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Wnt5a and Wnt11 interact in a maternal Dkk1-regulated fashion to activate both canonical and non-canonical signaling in *Xenopus* axis formation

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Wnt signaling in development and adult tissue homeostasis requires tight regulation to prevent patterning abnormalities and tumor formation. Here, we show that the maternal Wnt antagonist Dkk1 downregulates both the canonical and non-canonical signaling that are required for the correct establishment of the axes of the *Xenopus* embryo. We find that the target Wnts of Dkk activity are maternal Wnt5a and Wnt11, and that both Wnts are essential for canonical and non-canonical signaling. We determine that Wnt5a and Wnt11 form a previously unrecognized complex. This work suggests a new aspect of Wnt signaling: two Wnts acting in a complex together to regulate embryonic patterning.

KEY WORDS: Dkk1, Wnt11, Wnt5a, *Xenopus*, Complex

INTRODUCTION

The family of Wnt proteins share a signal sequence, several highly charged residues and glycosylation sites, and a characteristic distribution of 22 cysteines. They were initially classified into two groups, canonical and non-canonical: one subset of Wnts (e.g. Wnt1, Wnt3a, Wnt8) could induce a secondary axis with a head when overexpressed on the ventral side of *Xenopus* embryos, and could transform mouse mammary epithelial cells, whereas the other subset (e.g. Wnt4, Wnt5a, Wnt11) could not (Du et al., 1995). Further studies suggested that the two classes also showed differences in their targets: the Wnt1 group stabilized β-catenin (the canonical Wnt pathway), and the second group activated either or both Jun NH_2-terminal kinase (JNK) and calcium/calmodulin dependent kinase 2, and caused convergent extension movements and/or cellular polarization (the non-canonical group) (Kikuchi et al., 2007).

Recent studies suggest that the above classification of Wnts is too rigid, as individual Wnts can activate both canonical and non-canonical pathways, depending on context. Wnt11 regulates cell movements in *Xenopus* and zebrafish gastrulation and neurulation by non-canonical Wnt pathways (De Calisto et al., 2005; Tada et al., 2002; Tada and Smith, 2000), but also activates a canonical β-catenin-dependent pathway in establishing the dorsal/ventral axis (Tao et al., 2005). Wnt5a activates non-canonical signaling in rat cardiac myocytes and *Xenopus* gastrulation, whereas it activates canonical signaling for self-renewal in mouse embryonic stem cells (Katoh and Katoh, 2007). In addition, purified Wnt5a activates canonical signaling in the presence of the frizzled 4 receptor, and suppresses it in the presence of the Ror2 receptor (Mikels and Nusse, 2006). One possibility that has not been considered previously is that Wnt proteins may act together to activate signaling pathways.

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The current model for Wnt function in dorsal/ventral axis signaling in *Xenopus* embryos is that, after fertilization, vegetally localized *Wnt11* mRNA is moved by cortical rotation movements and becomes enriched on the dorsal versus the ventral side of the fertilized egg (Tao et al., 2005). During cleavage, Wnt11 is secreted by dorsal vegetal cells and activates signaling through a canonical Wnt pathway, so that on the dorsal side, newly synthesized β-catenin is more stable, interacts with XTcf3 in dorsal nuclei and activates Wnt target gene expression at MBT. However, although there is more *Wnt11* mRNA dorsally at the 32-cell stage, there is a significant amount of ventral *Wnt11* mRNA (Tao et al., 2005), which raises the question why is there no *Wnt11* target gene activation on the ventral side of the embryo? This study was initiated to determine whether the maternally encoded Wnt antagonist Dkk1 plays an important role in limiting the site and amount of activation of maternal Wnt target genes.

Dkk1 belongs to a small family of 24-29 kDa secreted glycoproteins (Niehrs, 2006). Zygotic *Dkk1* mRNA is necessary and sufficient for head formation in *Xenopus* (Glinka et al., 1998), and is implicated as a tumor suppressor in mammals (Niehrs, 2006). Dkk1 acts as a Wnt antagonist indirectly, by preventing the Wnt11-dependent interaction of LRP and frizzled (Bafico et al., 2001; Semenov et al., 2001). Hitherto, Dkk1 function has been considered to be specific for canonical rather than non-canonical Wnt signaling (Semenov et al., 2001), although recently it was shown to bind glypican 4 and to activate non-canonical signaling (Caneparo et al., 2007). Here, we demonstrate that maternal Dkk1 antagonizes both canonical and non-canonical signaling to regulate the dorsal/ventral patterning of the early *Xenopus* embryo.

As Wnt5a has been shown to affect non-canonical Wnt signaling during gastrulation in *Xenopus* (Moon et al., 1993), we reasoned that maternal Wnt5a may be the non-canonical Wnt regulated by maternal Dkk1. Surprisingly, depletion of maternal Wnt5a resulted in the same ventralized phenotype as that we reported previously for maternal Wnt11 depletion (Tao et al., 2005). This result suggested that both Wnt11 and Wnt5a are required for axis formation in the early embryo. We show here that Wnt5a acts together with Wnt11 in the initial signaling event that activates canonical, β-catenin-dependent, *Xnr3*, *siamois* and *Xnr5* expression, as well as in non-canonical JNK activation of the morphogenetic movements of

gastrulation. We demonstrate that Wnt11 and Wnt5a interact in both biochemical and functional tests. The results suggest that complexes formed between Wnt5a homodimers and Wnt11 homodimers are required to activate the dorsal signaling pathway in the early embryo.

MATERIALS AND METHODS

Oocytes and embryos

Oocytes and embryos were cultured as described (Tao et al., 2005). Antisense oligos were injected on day 1, oocytes were matured on day 3 and fertilized on day 4. For rescue experiments, mRNA was injected on day 3. Antisense oligos were: Dkk1 AS, 5′-g*g*a*gttggtcatcat*g*a*c-3′; Wnt11 AS, 5′-g*t*c*ggagccattggt*a*c*t-3′; Wnt5a AS, 5′-c*c*a*gacagttgg-ctg*c*a*c-3′; and β-catenin morpholino, 5′-tttcaaccgtttccaaagaaccagg-3′. Where * indicates a phosphorothioate bond.

Quantitative RT-PCR and in situ hybridization

Total RNA from oocytes, explants and early embryos was isolated using the protocol of Tao et al. (Tao et al., 2005). Real-time RT-PCR were performed using a LightCycler (Roche). Water-blank and RT-minus controls were included in all runs. All RT-PCR results are presented as percentage compared with the level in uninjected embryos after normalization to the expression of ornithine decarboxylase (ODC). Whole-mount in situ hybridization was as described (Birsoy et al., 2006).

Luciferase assay

TOPflash DNA (Upstate; 50 pg), together with 25 pg pRLTK DNA, was injected into two dorsal or ventral cells at the four-cell stage. Three replicate samples each of three embryos were frozen for each group at the early gastrula stage and assayed using Promega luciferase assay system.

Western blot

Western blots were carried out as described previously (Birsoy et al., 2006). Antibodies concentrations were: rabbit anti-pJNK, 1/750; rabbit anti-total JNK, 1/750; rabbit anti-pSmad1, 1/500; rabbit anti-pSmad2, 1/500 (all from Cell Signaling); and mouse anti-tubulin (DM1A, Neomarker), 1/5000.

Dimerization assays

Tagged mRNAs were injected at doses 100 pg-1 ng, and embryos were frozen at stage 10 in batches of five, lysed in 50 μl (10 μl/embryo) of ice-cold PBS-Triton buffer (1×PBS, pH 7.4; 1% Triton X-100) supplemented with Iodoacetamide (IAA, 10 mM). Clear lysate was obtained by a 10-minute centrifugation (14,400 ***g*** at 4°C), and was mixed either with sample buffer without β-ME (non-reducing condition) or with sample buffer containing 10% β-ME (reducing condition). Samples were processed by standard SDS-PAGE and western blotting. Antibodies: rat anti-HA (clone 3F10, Roche), 1/2000; rabbit anti-Myc (Cell Signaling), 1/1000; rabbit anti-Flag (Sigma), 1/2000; mouse anti-GFP (clone B-2, Santa Cruz Biotechnology), 1/500.

Co-immunoprecipitation

Embryos were frozen at stage 10 in batches of 50 and lysed with 1 ml (20 μl/embryo) ice-cold PBS-Triton buffer. The homogenate was spun at 500 ***g*** for 5 minutes at 4°C; the supernatant was collected in a new tube and spun at 14,400 ***g*** for 10 minutes at 4°C. The clear lysate was mixed with protein-A or protein-G agarose beads coated with the antibody of interest and incubated for 2 hours at 4°C. The beads were pelleted, washed four times with ice-cold lysis buffer, mixed with minimum volume of SDS-PAGE sample buffer and processed through standard electrophoresis and western blot protocol using ice-cold CAPS buffer (0.2% CAPS, 15% methanol, pH 10.5) for wet transfer.

