Jun NH_2-terminal kinase (JNK) prevents nuclear β-catenin accumulation and regulates axis formation in *Xenopus* embryos

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Jun NH_2-terminal kinases (JNKs) regulate convergent extension movements in *Xenopus* embryos through the noncanonical Wnt/planar cell polarity pathway. In addition, there is a high level of maternal JNK activity spanning from oocyte maturation until the onset of gastrulation that has no defined functions. Here, we show that maternal JNK activation requires Dishevelled and JNK is enriched in the nucleus of *Xenopus* embryos. Although JNK activity is not required for the glycogen synthase kinase-3-mediated degradation of β-catenin, inhibition of the maternal JNK signaling by morpholino-antisense oligos causes hyperdorsalization of *Xenopus* embryos and ectopic expression of the Wnt/β-catenin target genes. These effects are associated with an increased level of nuclear and nonmembrane-bound β-catenin. Moreover, ventral injection of the constitutive-active *Jnk* mRNA blocks β-catenin-induced axis duplication, and dorsal injection of active *Jnk* mRNA into *Xenopus* embryos decreases the dorsal marker gene expression. In mammalian cells, activation of JNK signaling reduces Wnt3A-induced and β-catenin-mediated gene expression. Furthermore, activation of JNK signaling rapidly induces the nuclear export of β-catenin. Taken together, these results suggest that JNK antagonizes the canonical Wnt pathway by regulating the nucleocytoplasmic transport of β-catenin rather than its cytoplasmic stability. Thus, the high level of sustained maternal JNK activity in early *Xenopus* embryos may provide a timing mechanism for controlling the dorsal axis formation.

nucelocytoplasmic transport | Wnt

The Wnt signaling pathway comprises the Wnt/β-catenin and the β-catenin-independent (noncanonical) branches (1, 2). In the canonical Wnt/β-catenin pathway, Wnt binds to its coreceptors Frizzled and LRP5/6 to activate Dishevelled (Dsh) and inhibits the proteaosomal degradation of β-catenin (3). Accumulated β-catenin then associates with Pygopus and Legless/BCL-9 to enter the nucleus and activate the Lef/TCF family of transcription factors (4, 5). The noncanonical Wnt signaling is subdivided into the Wnt/calcium and the Wnt/c-Jun NH_2-terminal kinase (JNK) pathways that may share common signaling transducers (2, 6, 7). Interestingly, one of the earliest observations concerning noncanonical Wnt signaling was its ability to inhibit the canonical Wnt pathway (7, 8). Recent studies further show the Wnt/calcium/NFAT pathway promotes ventral cell fate in *Xenopus* embryos (9), and noncanonical Wnt-5A induces the degradation of β-catenin (10). These findings have led to a generalized concept that noncanonical Wnt may antagonize canonical Wnt signaling, but whether JNK inhibits the canonical Wnt/β-catenin pathway has not been determined (11).

JNK was originally identified by its ability to phosphorylate c-Jun and mediate stress-induced cell death (12, 13). Moreover, JNK is downstream of the Dsh-mediated noncanonical Wnt pathway in establishing the planar cell polarity in *Drosophila* (14). JNK is implicated in gastrulation and convergent extension in *Xenopus* (15, 16). In addition, there is an earlier phase of maternal *Xenopus* JNK activity spanning from oocyte maturation to the onset of gastrulation that has no defined functions (17). Because many substrates of JNK are regulators of transcription, but large-scale transcription does not occur before midblastula transition in *Xenopus* embryos, the maternal JNK signaling may have nongenomic (transcriptional) functions. It has been suggested that a high level of JNK activity may induce the degeneration of unhealthy oocytes (17). However, this suggestion cannot explain the functions of maternal JNK signaling in healthy *Xenopus* embryos (18).

In the present study, we have used both gain- and loss-of-function approaches to investigate the role of maternal JNK signaling in *Xenopus* development. Our results indicate that JNK antagonizes canonical Wnt signaling by reducing nuclear β-catenin in both *Xenopus* embryos and mammalian cells.

