11 and LRP6 in regulating axin and β-catenin in the oocyte.

DISCUSSION

LRP6 is necessary and sufficient for axis formation

We found, by loss- and gain-of-function analysis, that LRP6 is essential in a canonical Wnt signaling pathway, lying upstream of β-catenin and downstream of maternal Wnt11. Unlike LRP6 function in the mouse (Kelly et al., 2004), we found no evidence of redundancy with LRP5, which continued to be expressed in LRP6-depleted oocytes, but did not rescue the effect of LRP6 depletion.

How does maternal LRP6 function?

Both the sequestration (Nusse, 2005) and nuclear shuttling (Cong and Varmus, 2004) models of axin function predict that LRP6 depletion or over-expression in oocytes and embryos would cause endogenous axin protein to change in localization rather than in total amount. We show here that LRP6 loss of function caused increases in axin levels in oocytes and embryos, supporting a role for LRP6 in the degradation of axin. However, we cannot rule out that LRP6 function in early embryos also involves the sequestration of axin.

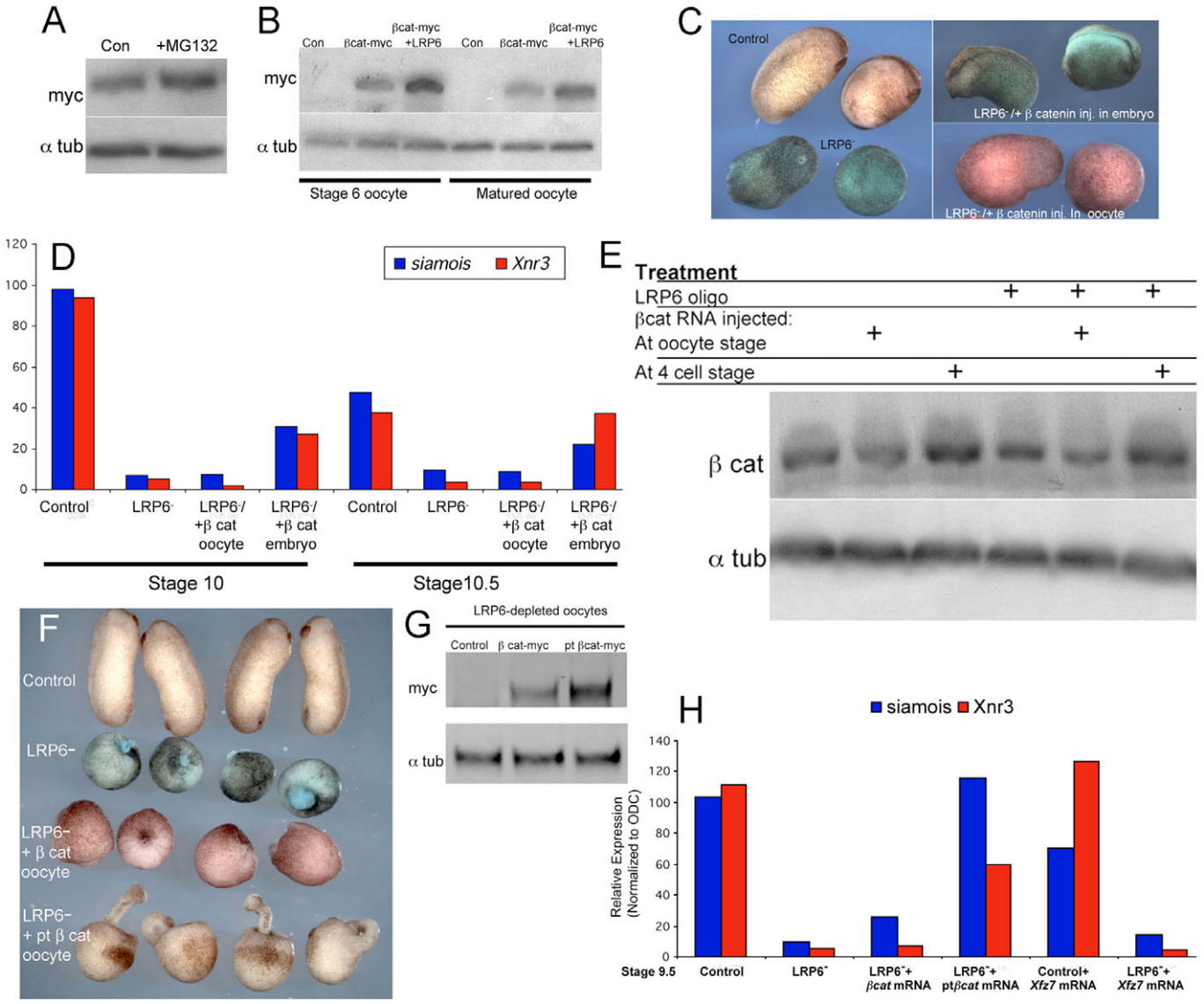

Fig. 7. Exogenous β-catenin is degraded in oocytes. (**A**) Western blot of wild-type unmatured oocytes injected with 100 pg myc-tagged *β-catenin* mRNA (Con) and oocytes injected with 100 pg myc-tagged *β-catenin* mRNA and treated with the proteasome inhibitor MG132 (+MG132), frozen after 3 days in culture and probed with an anti-myc antibody. Myc-tagged β-catenin protein expression was enhanced in the presence of the proteasome inhibitor (44% increase compared with control levels), suggesting that β-catenin is degraded by a proteasomal pathway. (**B**) Western blot of wild-type oocytes injected with 100 pg myc-tagged *β-catenin* mRNA (βcat-myc) compared to oocytes co-injected with 100 pg myc-tagged *β-catenin* mRNA together with 400 pg *LRP6* mRNA (βcat-myc +LRP6) frozen after 3 days in culture and probed with an anti-myc antibody. One group of oocytes was matured for 8 hours after progesterone stimulation (Matured oocyte) before