Generation of Wnt11ΔC and Wnt11-GFP

Wnt11ΔC was generated as a flag-tagged *Eco*RI/*Xho*I fragment by PCR amplification of nucleotides 1-846 (amino acids 1-282) of Wnt11 ORF and inserted in the pCS107 vector. For Wnt11-GFP, the full Wnt11 ORF was amplified as an *Eco*RI/*Bam*HI fragment and inserted behind and in frame with the GFP gene in the pEGFP-N1 vector. Wnt11 and GFP were excised as a single fragment by *Eco*RI/*Not*I digestion, and inserted into pCS107 vector. Wnt11ΔC and Wnt11-GFP constructs were linearized (*Nsi*I) and transcribed with Sp6 polymerase.

RESULTS

Dkk1 inhibits canonical Wnt signaling in axis formation

RT-PCR analysis of cDNA from stage 6 *Xenopus* oocytes showed that maternal *Dkk1* mRNA was expressed at a low level compared with zygotic expression levels (Fig. 1A). Unlike *VegT* mRNA, *Dkk1* mRNA was not localized in the oocyte or early embryo but was expressed equally in animal/vegetal, and dorsal/ventral half oocytes, as well as right and left halves of four-cell stage embryos (Fig. 1B,C).

An antisense oligonucleotide was effective in depleting *Dkk1* mRNA to less than 20% of control levels in stage 6 oocytes (Fig. 1D). In a temporal expression series comparing sibling control and Dkk1-depleted oocytes and embryos, *Dkk1* mRNA expression remained low in oligo-injected embryos until the onset of zygotic transcription, when zygotic *Dkk1* mRNA was expressed in both control and oligo-injected groups (Fig. 1A).

When sibling control oocytes and oocytes injected with 5, 7.5 and 10 ng of Dkk1AS oligo were fertilized, they developed normally to the late blastula stage, when Dkk1-depleted embryos underwent abnormal shape changes. During gastrulation and neurulation, devitellined embryos cultured on agar became extremely elongated compared with uninjected sibling controls (Fig. 2A). These effects were specifically due to *Dkk1* mRNA depletion, as they were partially rescued by injecting human *Dkk1* mRNA (20 pg) into Dkk1-depleted oocytes before maturation (Fig. 2B). As Dkk1 is a Wnt antagonist, we tested whether its depletion upregulated the maternal canonical Wnt signaling pathway. Fig. 2C shows that Dkk1

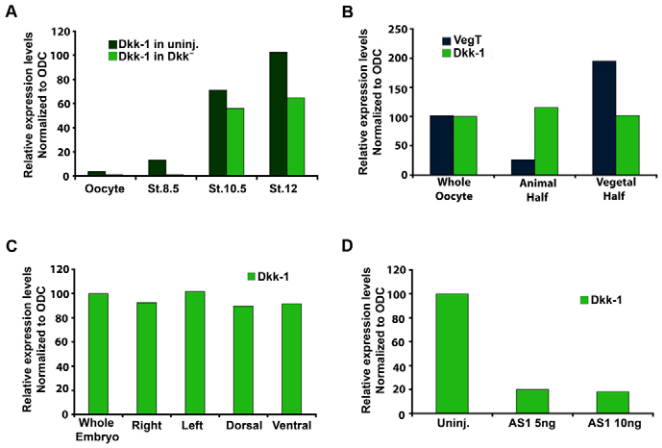

Fig. 1. Maternal Dkk1 is ubiquitously expressed. (**A**) Control uninjected (uninj.) and sibling antisense oligo (AS1 5 ng)-injected oocytes were frozen as mature oocytes, mid- (stage 8.5), early (stage 10.5) and late gastrula (stage 12) stage oocytes, and assayed by real-time RT-PCR for the relative expression of *Dkk1* mRNA. (**B**) Batches of two whole and four animal- or four vegetal-half uninjected stage 6 oocytes were assayed by real-time RT-PCR for the relative expression of *VegT* and *Dkk1* mRNA. (**C**) Batches of two whole and four right-, left-, dorsal- or ventral-half four-cell stage embryos were assayed by real-time RT-PCR for the relative expression of *Dkk1* mRNA. (**D**) Control uninjected (uninj.) and sibling antisense oligo (AS1 5 ng, 10 ng)-injected oocytes were frozen after 48 hours in culture as mature oocytes and assayed for maternal *Dkk1* mRNA expression.

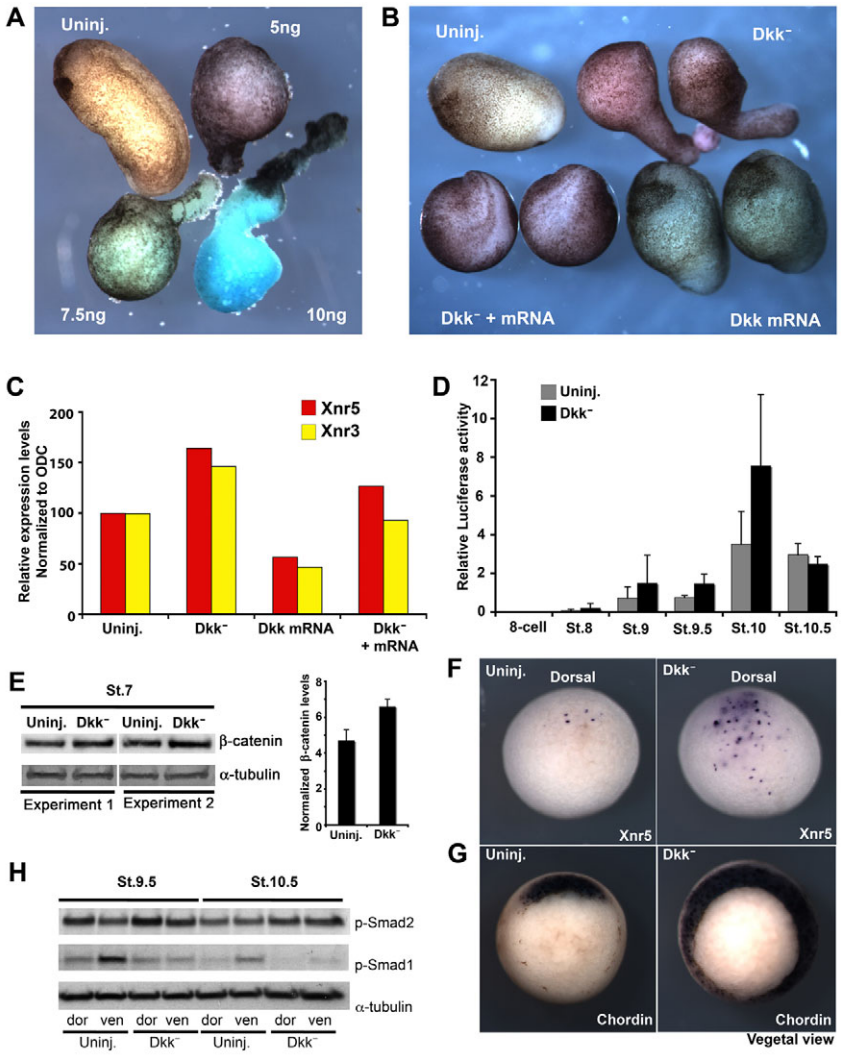

Fig. 2. Maternal Dkk1 inhibits canonical Wnt signaling. (**A**) Embryos derived from sibling control (uninj.) and Dkk1-depleted oocytes (5, 7.5 and 10 ng oligo injected) at the early tailbud stage. This phenotype was seen in seven experiments in a total of 85% of cases (124/165). (**B**) The phenotype of Dkk1-depleted (Dkk⁻) embryos was partially rescued by the reintroduction of 20 pg human *Dkk1* mRNA (Dkk⁻+mRNA) before fertilization. Here, Dkk1-depleted embryos (14/17) had the elongated phenotype shown compared with 4/14 for Dkk1⁻+mRNA and 0/24 uninjected; 20 pg human *Dkk1* mRNA alone (Dkk mRNA) caused enlargement of head structures (16/18). The experiment was repeated with a similar result. (**C**) The relative expression levels of *Xnr3* and *Xnr5* in control (uninj.), in Dkk1 depleted (Dkk⁻), in 20 pg human *Dkk1* mRNA (Dkk mRNA) and in Dkk1 depleted+20 pg human *Dkk1* mRNA injected (Dkk⁻+mRNA) embryos assayed by real-time RT-PCR at the late blastula stage; siblings of those shown in B. (**D**) TOPflash reporter activation after injection into two dorsal cells of four-cell stage control embryos compared with sibling Dkk1-depleted embryos frozen at the eight-cell, mid- (stage 8), late blastula (stages 9, 9.5) and early gastrula stages (stages 10, 10.5). (**E**) Western blot of total β-catenin protein in control and Dkk1-depleted sibling early blastulae (stage 7), using α-tubulin as a loading control. Quantitation is shown on the right. (**F**,**G**) In situ hybridization of sibling control and Dkk1-depleted early gastrulae (*Xnr5*, F; *chordin*, G). (**H**) Western blot of phospho-Smad2 and -Smad1 proteins in control and Dkk1-depleted sibling at late blastulae (stage 9.5) and early gastrulae (stage 10.5). Before freezing, embryos were hemisected into batches of four dorsal (dor) and four ventral (ven) halves.

depletion caused the upregulation of direct Wnt target genes expression (*Xnr3* and *Xnr5*) compared with their expression in sibling control uninjected embryos, which was rescued by human *Dkk1* mRNA (20 pg) (Fig. 2C).