Results

Maternal JNK Is Evenly Distributed in *Xenopus* Embryos and Enriched in the Nucleus. Previous work has shown *Xenopus* JNK activity from oocyte maturation until the beginning of gastrulation at stage 10 (17). However, that study did not address whether the regulation of JNK activity involves protein synthesis and/or degradation. To examine this issue, we applied an *in vitro* kinase assay and immunoblot to analyze the same number of oocytes and *Xenopus* embryos at different stages. Our analysis revealed a constant level of JNK protein (Fig. 1*A*). Thus, the regulation of maternal JNK signaling occurs at the activity level (Fig. 1*B*). Next, we examined the spatial distribution of JNK protein and activity in 16-cell and stage-7 *Xenopus* embryos and found no dorsoventral difference (Fig. 1*C*). It has been shown in mammalian cells that Wnt-5A, Wnt-11, and UV irradiation stimulate JNK activity (16). However, UV irradiation of *Xenopus* embryos at one-cell stage or injection of *Wnt-5A* or *Wnt-11* mRNA did not prevent the down-regulation of JNK activity in gastrulation (Fig. 1*D*). Using immunocytochemistry to examine the subcellular distribution of JNK protein, we found that JNK is concentrated in the nucleus of stage-8 *Xenopus* embryos (Fig. 1*E*). These results show that maternal *Xenopus* JNK is evenly distributed in pregastrulation embryos and enriched in the nucleus.

JNK Is Not Required for Glycogen Synthase Kinase-3-Mediated β-Catenin Degradation *per se*. The high level of JNK activity in *Xenopus* eggs and pregastrulation embryos prompted us to examine whether

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Abbreviations: JNK, Jun NH_2-terminal kinase; Dsh, Dishevelled; MO, morpholino.

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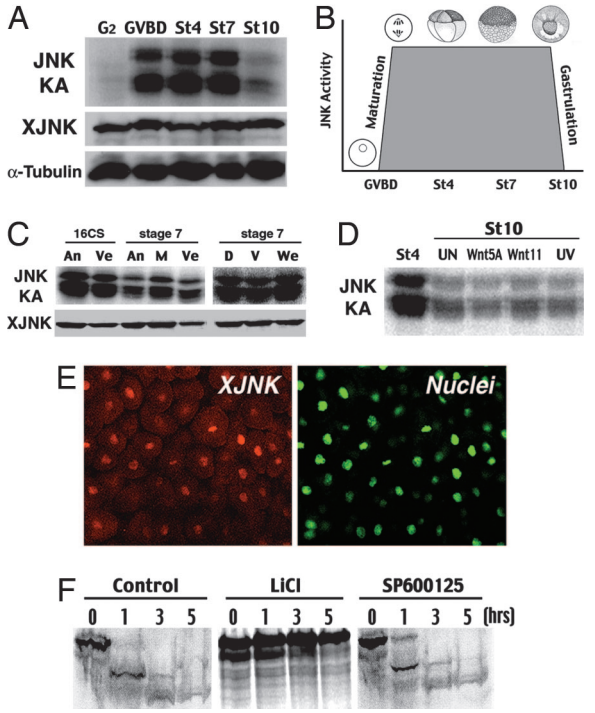

Fig. 1. Characterization of maternal JNK signaling in *Xenopus* embryos. (*A*) The kinase assay (KA) shows a rapid rise of JNK activity during germinal vesicle breakdown (GVBD) that lasts until stage (St) 10 despite a constant level of JNK protein expression. XJNK, *Xenopus* JNK. (*B*) Schematic diagram of maternal JNK signaling during early *Xenopus* development. (*C*) Dissecting 16-cell (16CS) and stage-7 embryos shows no obvious regionalization of JNK activity. An, animal half; Ve, vegetal half; M, marginal zone; D, dorsal half; V, ventral half; We, whole embryo. (*D*) UV irradiation of one-cell *Xenopus* embryos or injection of Wnt5A or Wnt11 mRNA at the four-cell stage did not prevent the decline of JNK activity at stage 10. (*E*) Immunostaining shows that JNK is concentrated in the nucleus in stage-8 *Xenopus* embryos. The nucleus is counterstained with SYTOX green dye. (Magnification: ×6,300.) (*F*) The addition of lithium chloride (25 mM) effectively blocks β-catenin degradation in an *in vitro* assay using the *Xenopus* egg extracts. In contrast, the addition of JNK inhibitor SP600125 (20 μM) does not prevent β-catenin degradation.