freezing. Myc-tagged β-catenin protein expression was enhanced in the presence of LRP6, both before (increased by 32%) and after (increased by 62%) maturation. (**C**) The ventralized phenotype of LRP6-depleted embryos (bottom left; 6/7 ventralized) was rescued by 50 pg *β-catenin* mRNA when injected at the 4-cell stage (top right; 0/15 ventralized), but not by injection of 50 pg *β-catenin* mRNA in the stage-6 oocyte before maturation (bottom right; 7/7 ventralized; 16 died at cleavage stage). (**D**) The expression of the Wnt target genes *siamois* and *Xnr3* assayed by real-time RT-PCR at the beginning and early gastrula stages (stage 10 and 10.5) in embryos that were siblings to those shown in C. Expression of Wnt target genes was severely reduced in LRP6-depleted embryos, and were partially rescued by the injection of *β-catenin* mRNA after, but not before, fertilization. (**E**) Western blot of sibling embryos to those shown in C, frozen at the 64-cell stage and analyzed for total β-catenin protein. Injection of LRP6 oligo reduced β-catenin levels, which was rescued by the injection of *β-catenin* mRNA at the 4-cell stage, but not by injection of *β-catenin* mRNA in the oocyte. (**F**) Stabilized *β-catenin* mRNA (ptβcat-myc, 20 pg) rescued LRP6 depletion when injected into oocytes (8/12 cases dorsalized), in comparison to 20 pg wild-type *β-catenin* mRNA (8/8 ventralized). Embryos at tailbud stage. (**G**) Western blot of lysates of LRP6-depleted oocytes that were siblings to those used in F. Protected β-catenin protein accumulates after 48 hours in culture, more so than wild type β-catenin. (**H**) The expression of the Wnt target genes *siamois* and *Xnr3*, assayed by real-time RT-PCR at stage 9.5 in sibling embryos to those in F. Wnt target genes were severely reduced in expression in LRP6-depleted embryos, and were partially rescued by the injection of pt*β-catenin* mRNA before fertilization. *Xfz7* mRNA did not rescue *siamois* and *Xnr3* expression.

When does Wnt11-LRP6 function in dorsal axis formation?