To confirm that Dkk1 depletion upregulated canonical Wnt signaling, we injected the TOPflash reporter in control and Dkk1-depleted embryos. Dkk1 depletion increased TOPflash activity in late blastula and early gastrula stage embryos compared with controls (Fig. 2D). In addition, loss of Dkk1 activity increased the amount of stable β-catenin protein in early blastula stage embryos (Fig. 2E).

Increased expression of *Xnr3* and *Xnr5* in Dkk1-depleted embryos could be due to enhanced Wnt signaling in the correct site of expression, or to Wnt signal to ectopic sites, or both. Whole-mount in situ hybridization analysis at the early gastrula stage showed that *Xnr5* mRNA was enriched in the usual dorsal vegetal location, and was also ectopically expressed in the ventral vegetal area in Dkk1-depleted embryos (Fig. 2F). *Xnr3* mRNA was present in a narrower longer area of the dorsal equatorial and vegetal region compared with sibling uninjected control early gastrulae (see Fig. S1 in the supplementary material). Confirming the ectopic activation of canonical signaling, TOPflash reporter activity was enhanced in ventral half embryos at the late blastula stage (see Fig. S2 in the supplementary material) and *Xnr5* was increased on the ventral side by RT-PCR (see Fig. S3 in the supplementary material).

Increased activation of the direct Wnt target genes should cause enhanced activation of secondary targets (Xanthos et al., 2002), which was seen for the dorsal markers *chordin*, *goosecoid* and *Hex* (Fig. 2G and data not shown). These experiments show that Dkk1 regulates both the level and position of expression of canonical Wnt target genes.

Xnrs activate each other's expression, increasing Smad2 signaling (Hyde and Old, 2000; Takahashi et al., 2000). We therefore analyzed the Smad2 phosphorylation state of Dkk1-depleted and sibling control embryos at the late blastula and early gastrula stages, using dissected dorsal and ventral halves. P-Smad2 was increased in both dorsal and ventral half embryos compared with controls (Fig. 2H).

Increased levels of chordin expression in Dkk1-depleted embryos might abrogate signaling in the BMP pathway. Fig. 2H shows that the level of phosphorylated Smad1 was decreased in ventral and dorsal Dkk1-depleted half embryos compared with controls. These experiments show that the loss of Wnt antagonism caused by maternal Dkk1 depletion has profound effects on other signaling networks within 2 hours of MBT.

Dkk1 inhibits non-canonical Wnt signaling in axis formation

To determine whether the observed effects of Dkk1 depletion were due only to the hyper-activation of the canonical Wnt signaling pathway, we compared embryos depleted of both Dkk1 and β-catenin with those depleted of each alone. If the phenotype caused by Dkk1 depletion were completely dependent on canonical signaling, double-depleted embryos should phenocopy the ventralized β-catenin-depleted phenotype. However, Fig. 3A shows that some aspects of the Dkk1 depletion phenotype were β-catenin

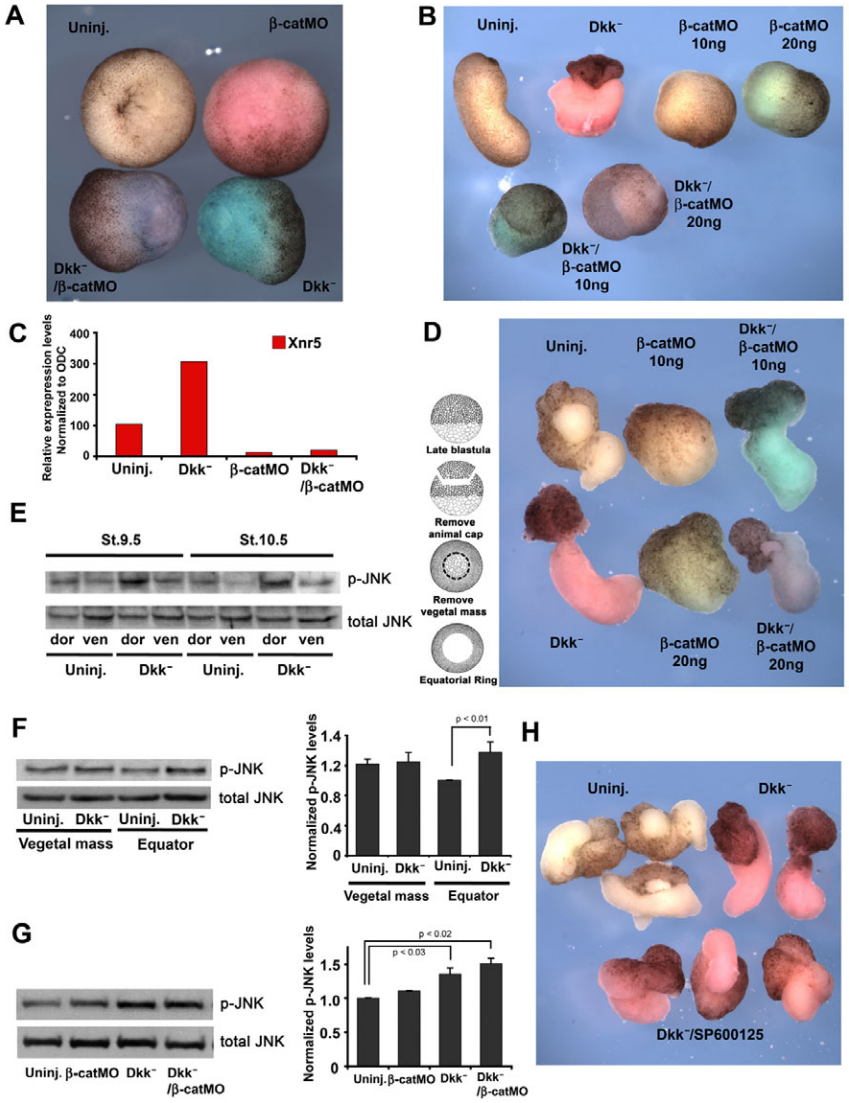

Fig. 3. Maternal Dkk1 inhibits non-canonical Wnt signaling. (**A**) Embryos derived from sibling control, β-catenin-depleted (β-catMO), Dkk1-depleted and Dkk1/β-catenin-depleted oocytes at the early gastrula stage. The Dkk1-depleted abnormal shape change was seen in 22/26 cases, and was present in 0/25 β-catenin-depleted, 18/21 Dkk/β-catenin-depleted cases and 2/24 controls. The experiment was repeated with similar results. (**B**) Embryos derived from sibling control, Dkk1-depleted, β-catenin-depleted and Dkk/β-catenin-depleted oocytes at the early tailbud stage. 18/20 Dkk1-depleted embryos had the phenotype shown, whereas β-catenin-depleted embryos were ventralized (20/20, 10 ng MO; 18/18, 20 ng MO). Dkk/β-catenin-depleted embryos were also ventralized (19/20 Dkk⁻/10ng MO; 17/17 Dkk⁻/20ng MO) and 24/24 controls were normal. (**C**) The relative expression levels of *Xnr5* in control, Dkk1-depleted, β-catenin-depleted and Dkk1/β-catenin-depleted embryos assayed by real-time RT-PCR at the early gastrula stage. (**D**) Equatorial zones from control, Dkk1-depleted, β-catenin-depleted and Dkk1/β-catenin-depleted dissected at the mid-blastula stage and cultured until the late neurula stage. Dkk1-depleted (9/9) and Dkk1/β-catenin-depleted explants (10/11 Dkk⁻/10 ng MO; 11/11 Dkk⁻/20 ng MO) were hyper-elongated compared with control, whereas β-catenin-depleted embryos were round (8/8 with 10 ng MO; 9/9 with 20 ng MO). The experiment was repeated with similar results. (**E**) Western blot of p-JNK-1 protein in control and Dkk1-depleted sibling at late blastulae (stage 9.5) and early gastrulae (stage 10.5), hemisected into dorsal (dor) and ventral (ven) halves before freezing. A total JNK antibody was used as loading control. (**F**) Western blot of p-JNK-1 protein in control and Dkk1-depleted vegetal masses compared with equatorial explants. Ten vegetal masses and five equators were compared. Quantitation is shown on the right. $P<0.01$, Student's *t*-test. (**G**) Western blot of p-JNK-1 protein in control, β-catenin-depleted, Dkk1-depleted and Dkk1/β-catenin-depleted (Dkk⁻/β-catMO) late blastulae. Quantitation is shown on the right. $P<0.03$, Student's *t*-test. (**H**) Equatorial zones from control, Dkk-depleted and JNK inhibitor SP600125-treated equatorial zones dissected at the mid-blastula stage and cultured until the late neurula stage. 0/10 uninjected, 10/10 Dkk⁻ and 1/10 Dkk⁻/SP600125-treated explants had the Dkk1-depleted shape. The experiment was repeated with similar results.