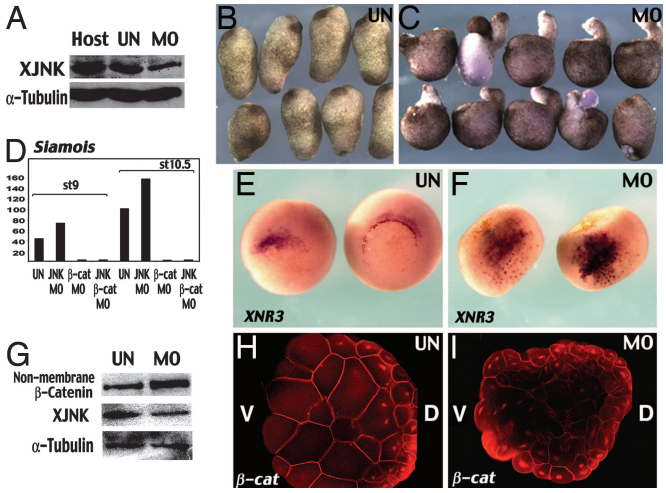

Fig. 2. Loss-of-function analysis of maternal JNK in *Xenopus* development. (*A*) Immunoblot shows the reduction of JNK protein in stage-7 embryos derived from the oocytes injected with 20 ng of MO-modified JNK antisense oligo (MO). UN, uninjected; XJNK, *Xenopus* JNK. (*B* and *C*) The morphology of host-transferred tailbud-stage embryos derived from the uninjected oocytes (*B*) or oocytes injected with 30 ng of JNK MO (*C*). JNK MO-injected embryos are hyperdorsalized with prominent elongation of the notochord. (*D*) The expression of *Siamois* mRNA is increased in host-transfer embryos derived from JNK MO-injected oocytes (30 ng), but drastically reduced in β-catenin-MO and dual JNK/β-catenin MO-injected oocytes. (*E* and *F*) Whole-mount *in situ* hybridization shows the expression of *Xnr3* mRNA is restricted around the dorsal lip in stage 10–10.5 embryos derived from uninjected oocytes (*E*), but is much more widely and strongly expressed in embryos derived from oocytes injected with 30 ng of JNK MO (F). (*G*) Cell fractionation and immunoblot shows an increase of soluble β-catenin and a reduction of JNK in the JNK MO-injected and host-transferred stage-7 embryos. (*H* and *I*) Immunostaining shows dorsal enrichment of β-catenin in stage-8 control embryos (*H*) and expanded expression of nuclear β-catenin in the embryos derived from oocytes injected with 30 ng of JNK MO (*I*). V, ventral; D, dorsal. (Magnifications: ×2,000.)

JNK is required for β-catenin degradation by using an *in vitro* assay (19). We found that the addition of JNK-specific inhibitor SP600125 (20) did not affect the degradation of β-catenin, in contrast to the effect of lithium chloride (25 mM) that completely blocked β-catenin degradation via inhibition of the glycogen synthase kinase-3 activity (Fig. 1*F*). To explore the function of JNK in oocytes, we expressed a constitutive-active form of JNK (FLAG-MKK7-JNK1 fusion protein, ref. 21) in full-grown meiotic-arrested oocytes that have little basal JNK activity. Although FLAG-MKK7-JNK1 produced a high level of JNK activity in oocytes without progesterone stimulation, it did not lead to germinal vesicle breakdown or oocyte degeneration even after an extended period of culture (40 h) (Fig. 6, which is published as supporting information on the PNAS web site). Together, these results show that JNK signaling is not required for β-catenin degradation *per se* and that JNK activation is insufficient to trigger oocyte maturation or degeneration (22).