These experiments show that LRP6 is essential for axis formation. However, because LRP6 is depleted by injecting antisense oligos into oocytes, we cannot distinguish whether LRP6 is required for axis-formation after fertilization, or before. The Wnt signaling event responsible for establishing the dorsal axis is likely to occur after fertilization, because blocking β-catenin synthesis after fertilization using a translation-inhibitory morpholino oligo causes ventralization if the morpholino is injected vegetally at the 2- to 4-cell stage (Heasman et al., 2000; Yang et al., 2002). If axis formation depended on oocyte-synthesized β-catenin that moved in vesicles to the dorsal side during the first cell cycle (Rowning et al., 1997), then blocking β-catenin translation at the 2-to 4-cell stage should not affect axis formation. Also, dorsal and/or ventral asymmetries of *Wnt11* mRNA and protein are evident after, but not before, fertilization (Tao et al., 2005; Schroeder et al., 1999), and a dorsal reduction of axin occurs specifically at the 8-cell stage (Fig. 5B), whereas β-catenin becomes dorsally nuclearly localized by the 16-cell stage (Larabell et al., 1997).

We show here that *LRP6* mRNA does not rescue LRP6-depleted embryos when injected after fertilization. One possibility is that there is insufficient time for LRP6 protein to be made and inserted onto the membrane in oligomeric complexes for the transduction of the Wnt11 signal. There is only a brief time-window after fertilization when the dorsal pathway can be activated by *β-catenin* mRNA, because *β-catenin* mRNA can only rescue β-catenin-depleted embryos when injected on the dorsal side at or before the 4-cell stage (Heasman et al., 2000; Yang et al., 2002). One possibility is that β-catenin is required for chromatin remodeling as a prerequisite for transcriptional activation. If Wnt11-LRP6 fails to stabilize dorsal β-catenin early, then such remodeling may not be successfully initiated. A second possibility is that a Wnt inhibitor, such as dickkopf, may be synthesized after fertilization and may actively block all but the earliest Wnt11-LRP6 interaction. Preliminary data suggest that *Dkk* mRNA is synthesized during oogenesis and may be a candidate for this role.

The fact that axin levels in wild-type embryos are not reduced dorsally after the 8-cell stage may be explained in any or all of three ways: first, synthesis of axin may outpace its degradation; second, axin may sequester, as well as degrade, β-catenin; and third, Wnt signaling may be downregulated by a Wnt inhibitor. Further experiments are needed to distinguish between these possibilities.

Steady-state Wnt signaling

We have previously assumed that Wnt signaling is 'activated' on the dorsal side of the embryo during the cleavage stages, leading to increased β-catenin levels on the dorsal side. However, this begs the question of what mechanism regulates the 'steady state' levels of β-catenin before such an activation? In the current model of canonical Wnt signaling, the assumption has been that expression of Wnt target genes or TCF reporter constructs are indicators of Wnt signaling (Boras-Granic et al., 2006; De Langhe et al., 2005; Hens et al., 2005), suggesting that their lack of expression coincides with Wnt pathway inactivation. An alternative model is that Wnt signaling regulates β-catenin constitutively (Fig. 6F), and that the changes that lead to the nuclear accumulation of β-catenin are due to either increased levels of Wnt signaling (Fig. 4C), or to the involvement of other pathway components, rather than de novo activation. In its simplest form, a constitutive low level of Wnt signaling would lead to low levels of axin degradation by LRP6 (Fig. 6F). This in turn would lead to high levels of axin, and lower β-catenin levels. In the embryo, increased Wnt signaling on the dorsal side would lead to increased levels of β-catenin, through the same mechanism. The data presented here provide evidence for the existence of a steady-state role for Wnt signaling in modulating axin levels. Specifically, Wnt11 depletion and LRP6 depletion both cause an increase in axin levels in the full-grown oocyte. Our study has shown, in several ways, that the effects on axin are important for β-catenin regulation in the oocyte. First, the amount of β-catenin increased in oocytes when axin was depleted using an antisense oligo (Fig. 6E). Second, exogenous β-catenin was degraded in oocytes in a proteasome-dependent fashion (Fig. 7A) and stabilized by *LRP6*-mRNA co-injection (Fig. 7B). And third, *β-catenin* mRNA rescues LRP6-depleted embryos when injected after fertilization, but not before (Fig. 7F).