independent. Specifically, at the late blastula and early gastrula stages, Dkk1/β-catenin-depleted embryos underwent the same abnormal shape changes as Dkk1-depleted siblings (Fig. 3A). The embryos began to elongate along the animal-vegetal axis compared with wild-type or β-catenin depleted siblings, and formed a constricted region in the equatorial zone, such that they developed a 'mushroom' shape at stage 9. By contrast, loss of canonical Wnt target gene expression occurred in Dkk1/β-catenin-depleted embryos, as it did in β-catenin-depleted siblings (Fig. 3C). In addition, by the neurula and tailbud stages, the Dkk1/β-catenin-depleted embryos phenocopied their ventralized β-catenin-depleted siblings, suggesting that later cell movements were dependent on signaling through β-catenin (Fig. 3B).

Convergent extension movements occur mainly in the equatorial zone. To compare these movements more closely in control, Dkk1-depleted, β-catenin-depleted and Dkk1/β-catenin-depleted embryos, the equatorial zones were dissected at the mid-blastula stage and cultured until siblings reached early tailbud (Fig. 3D). Dkk1 depleted equatorial zones underwent extreme elongation compared with controls, whereas β-catenin depleted explants were rounded in appearance. Equatorial zones from double-depleted embryos also elongated more than uninjected controls, phenocopying Dkk1 depletion rather than the rounded β-catenin-depleted morphology (Fig. 3D). Sibling double-depleted whole embryos were cultured to the tailbud stage, and Dkk1/β-catenin-depleted embryos showed the ventralized phenotype resembling β-catenin-depleted embryos (Fig. 3B). When dorsal and ventral equatorial zones were compared, the ventral marginal zones of wild-type sibling embryos did not elongate, whereas those of Dkk1-depleted embryos did (see Fig. S4 in the supplementary material).

The fact that Dkk1-depleted embryos continued to undergo abnormal gastrulation movements in the absence of β-catenin suggested that Dkk1 may also regulate non-canonical Wnt signaling. As JNK activation is a key component in non-canonical signaling (Schambony and Wedlich, 2007; Yamanaka et al., 2002), we next investigated JNK activity. We found that there was a sustained increase in the level of phospho-JNK1 compared with controls in the late blastula and early gastrula stages in Dkk1-depleted embryos (Fig. 3E). This increase was localized to the equatorial zone (presumptive mesoderm) compared with the vegetal mass (presumptive endoderm) in equatorial and vegetal explants (Fig. 3F).

As Dkk1/β-catenin-depleted embryos and explants underwent extreme morphogenetic movements, we asked whether they maintained higher levels of p-JNK compared with controls. Fig. 3G shows that Dkk1/β-catenin-depleted embryos have increased levels of p-JNK compared with uninjected sibling controls. To confirm the dependence on enhanced JNK1 activity, Dkk1-depleted equatorial zones were cultured with or without the JNK inhibitor SP600125. In the presence of SP600125, the excessive elongation of Dkk1-depleted equatorial zones was significantly reduced (Fig. 3H). Together, these experiments show that Dkk1 acts to limit the extent of non-canonical Wnt signaling, leading to morphogenetic movement via JNK-1 activity.

Maternal Wnt5a and Wnt11 are both required for canonical and non-canonical signaling in the early *Xenopus* embryo

As Wnt5a is involved in non-canonical Wnt signaling during gastrulation in *Xenopus* (Moon et al., 1993; Schambony and Wedlich, 2007), we reasoned that maternal Wnt5a may be the non-canonical Wnt regulated by maternal Dkk1. We designed an antisense oligonucleotide that was effective in depleting *Wnt5a* mRNA, and did not degrade maternal *Wnt11*, *β-catenin* or *Dvl2* mRNA (Fig. 4A). We confirmed that zygotic *Wnt5a* mRNA does not begin to accumulate until after gastrulation in control uninjected embryos (Fig. 4B) (Moon et al., 1993), such that any loss-of-function phenotypes caused before this time must be the result of the loss of maternal Wnt5a. Sibling control oocytes and maternal Wnt5a-depleted oocytes were fertilized and developed normally to the gastrula stage. Wnt5a-depleted embryos gastrulated without forming axial structures, showing the 'ventralized' phenotype typical of maternal Wnt11- and β-catenin-depleted embryos (Fig. 4C) (Heasman et al., 1994; Tao et al., 2005). Axis formation was partially rescued by the re-introduction of *Wnt5a* mRNA before fertilization (Fig. 4D). Wnt5a-depleted embryos had reduced expression of the canonical Wnt target genes *Xnr3* and *Xnr5*, which was rescued by the reintroduction of *Wnt5a* mRNA, showing the specificity of the phenotype (Fig. 4E). We confirmed that Wnt5a depletion affected canonical Wnt signaling as endogenous TOPflash activity was reduced at the late blastula stage after Wnt5a depletion (Fig. 4F). Furthermore, *Wnt5a* mRNA overexpression caused an upregulation of TOPflash, and this was completely dependent on the presence of β-catenin protein (Fig. 4G). These experiments show that maternal Wnt5a, like maternal Wnt11, is essential for activation of the canonical Wnt pathway required for axis formation.

We next asked whether maternal Wnt11 or Wnt5a is required for the non-canonical Wnt pathway. Embryos depleted of either Wnt11 or Wnt5a were analyzed at the early and late blastula stages for activated JNK activity. Fig. 4H shows that p-JNK1 levels were reduced by either Wnt11 or Wnt5a depletion, suggesting that both are required for non-canonical pathway activity.

To confirm whether both maternal Wnt11 and Wnt5a are essential for activating the signaling pathways leading to axis formation, we examined the effect of double depletions of Dkk1 together with either Wnt11 or Wnt5a. If Wnt11 and Wnt5a are both required, then depleting either should abrogate Dkk1 hyperactivation activity and cause ventralization and loss of p-JNK. If one Wnt signaling pathway is separate from the other, and antagonized by Dkk1, Dkk1 depletion should enhance the activity of the remaining Wnt. As shown in Fig. 4H-J, the double depletions of both Dkk1/Wnt11 and Dkk1/Wnt5a caused the same phenotype as the single Wnt depletion: i.e. reduced p-JNK1 and loss of expression of canonical Wnt target genes. The Dkk/Wnt-depleted embryos developed with a ventralized phenotype (see Fig. S5 in the supplementary material). This indicates that both maternal Wnt11 and 5a are required for activating the signaling pathways leading to axis formation.

As Pol2 transcription is not active until MBT (Dunican et al., 2008), and maternal Wnt11 is expressed in Wnt5a-depleted oocytes (Fig. 4A) and vice versa (data not shown), maternal Wnt5a is not upstream or downstream of maternal Wnt11 in a transcriptional sense before MBT. In addition, after MBT, the maternal depletion of Wnt5a does not affect the level of Wnt11 at the early gastrula stage (data not shown). One possible explanation of the similarity in phenotype caused by their depletion is that *Wnt5a* mRNA could maintain the localization of *Wnt11* mRNA in the vegetal cortex of the oocyte (Ku and Melton, 1993) or in its translocation to the dorsal side after fertilization (Schroeder et al., 1999). However, neither *Wnt11* mRNA distribution in the oocyte nor its dorsal enrichment at the 32-cell stage was altered by Wnt5a depletion (Fig. 4K,L; data not shown).