Knockdown of Maternal JNK Hyperdorsalizes *Xenopus* Embryos and Induces Wnt/β-Catenin Target Gene Expression. We next performed a loss-of-function analysis using antisense injection and the host-transfer technique to knock down maternal JNK (23). We generated morpholino (MO)-modified oligonucleotides against *Xenopus* JNK (hereafter referred to as JNK MO-oligos) that has been shown to be specific for JNK and induce convergent extension defects (16). Eggs derived from the JNK MO-oligos-injected oocytes (20–40 ng) were fertilized *in vitro* and developed normally until the gastrulation stage. Immunoblots confirmed the reduction of JNK protein in stage-7 host-transfer embryos (Fig. 2 *A* and *G*). However, JNK MO-oligos-injected embryos showed delayed gastrulation and exhibited "hyperdorsalized" phenotypes, including enlarged heads and elongated notochords by the tailbud stage (Fig. 2 *B* and *C*). These phenotypes are remarkably similar to those of Axin-depleted embryos that had increased Wnt/β-catenin signaling (24). Quantitative RT-PCR analysis showed that JNK MO-oligos-injected embryos had 50–60% higher expression of a Wnt/β-catenin target gene, *siamois* (25), in stage 9–10.5 embryos (Fig. 2*D*). In addition, the expression of the dorsal marker genes *Xnr3* and *Goosecoid* was increased in both dorsal and ventral halves of JNK MO-oligos-injected embryos (Fig. 7, which is published as supporting information on the PNAS web site). Moreover, whole-mount *in situ* hybridization showed more abundant and widespread distribution of the *Xnr3* transcript in stage 10–10.5 JNK MO-oligos-injected embryos, which was confined in the Spemann organizer in the control embryos (Fig. 2 *E* and *F*). These results suggest that knockdown of maternal JNK up-regulates the canonical Wnt/β-catenin pathway, leading to hyperdorsalization.

Three lines of evidence further support the notion that maternal JNK inhibits Wnt/β-catenin signaling. First, epistatic analysis showed the induction of *siamosis* was abolished in JNK/β-catenin double-depleted embryos, suggesting that JNK intercepts the canonical Wnt/β-catenin pathway (Fig. 2*D*). Second, when stage-6 embryos were subjected to cell fractionation