This work changes our assumption that Wnt signaling is an on or off switch, and raises the query as to whether similar constitutive Wnt regulation of axin occurs elsewhere in embryonic and adult tissues, in places that would not be revealed by studying transcriptional target activation.

Thanks to Dr Peter Klein for generously supplying the axin serum and to Dr Ethan Lee for sharing data prior to publication. Thanks to Drs Aaron Zorn, Frank Hess (Merck) and Doug Melton for plasmids, and to Stephanie Lang and Helbert Puck for technical assistance.

References

Bird, A. P. and Wolffe, A. P. (1999). Methylation-induced repression – belts, braces, and chromatin. *Cell* **99**, 451-454.

Boras-Granic, K., Chang, H., Grosschedl, R. and Hamel, P. A. (2006). Lef1 is required for the transition of Wnt signaling from mesenchymal to epithelial cells in the mouse embryonic mammary gland. *Dev. Biol.* **295**, 219-231.

Carter, M., Chen, X., Slowinska, B., Minnerath, S., Glickstein, S., Shi, L., Campagne, F., Weinstein, H. and Ross, M. E. (2005). Crooked tail (Cd) model of human folate-responsive neural tube defects is mutated in Wnt coreceptor lipoprotein receptor-related protein 6. *Proc. Natl. Acad. Sci. USA* **102**, 12843-12848.

Cong, F. and Varmus, H. (2004). Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of beta-catenin. *Proc. Natl. Acad. Sci. USA* **101**, 2882-2887.

Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A. and Niehrs, C. (2005). Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* **438**, 867-872.

De Langhe, S. P., Sala, F. G., Del Moral, P. M., Fairbanks, T. J., Yamada, K. M., Warburton, D., Burns, R. C. and Bellusci, S. (2005). Dickkopf-1 (DKK1) reveals that fibronectin is a major target of Wnt signaling in branching morphogenesis of the mouse embryonic lung. *Dev. Biol.* **277**, 316-331.

Dupont, S., Zacchigna, L., Cordenonsi, M., Soligo, S., Adorno, M., Rugge, M. and Piccolo, S. (2005). Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. *Cell* **121**, 87-99.

Hamada, F., Tomoyasu, Y., Takatsu, Y., Nakamura, M., Nagai, S., Suzuki, A., Fujita, F., Shibuya, H., Toyoshima, K., Ueno, N. et al. (1999). Negative regulation of Wingless signaling by D-axin, a Drosophila homolog of axin. *Science* **283**, 1739-1742.

Heasman, J. (2006). Patterning the early Xenopus embryo. *Development* **133**, 1205-1217.

Heasman, J., Kofron, M. and Wylie, C. (2000). Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. *Dev. Biol.* **222**, 124-134.

Hens, J. R., Wilson, K. M., Dann, P., Chen, X., Horowitz, M. C. and Wysolmerski, J. J. (2005). TOPGAL mice show that the canonical Wnt signaling pathway is active during bone development and growth and is activated by mechanical loading in vitro. *J. Bone Miner. Res.* **20**, 1103-1113.

Houston, D. W. and Wylie, C. (2002). Cloning and expression of Xenopus Lrp5 and Lrp6 genes. *Mech. Dev.* **117**, 337-342.

Kelly, O. G., Pinson, K. I. and Skarnes, W. C. (2004). The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. *Development* **131**, 2803-2815.

Kofron, M., Klein, P., Zhang, F., Houston, D. W., Schaible, K., Wylie, C. and Heasman, J. (2001). The role of maternal axin in patterning the Xenopus embryo. *Dev. Biol.* **237**, 183-201.

Landsberger, N. and Wolffe, A. P. (1995). Chromatin and transcriptional activity in early Xenopus development. *Semin. Cell Biol.* **6**, 191-199.

Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D. and Moon, R. T. (1997). Establishment of the dorso-ventral axis

in Xenopus embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.* **136**, 1123-1136.
Lee, E., Salic, A., Kruger, R., Heinrich, R. and Kirschner, M. W. (2003). The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. *PLoS Biol.* **1**, E10.
Li, Y., Lu, W., He, X., Schwartz, A. L. and Bu, G. (2004). LRP6 expression promotes cancer cell proliferation and tumorigenesis by altering beta-catenin subcellular distribution. *Oncogene* **23**, 9129-9135.
Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.
Mao, J., Wang, J., Liu, B., Pan, W., Farr, G. H., 3rd, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L. et al. (2001). Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. *Mol. Cell* **7**, 801-809.
Nusse, R. (2005). Cell biology: relays at the membrane. *Nature* **438**, 747-749.
Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J. and Skarnes, W. C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407**, 535-538.
Rowning, B. A., Wells, J., Wu, M., Gerhart, J. C., Moon, R. T. and Larabell, C. A. (1997). Microtubule-mediated transport of organelles and localization of beta-catenin to the future dorsal side of Xenopus eggs. *Proc. Natl. Acad. Sci. USA* **94**, 1224-1229.
Salic, A., Lee, E., Mayer, L. and Kirschner, M. W. (2000). Control of beta-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in Xenopus egg extracts. *Mol. Cell* **5**, 523-532.
Schneider, S., Steinbeisser, H., Warga, R. M. and Hausen, P. (1996). Beta-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. *Mech. Dev.* **57**, 191-198.
Schroeder, K. E., Condic, M. L., Eisenberg, L. M. and Yost, H. J. (1999). Spatially regulated translation in embryos: asymmetric expression of maternal Wnt-11 along the dorsal-ventral axis in Xenopus. *Dev. Biol.* **214**, 288-297.
Sumanas, S., Strege, P., Heasman, J. and Ekker, S. C. (2000). The putative wnt receptor Xenopus frizzled-7 functions upstream of beta-catenin in vertebrate dorsoventral mesoderm patterning. *Development* **127**, 1981-1990.
Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P. and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407**, 530-535.
Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z. and He, X. (2004). A mechanism for Wnt coreceptor activation. *Mol. Cell* **13**, 149-156.
Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C. C., Lin, X. and Heasman, J. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. *Cell* **120**, 857-871.
Tolwinski, N. S., Wehrli, M., Rives, A., Erdeniz, N., DiNardo, S. and Wieschaus, E. (2003). Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3beta activity. *Dev. Cell* **4**, 407-418.
Wehrli, M., Dougan, S. T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A. and DiNardo, S. (2000). arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature* **407**, 527-530.
Wiechens, N., Heinle, K., Englmeier, L., Schohl, A. and Fagotto, F. (2004). Nucleo-cytoplasmic shuttling of Axin, a negative regulator of the Wnt-beta-catenin Pathway. *J. Biol. Chem.* **279**, 5263-5267.
Willert, K., Logan, C. Y., Arora, A., Fish, M. and Nusse, R. (1999a). A Drosophila Axin homolog, Daxin, inhibits Wnt signaling. *Development* **126**, 4165-4173.
Willert, K., Shibamoto, S. and Nusse, R. (1999b). Wnt-induced dephosphorylation of axin releases beta-catenin from the axin complex. *Genes Dev.* **13**, 1768-1773.
Yang, J., Tan, C., Darken, R., Wilson, P. and Klein, P. (2002). Beta catenin/TCF regulated transcription prior to the mid-blastula transition. *Development* **129**, 5743-5752.
Yost, C., Torres, M., Miller, J., Huang, E., Limelman, D. and Moon, R. (1996). The axis-inducing activity, stability, and subcellular localization of beta catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. *Genes Dev.* **10**, 1443-1454.
Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J. and He, X. (2005). A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* **438**, 873-877.
Zuck, M. V., Wylie, C. C. and Heasman, J. (1998). Maternal mRNAs in Xenopus embryos: an antisense approach. In *A comparative methods approach to the study of oocytes and embryos*, (ed. J. D. Richter), pp. 341-354. Oxford: Oxford University Press.