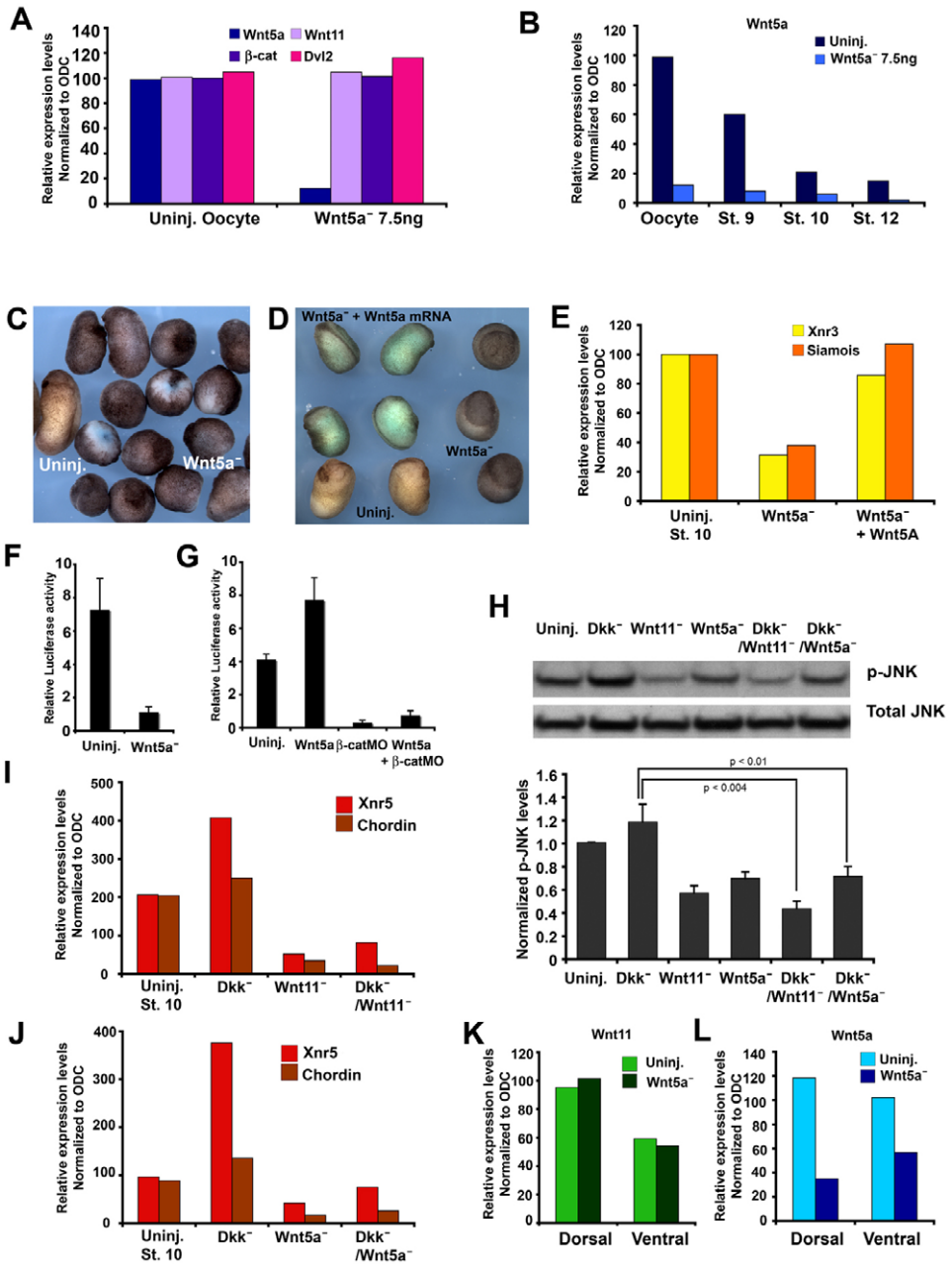

Fig. 4. Maternal Wnt5a is essential for both canonical and non-canonical Wnt signaling. (**A**) Control uninjected and sibling, Wnt5a antisense oligo-injected oocytes were assayed by real-time RT-PCR for the relative expression of *Wnt5a*, *Wnt11*, *β-catenin* and *Dishevelled 2* (*Dvl2*) mRNA. (**B**) Real-time RT-PCR assay of the relative expression of *Wnt5a* in uninjected and Wnt5a-depleted oocytes at blastulae (stage 9) and gastrulae (stages 10, 12). (**C**) Embryos derived from sibling control and Wnt5a-depleted oocytes at the early tailbud stage. This phenotype was seen in four experiments in a total of 99% of cases (102/104); controls were normal in 99% of cases (91/92). (**D**) The phenotype of Wnt5a depleted (Wnt5a⁻) embryos was partially rescued by the reintroduction of 25 pg *Xenopus* Wnt5a mRNA (Wnt5a⁻+Wnt5a mRNA) before fertilization. Wnt5a-depleted early tailbud stage embryos (5/6) had the ventralized phenotype shown compared with 0/6 Wnt5a⁻+25 pg *Wnt5a* mRNA embryos and 0/10 Wnt5a⁻+100 pg *Wnt5a* mRNA embryos; 11/11 controls were normal. The experiment was repeated with a similar result. (**E**) The relative expression levels of *Xnr3* and *siamois* in control, Wnt5a-depleted and Wnt5a-depleted+100 pg *Xenopus Wnt5a* mRNA-injected embryos assayed by real-time RT-PCR at the early gastrula stage. (**F**) TOPflash reporter activation in control and Wnt5a-depleted embryos frozen at the late blastula stage. (**G**) TOPflash reporter activation in *Wnt5a* mRNA overexpressing embryos (100 pg), β-catenin-depleted embryos and sibling embryos injected with both *Wnt5a* mRNA and β-catenin MO. (**H**) Western blot of p-JNK-1 protein in control, Dkk1-depleted, Wnt11-depleted, Wnt5a-depleted, Dkk1/Wnt11- and Dkk1/Wnt5a-depleted late blastulae. Quantitation is shown below. $P<0.01$, Student's *t*-test. (**I**) The relative expression levels of *Xnr5* and *chordin* in control, Dkk1-depleted, Wnt11–depleted and Dkk1/Wnt11-depleted embryos assayed by real-time RT-PCR at the early gastrula stage. (**J**) The relative expression levels of *Xnr5* and *chordin* in control, Dkk1-depleted, Wnt5a-deleted and Dkk1/Wnt5a-depleted embryos assayed by real-time RT-PCR at the early gastrula stage. (**K**,**L**) Batches of two whole and four dorsal- or ventral-half, 32-cell stage control and Wnt5a-depleted embryos assayed by real-time RT-PCR for the relative expression of *Wnt11* mRNA (K) and *Wnt5a* mRNA (L).

Wnt11 and Wnt5a interact in physical and functional complexes

Next we tested whether Wnt11 and Wnt5a physically interact in co-immunoprecipitation assays after co-injection of *Wnt11-HA* and *Wnt5a-Myc* mRNA. Fig. 5A shows that Wnt11-HA protein was detected in complexes with Wnt5a-Myc and that the immunoprecipitation of Wnt11-HA by the anti-Myc antibody was Wnt11 specific, as the Nodal-related protein Xnr2-HA did not interact. To test whether Wnt11 and Wnt5a non-specifically interacted during synthesis, we repeated the immunoprecipitation after co-injecting Wnt11-HA and Wnt5-Myc either into the same blastomere or into two adjacent blastomeres of four-cell stage embryos (Fig. 5B). Fig. 5C shows that Wnt11-HA protein was detected in complexes with Wnt5a-Myc, even when the two mRNAs were injected into two separate cells (left panel). Similarly, Wnt5a-HA was detected in complexes with Wnt11-GFP (right panel). This suggests that Wnt11 and Wnt5a can interact extracellularly after secretion, as the Wnt proteins were detected in complexes even when the mRNAs were injected in different cells.

As a functional test of the interaction of Wnt11 and Wnt5a, we analyzed the effect of injection of *Wnt11-HA* mRNA alone, *Wnt5a-Myc* alone or both mRNAs injected together into oocytes on TOPflash activity measured on the dorsal side at the mid-blastula stage (Fig. 5D). Fig. 4G shows that high doses of *Wnt5a* mRNA (100 pg) enhanced TOPflash activity above control levels. Similar results were seen for *Wnt11* mRNA (Tao et al., 2005). However, when more physiological doses of the mRNAs were injected (2.5-10 pg), either Wnt alone repressed endogenous TOPflash activity at the late blastula stage, whereas co-injection of a mixture of 5 pg of Wnt11 and 2.5 pg Wnt5a mRNAs doubled TOPflash activity compared with the endogenous Wnt signal (Fig. 5E). This result was repeated three times and suggests that, at physiological levels, complexes of exogenous Wnt11+ 5a enhances endogenous canonical signaling, whereas exogenous Wnt11 alone or Wnt5a alone interferes with the endogenous Wnt complex stoichiometry and represses signaling.