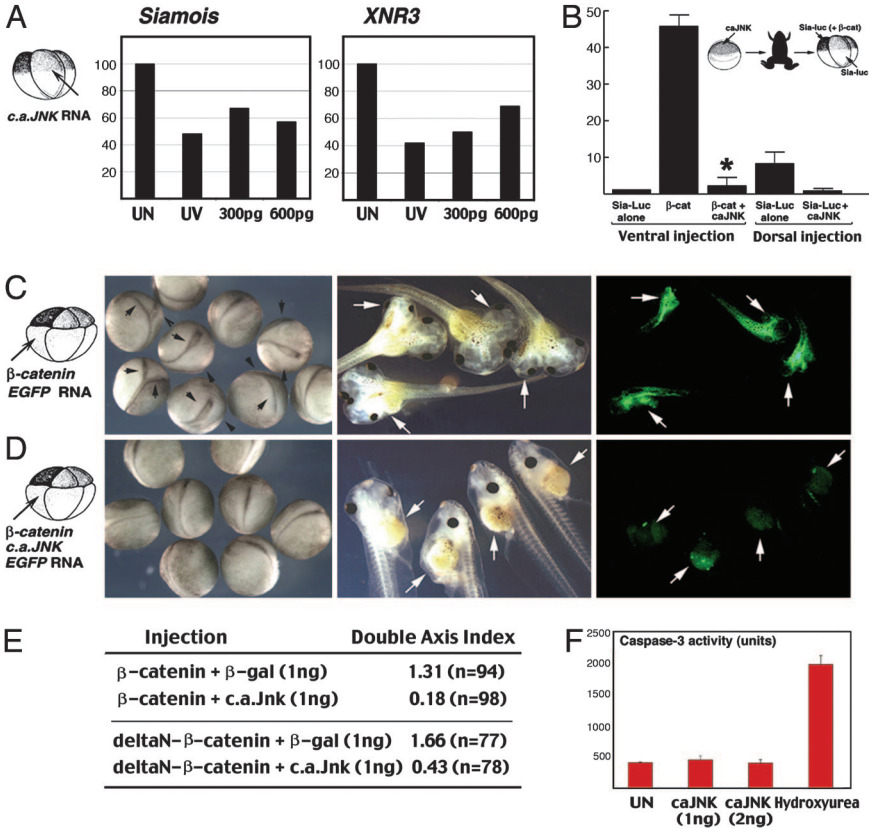

Fig. 3. JNK activation inhibits β-catenin-induced transcription and axis formation. (*A*) Injection of 300–600 pg of constitutive-active *Jnk* mRNA into the dorsal marginal zone of four-cell *Xenopus* embryos reduces the *Siamois* and *Xnr3* expression in stage-10 embryos, similar to the effect of UV irradiation at the one-cell stage. UN, uninjected. (*B*) Injection of 1.5 ng of active *Jnk* mRNA into oocytes decreases the expression of luciferase at stage 10 after ventral coinjection of β-catenin mRNA and Sia-luc plasmid (⋆, $P = 0.0059$ using *t* test) or dorsal injection of Sia-Luc plasmid into host-transfer embryos ($P = 0.2$ using *t* test) at the four-cell stage. (*C* and *D*) Axis duplication assay by ventral injection of *EGFP* (300 pg) and β-catenin (1 ng) with either β-gal (1 ng) or active *Jnk* mRNA (1 ng) into four-cell *Xenopus* embryos. The progeny of β-catenin/β-gal-injected blastomere populate the ectopic axis (arrow in *C*), but those of β-catenin/active *Jnk*-injected blastomore develop into endodermal tissues (arrow in *D*). (*E*) Coinjection of 1 ng active *Jnk* mRNA blocks axis duplication induced by ventral injection of WT β-catenin (40 pg) or stabilized ΔN-β-catenin (7.5 pg). (*F*) Hydroxyurea (30 mM)-treated, but not active *Jnk* mRNA (2 ng)-injected embryos had increased caspase-3 protease activity. (Magnifications: *C* and *D*, ×10.)

and immunoblot analysis, an increased amount of nonmembrane-bound β-catenin was detected in the embryos derived from JNK MO oligos-injected oocytes (Fig. 2*G*). This result shows that JNK depletion increased the cytosolic and nuclear pool of β-catenin. Finally, whole-mount immunocytochemistry shows that nuclear β-catenin staining expands to the ventral side of stage-8 JNK-depleted embryos (Fig. 2 *H* and *I*). Together, these results indicate that JNK negatively regulates canonical Wnt/β-catenin signaling in *Xenopus* embryos.