Wnt11 and Wnt5a form homodimers but not heterodimers

Previous studies purifying Wnt1 (Int-1) and Wnt8 protein showed that they form large complexes (>600 KkDa) in conditioned medium (Dann et al., 2001; Papkoff, 1989), and size exclusion chromatography assays on Wnt3a suggested the active form was monomeric (Willert et al., 2003). However, no previous studies have tested the interaction of two Wnts in functional and biochemical assays. In addition, the Wnt receptor Frizzled forms dimers, and ligand-induced multimerization of Frizzled receptors drives signal transduction (Carron et al., 2003; Dann et al., 2001). Therefore, we asked whether Wnt ligands form homo- and heterodimers. Overexpression of *Wnt11-HA* mRNA produced both a monomer-size band (50 kDa) and a dimer-size band (100 kDa) when the gastrula lysate was analyzed in non-reducing conditions (Fig. 6A). The dimer was lost when reducing agent β-

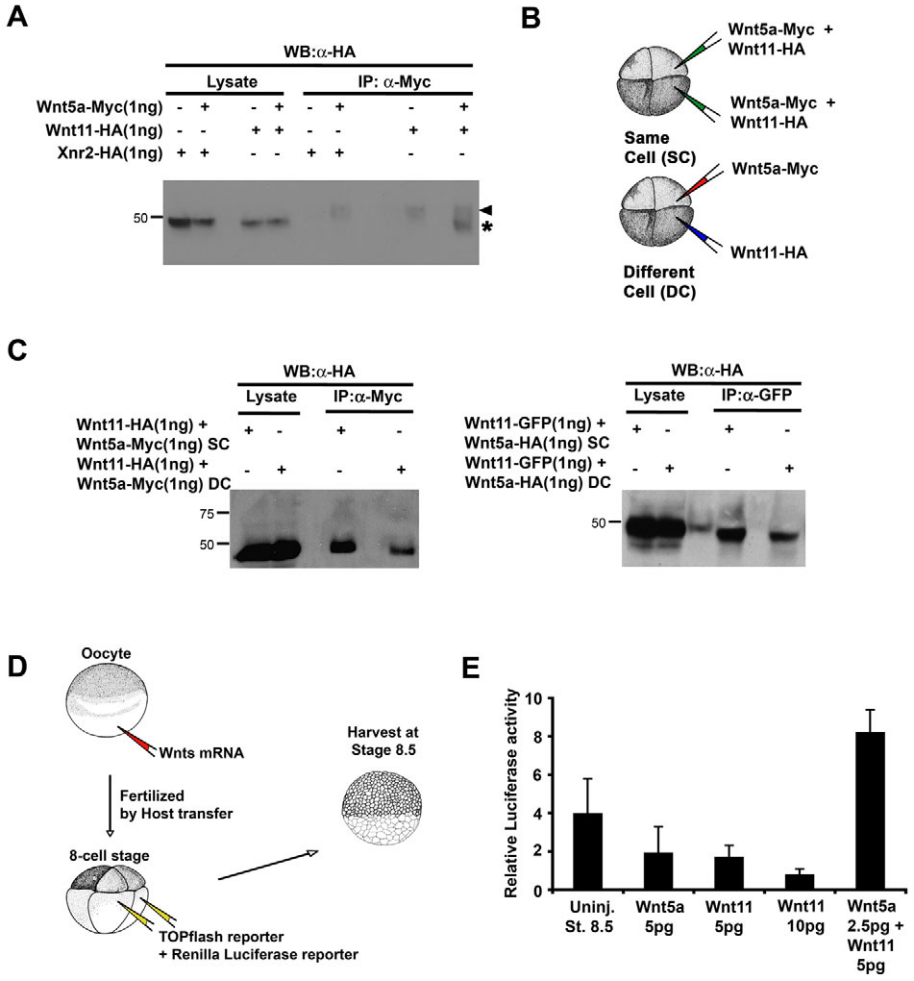

Fig. 5. Interactions between Wnt11 and Wnt5a. (**A**) *Wnt11-HA* or *Xnr2-HA* mRNA (1 ng) was injected either alone or together with *Wnt5a-Myc* mRNA (1 ng) in the same cells at the two-cell stage. Immunoprecipitation was performed with anti-Myc antibody. Co-immunoprecipitation shows Xnr2-HA present in the lysate (lanes 1-2) but absent in the anti-Myc IP-complex (lanes 5-6); Wnt11-HA was present in the whole lysates (lanes 3-4) and precipitated with Wnt5a-Myc (lanes 7-8). Arrowhead, IgG band; asterisk, Wnt11-HA band. (**B**) Schematic description of same cells (SC) and different cells (DC) injected with tagged Wnt11 and Wnt5a mRNAs at the four-cell stage. (**C**) Co-immunoprecipitation shows Wnt11-HA present in the whole lysates (lanes 1-2) and precipitated with anti-Myc from both SC and DC injections (lanes 3-4). Wnt5a-HA is detected in whole lysates (lanes 5-6) and after anti-GFP co-IP from SC and from DC injections (lanes 7-8). (**D**) Schematic description of the TOPflash reporter assay after Wnt mRNA overexpression in the oocyte. (**E**) TOPflash Luciferase activity measure in blastulae derived from control (uninj), Wnt5a-injected (Wn5a 5 pg), Wnt11 injected (Wnt11 5 and 10 pg) or co-injected (Wnt5a 2.5 pg+Wnt11 5 pg) oocytes.