Hyperactivation of JNK Blocks β-Catenin-Induced Transcription and Axis Duplication. We next examined the roles of JNK signaling in axis formation in *Xenopus* by using the gain-of-function approach. First, constitutive-active *Jnk* mRNA was injected into the dorsal marginal zone of four-cell embryos to assess the consequence of maintaining the JNK activity during gastrulation. We found that dorsal expression of active JNK decreases *Siamois* and *Xnr3* transcripts to a level close to UV irradiation-induced ventralization (Fig. 3*A*). Dorsally active *Jnk*-injected embryos also displayed a short, curved body axis with incomplete blastopore closure, consistent with the report of convergent extension defects caused by alteration of JNK signaling (16). In contrast, ventral injection of constitutive-active *Jnk* mRNA produces no discernible defects. Second, a plasmid encoding *luciferase* driven by the Siamois promoter (Sia-luc) was injected into embryos derived from control or active-*Jnk* mRNA–injected oocytes to assess the endogenous and β-catenin-induced transcription (Fig. 3*B*). Control experiments show that coinjection of β-catenin mRNA into four-cell embryos significantly increased the luciferase activity derived from the Sia-luc plasmid, but not the TOPFLASH plasmid that has mutated β-catenin/TCF-binding sites (data not shown). We found that coinjection of β-catenin mRNA (100 pg) with the Sia-Luc plasmid into ventral animal blastomeres causes a 46-fold increase of the luciferase activity in control embryos compared with 2.7-fold increase in the embryos derived from 1.5 ng of constitutive-active *Jnk* mRNA-injected oocytes ($P = 0.0059$; Fig. 3*B*). JNK activation also reduces the dorsal-ventral difference of Sia-luc-mediated Luciferase activity that reflects endogenous Wnt/β-catenin signaling (Fig. 3*B*). These results show that JNK effectively inhibits endogenous and β-catenin-induced transcription of the canonical Wnt target genes.

We also examined the interactions between JNK and canonical Wnt/β-catenin signaling in axis duplication. The mRNA of WT (40 pg) or stabilized ΔN-β-catenin (7.5 pg) was coinjected with either β-gal or constitutive-active *Jnk* mRNA into one ventral vegetal blastomere of eight-cell embryos. The double-axis formation of tailbud embryos was scored as a 2 (complete axis with head and cement gland), 1 (truncated or fused second axis), or 0 (normal embryos). We found that β-catenin/β-gal coinjection induces a complete or partial secondary axis in 78.7% of embryos (mean index = 1.31, $n = 94$). In contrast, coinjection with active *Jnk*