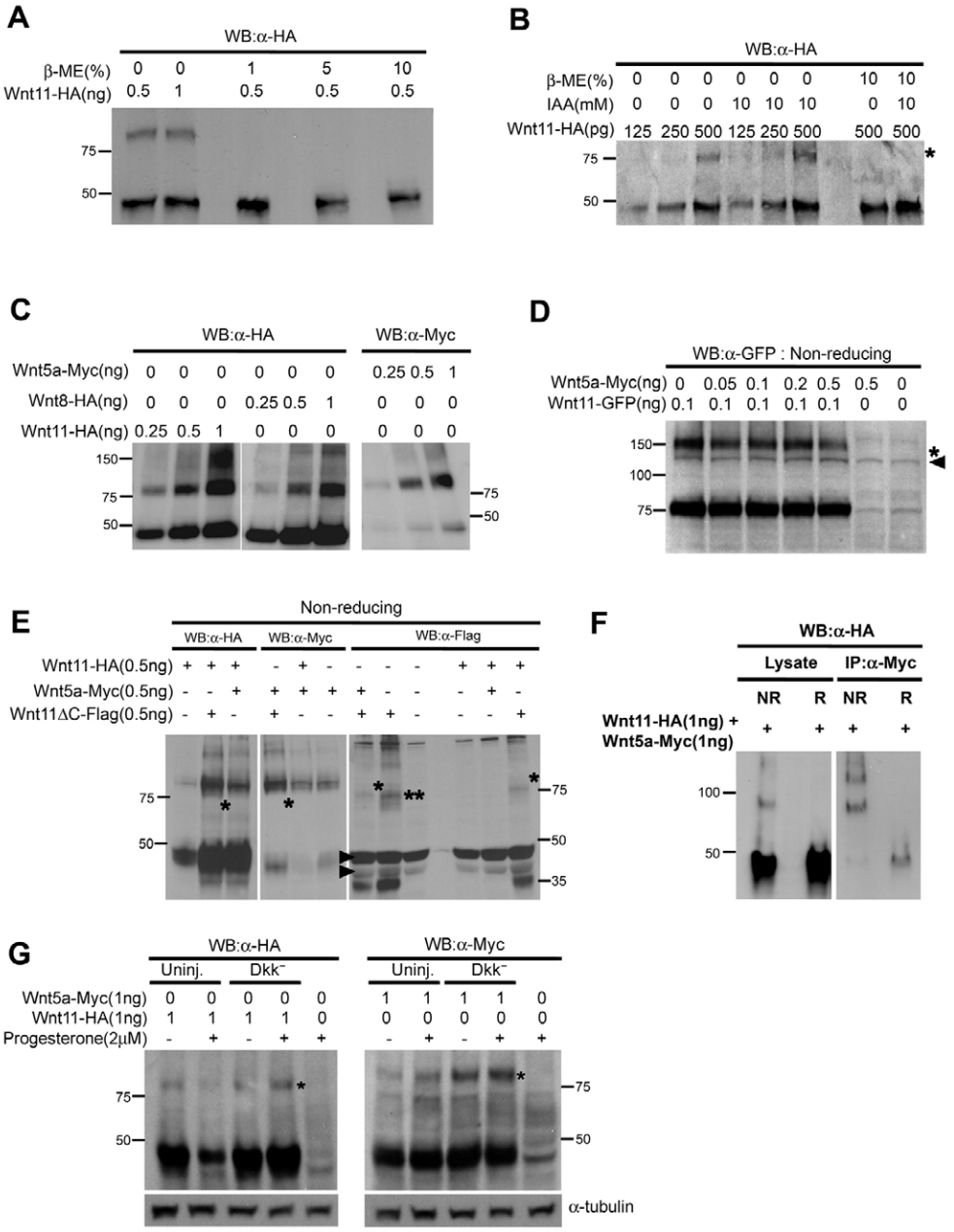

Fig. 6. Wnt proteins form homodimers. (**A**) Western blot analysis of gastrulae injected at the two-cell stage with 0.5 ng or 1 ng of *Wnt11-HA* mRNA. Lanes 1 and 2, no reducing agent; bands of Wnt11-HA are present at 50 kDa (monomer) and 100 kDa (dimer). Lanes 3-5, β-ME added (1, 5, 10%); only the 50 kDa band is visible. (**B**) Western blot of 125, 250 or 500 pg *Wnt11-HA* mRNA injected embryos+iodoacetamide (IAA, 10 mM). There is a 100 kDa band (*) in the 250 and 500 pg lanes both in the absence (lanes 2, 3) and presence (lanes 5, 6) of IAA. β-ME converts to monomers (lanes 7, 8). (**C**) Western blot of 0.25, 0.5, 1 ng *Wnt11-HA*, *Wnt8-HA* or *Wnt5a-Myc* mRNA-injected embryos with IAA-containing buffer and non-reducing electrophoresis. Wnt11 (lanes 1-3), Wnt8 (lanes 4-6) and Wnt5a (lanes 7-9) form dimers. (**D**) Anti-GFP western blot analysis of embryos injected with 100 pg of *Wnt11-GFP* mRNA alone or +50, 100, 200, 500 pg of *Wnt5a-Myc* mRNA; non-reducing conditions. Wnt11-GFP alone (lane 1) produces a 75 kDa band (monomer) and a 150 kDa band (dimer). Co-injection with increasing doses of *Wnt5a* mRNA (lanes 2-5) showed no band of the expected 125 kDa (*) of Wnt5a-Myc/Wnt11-GFP heterodimer. Lanes 6 and 7 show that the signal in lanes 1-5 is specific to Wnt11-GFP. Arrowhead indicates a non-specific band. (**E**) Anti-HA WB in non-reducing conditions where 0.5 ng of *Wnt11-HA* mRNA alone (lane 1) produced 50 and 100 kDa bands. Co-injection with 0.5 ng of Wnt11ΔC-*Flag* mRNA (30 kDa) (lane 2) did not produce the 80 kDa band (*) expected for Wnt11ΔC-Flag/Wnt11-HA heterodimer. Similarly, the pattern of Wnt5a-Myc bands (lane 6) was not changed by the co-injection of Wnt11ΔC-Flag (lane 4); *expected positions for a Wnt5a-Myc/Wnt11ΔC-Flag heterodimer (80 kDa). **Wnt11ΔC-Flag homodimer. No heterodimer bands were detected in co-injection samples (lanes 7, 12, asterisks) compared with Wnt11ΔC-Flag alone (lane 8). Arrowheads indicate non-specific bands. (**F**) Analysis of Wnt11-HA and Wnt5a-Myc Co-IP under non-reducing conditions (NR) shows that only dimers (100 kDa) and oligomers (>100 kDa) of Wnt11-HA were precipitated by Wnt5a-Myc (lane 3); under reducing conditions (R), the IP product was seen a single monomer band (lane 4). (**G**) Western blot under non-reducing conditions of control and Dkk1-depleted oocytes injected with 1 ng *Wnt11-HA* or *Wnt5a-Myc* mRNAs. Dimer forms of Wnt proteins are marked with asterisks. Repeated experiments showed similar results.

mecaptoethanol (β-ME) was added in the sample buffer, suggesting that the Wnt11 protein dimerizes by forming disulphide bonds. To test whether the disulphide bonds formed during the sample lysis, we supplemented the lysis buffer with 10 mM iodoacetamide (IAA), which inactivates free cysteine side-chains. Fig. 6B shows that the dimer-sized band was still detected under these conditions. Wnt5a and Wnt8 proteins also formed dimers in non-reducing conditions (Fig. 6C), and for Wnt5a, dimers predominated over monomers.

We next asked whether Wnt11 and Wnt5a interact by forming heterodimers. As Wnt11-HA and Wnt5a-Myc migrate at about the same molecular weight, homodimers and heterodimers would be difficult to distinguish. Therefore we generated a C-terminal truncated flag-tagged Wnt11 (Wnt11ΔC, 30 kDa) (Tada and Smith, 2000) and a GFP-tagged Wnt11 (Wnt11-GFP, 75 kDa). As shown in Fig. 6E, when Wnt11ΔC was overexpressed, homodimers still formed (suggesting that the C terminus is not required for dimerization), but Wnt11ΔC did not form heterodimers of the expected size (marked by an asterisk in Fig. 6E) with either full-length Wnt11-HA or Wnt5a-Myc.

To test whether GFP-tagged Wnt11 and Wnt5a-Myc heterodimerized, we injected various doses of *Wnt5a-Myc* mRNA together with 100 pg of *Wnt11-GFP* mRNA. Fig. 6D shows that Wnt11-GFP dimerizes when injected alone, but there was no change in the ratio of monomer to dimer or in their amounts in the presence of Wnt5a. Moreover, no band equivalent to Wnt11-GFP and Wnt5a-Myc heterodimer (125kDa) could be detected in co-injection samples (Fig. 6D). Thus, although dimerization may be a general property of Wnt ligands, we found no evidence suggesting that Wnt11 and Wnt5a form heterodimers. However, the fact that there was no cross-binding between Wnt11 and Wnt5a, even when the proteins were synthesized together in the same cells, strongly argues against the idea that Wnt proteins are 'sticky' and interact non-specifically because of their many cysteine residues.

To determine which molecular form of Wnt11 complexes with Wnt5a, we analyzed the immunoprecipitate of Wnt 11-HA and Wnt5a-myc in the presence or absence of reducing agent. Fig. 6F shows that, in non-reducing conditions, Wnt5a-Myc mainly immunoprecipitates dimers and oligomers of Wnt11-HA, and not monomers. When disulphide bonds were broken by β-ME addition, only monomeric Wnt11-HA was seen. This suggests that the co-IP between Wnt11 and Wnt5a occurs between Wnt11 and Wnt5a dimers or oligomers, and not between monomers.

Finally, as maternal Dkk1 acts as a Wnt antagonist, we asked how Dkk1 depletion affected the levels of dimer and/or monomer form of Wnt11-HA or Wnt5a-Myc protein when each mRNA was overexpressed in oocytes. Here, matured, non-matured uninjected and Dkk1-depleted oocytes were compared, as oocyte maturation often stimulates protein synthesis (Richter et al., 1982). When *Wnt11-HA* mRNA and *Wnt5a-myc* mRNA were injected into Dkk1-depleted oocytes, more Wnt protein, particularly dimeric Wnt protein (*) was detected in non-reducing conditions compared with injection into control oocytes (Fig. 6G). Thus, loss of Dkk1 caused an increase in the total amount of Wnt dimer, presumably by increasing Wnt protein stability or reducing its turnover. This was not due to increased *Wnt* transcription as the stage 6 oocyte is transcriptionally inactive.

Taken together, Figs 5 and 6 suggest that secreted homodimers of Wnt11 and homodimers of Wnt5a form non-covalently linked higher molecular weight complexes to activate the canonical and non-canonical processes involved in axis formation (Fig. 7).

DISCUSSION

Dkk1 regulates the canonical and non-canonical signaling activated by both maternal Wnt11 and Wnt5a

Here, we show that maternal Dkk1 both prevents canonical Wnt signaling in ectopic sites in the embryo and regulates excessive canonical Wnt signaling in the correct location. More surprisingly, Dkk1 also antagonizes non-canonical Wnt signaling, as evidenced by Dkk1/β-catenin loss-of-function experiments where Dkk1 depletion caused increased JNK-1 phosphorylation and enhanced early morphogenetic movements in the absence of β-catenin (Fig. 3D,G). We show that maternal Wnt5a, as well as maternal Wnt11, is required to activate canonical and non-canonical signaling, because maternal Wnt5a loss of function phenocopied Wnt11 loss of function. In addition, loss of either ligand prevented the upregulation of both canonical and non-canonical signaling caused by Dkk1 depletion, arguing that the ligands act together. If Wnt11 were regulating canonical signaling, and Wnt5a non-canonical signaling, then when Wnt11 was depleted, the early convergence extension movements and JNK activity should be preserved in the Dkk/Wnt11 double depletions.

Previous studies have suggested similarities in Wnt11 and Wnt5a function. When Wnt11 was depleted from mesodermal stem cells, Wnt5a was able to substitute for Wnt11 and rescue a broad range of blood cell types (Brandon et al., 2000). Overexpressed Wnt11 and Wnt5a activate canonical or non-canonical signaling in different co-receptor contexts (Heisenberg et al., 2000; Mikels and Nusse, 2006; Tao et al., 2005). In addition, both Wnt11 and Wnt5a physically interact with the frizzled 7 receptor and FRL1 co-receptor (Djiane et al., 2000; Tao et al., 2005). Here, we suggest that that, at least in the signaling pathway involved in dorsal axis formation, Wnt11 and Wnt5a form a complex and function together.

The extent to which the effects of maternal RNA depletion of Dkk1, Wnt5a and Wnt11 last beyond the onset of zygotic transcription

For canonical signaling, the earliest effects caused by Dkk1, Wnt5a and Wnt11 depletions were on the stabilization of β-catenin (stage 8), on Tcf3-reporter activity (TOPflash activity; stage 8.5), and on *siamois*, *Xnr3* and *Xnr5* expression (stage 9). These changes are known to be dependent on Wnt signaling during the cleavage stages (Xanthos et al., 2002; Yang et al., 2002; Tao et al., 2005). Thus, Dkk1, Wnt5a and Wnt11 depletion first causes effects pre-MBT. At MBT, once the XTcf-regulated transcriptional network is activated, the indirect effects of the up- (in the case of Dkk1 depleted) and downregulation (for Wnt11/5a) of these canonical Wnt targets become complex, including effects on Nodal signaling and on BMP antagonists (chordin), which in turn affect gastrulation movements. In addition, after the depletion of the maternal pools of Dkk1, Wnt5a and Wnt11, the total amount of mRNA (zygotic+ maternal) during gastrulation remains lower than in wild-type sibling embryos. Thus, some effects may be due to the lack of direct maternally encoded Wnt signaling/signaling antagonism during gastrulation. This is particularly true for Wnt5a, as zygotic transcripts do not begin to accumulate until the end of gastrulation (Moon et al., 1993), compared with early gastrula for Wnt11 and Dkk1. Here, we have concentrated on examining further the mechanism of the interaction of Wnt11 and Wnt5a with regards to canonical signaling, as the TOPflash assay is an early and direct measure. However, it will be equally important to establish whether, when maternal Wnt5a signals during gastrulation, it is also acting in a complex with Wnt11.

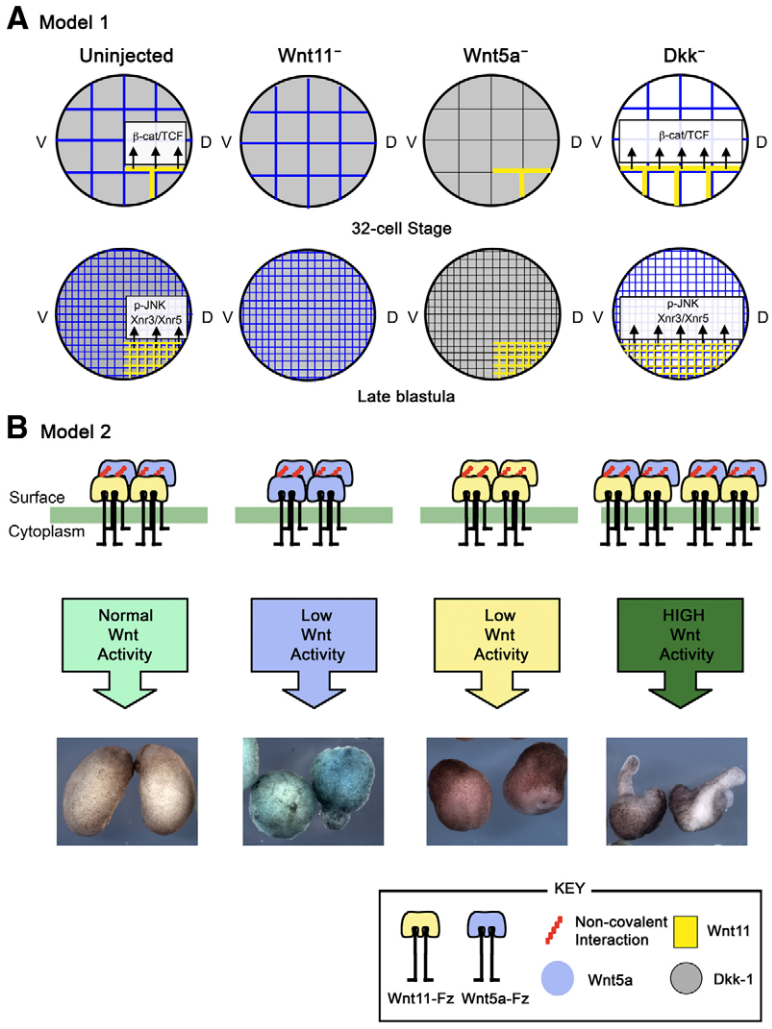

Fig. 7. Models of Wnt11/5a signaling activity in dorsal axis formation. (**A**) Model 1. The relative distribution of Wnt11 and Wnt5a protein in eight-cell stage and Wnt signaling activity at late blastula stage embryos as described in the text. (**B**) Model 2. Possible composition of the Wnt11/5a signaling complexes, as described in the text.

The mechanism of interaction of Wnt11 and Wnt5a

Unlike *Wnt11* mRNA, *Wnt5a* mRNA is not a localized mRNA in oocytes or early embryos. The simplest hypothesis to explain the dependence of dorsal signaling on both Wnt11 and Wnt5a is that, in the cleavage stage embryo, the dorsal area where Wnt11 is enriched and Wnt5a is co-expressed, is the only quadrant where a threshold is passed allowing transduction of the Wnt signal (Fig. 7). In this model, the crucial asymmetry that sets up the dorsal dominance of gastrulation movement and of organizer gene expression is the cortical rotation movement of the first cell cycle that sweeps vegetally localized *Wnt11* mRNA away from the sperm entry point, leading to more Wnt11 protein (shown in yellow) being secreted by the dorsal vegetal quadrant. Wnt5a protein (shown in blue) is a necessary but constitutive signaling partner in the dorsal axis pathway. The size of the dorsal Wnt signaling zone is further limited by the activity of secreted Dkk1 protein. We assume here that Wnt 11/Wnt5a interaction and Dkk antagonism occur extracellularly, as they are all secreted proteins. In addition, we show that Wnt11 and Wnt5a physically interact by co-immunoprecipitation studies, where the mRNAs are injected into different cells, so that the proteins can only come together after secretion.

The most intriguing question raised here is how do Wnt11 and Wnt5a interact? Although both Wnts form homodimers, we ruled out the simple model of heterodimerization. Although this finding leaves the question of mechanism unanswered, it provides evidence that the Wnt5a/11 co-immunoprecipitation is not simply a result of non-specific stickiness of these cysteine-rich proteins in the ER, because if it were then Wnt11 and Wnt5a would stick together. We favor the hypothesis that Wnts are synthesized and secreted as homodimers held together by disulphide bonds, and that they form, at the cell surface, non-covalently linked tetrameric and oligomeric complexes made up of homodimers of Wnt11 together with homodimers of Wnt5a (Fig. 7). In support of this, co-immunoprecipitation experiments show that the fraction of the Wnt11-HA protein pool that interacts with Wnt5a-myc consists of dimers and oligomers but not monomers.

As dimerization is important for the receptor Frizzled to activate downstream signaling, proper dimerization of Frizzled may be a prerequisite for such a multimeric complex to be fully active. One suggestion is that the minimum active Wnt signaling unit during endogenous dorsal signaling at the cleavage stage consists of a multimeric complex of four Wnts (a homodimer of Wnt11 and a homodimer of Wnt5a) bound to eight frizzled proteins (four dimers) (Fig. 7).

When either Wnt11 or Wnt5a is overexpressed (100-500 pg), each hyperactivates TOPflash and causes hyperdorsalization, showing that either protein alone acting in excess can activate the canonical pathway. It is likely that the excess exogenous protein dominates over the endogenous Wnt11/5a mechanism, which is

likely to be acting with much smaller amounts of protein. Frizzled 7 is ubiquitous in the early *Xenopus* embryo, and it interacts with both Wnt11 and Wnt5a (Witzel et al., 2006) (and data not shown). The endogenous Wnt pool is very sensitive to exogenously supplied Wnt protein, as 2.5-10 pg of injected *Wnt11* or *Wnt5a* mRNA inhibited endogenous signaling, whereas a mixture of 2.5 pg *Wnt5a* + 5 pg of *Wnt11* mRNA increased TOPflash above endogenous levels. This suggests that, at physiological doses, the exogenous individual Wnt may act in an inhibitory fashion on the endogenous signaling complex, whereas exogenous complexes containing both Wnt11 and Wnt5a homodimers synergize with the endogenous signal.

In summary, we have shown here a novel aspect of Wnt signaling: two Wnts required for both canonical and non-canonical signaling in a pathway that is essential for establishing the dorsal asymmetry of the embryo. We have suggested that the mode of activity is via non-covalently linked tetrameric and oligomeric complexes made up of homodimers of Wnt11 together with homodimers of Wnt5a. An important issue remaining is whether this is a paradigm for Wnt signaling activity in many other embryonic and adult tissues where two Wnts are expressed in overlapping territories.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/22/3719/DC1

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