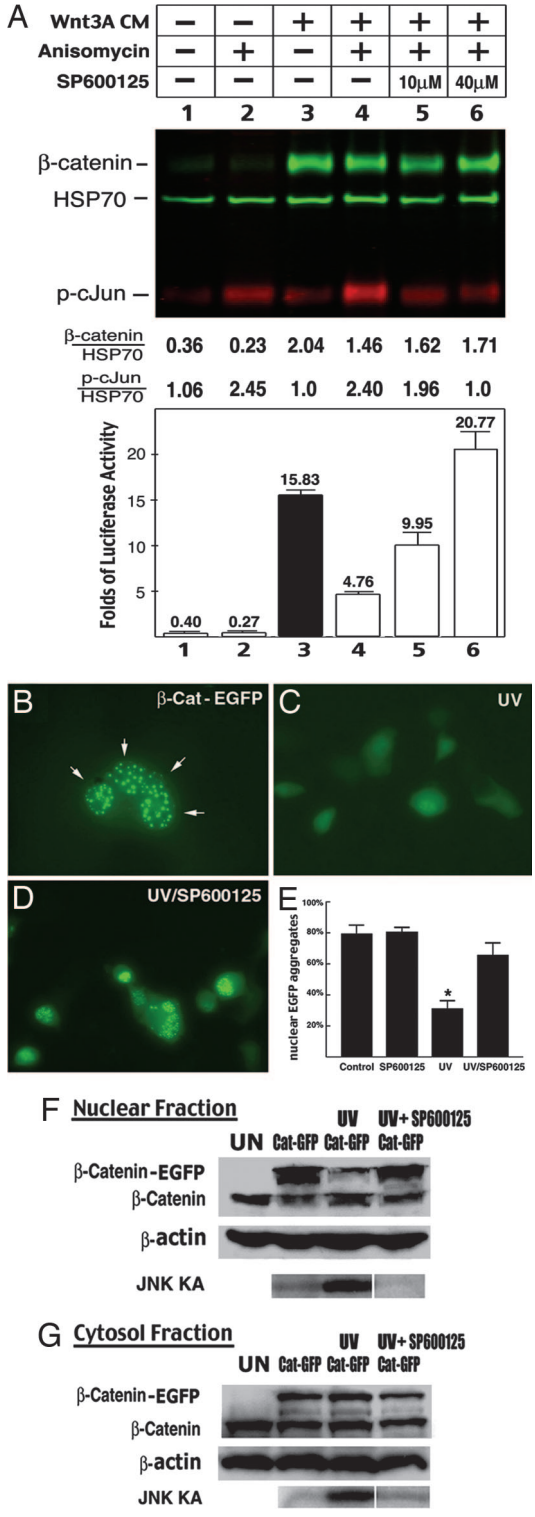

Fig. 4. JNK activation expels nuclear β-catenin. (*A*) L cells transfected with the superTOPFLASH plasmid were treated with Wnt-3A conditional medium (CM), anisomycin (67 ng/ml), and/or a JNK inhibitor, SP600125 (10, 40 μM), to assess the effect of JNK signaling activation (indicated by the phospho-cJun level on the immunoblot) on the protein level of β-catenin (normalized to HSP70) and β-catenin/TCF-mediated gene expression (folds of the fireflyluceferase activity normalized to the *Renilla* luciferase activity) at 24 h after various treatments. Quantification of the β-catenin/HSP70, phospho-cJun/HSP70 ratios, and the folds of luciferase activity are shown as the average and SD from three sets of independent experiments. (*B–D*) Transfection of β-catenin-EGFP plasmid to COS-1 cells caused fluorescent aggregates in the nucleus (*B*), which were eliminated after UV irradiation (100 J/m^2) (*C*) but were retained inside the nucleus by application of JNK inhibitor SP600125 (10 μM) after UV irradiation (*D*). (*E*) The percentage of β-catenin-EGFP-expressing COS-1 cells that have prominent nuclear aggregates in control (79.3% ± 5.1%; mean ± SD), SP600125 alone (80.5% ± 3%), UV irradiation (31.8% ± 5.7%), and UV irradiation with SP600125 (66% ± 7.4%) in three sets of experiments. *, $P < 0.0001$ versus the rest with a *t* test. (*F* and *G*) The nuclear and cytosol fractions of HEK293T cells after transfection with β-catenin-EGFP plasmids were examined by immunoblots to quantify the effect of UV-induced nuclear β-catenin and JNK kinase activity (KA). Anti-β-actin was used as a control for protein loading. This analysis shows UV causes a JNK activity-dependent depletion of nuclear β-catein-EGFP. Shown is representative result from three sets of independent experiments. UN, uninjected. (Magnifications: *B–D*, ×4,000.)

mRNA induced partial secondary axis in only 13.2% of embryos (average index = 0.18, $n = 98$). Similarly, injection of stabilized ΔN-β-catenin induced an average double axis index of 1.66 ($n = 77$), whereas coinjection with active *Jnk* mRNA reduced the double-axis index to 0.43 ($n = 78$) (Fig. 3*E*). Because JNK often induces apoptosis in mammalian cells, we also coinjected 300 pg of EGFP mRNA with β-catenin/β-gal or β-catenin/*Jnk* mRNA to label the progeny of injected blastomere. This lineage-tracing experiment showed that the progeny of β-catenin/β-gal-injected ventral blastomere contributed to the ectopic axis, whereas those of β-catenin/*Jnk*-injected blastomeres survived and developed into endodermal tissues as predicted by the fate map (Fig. 3 *C* and *D*). Moreover, the caspase-3 activity was not induced by ventral injection of 1–2 ng of constitutive-active *Jnk* mRNA (Fig. 3*F*). These results suggest that active *Jnk* mRNA does not induce apoptosis of the injected blastomeres to inhibit the β-catenin-induced axis duplication. Together, these gain-of-function analyses support the notion that JNK directly inhibits the Wnt/β-catenin signaling for axis formation in *Xenopus* embryos.

JNK Inhibits Wnt/β-Catenin-Mediated Transcription and Exports β-Catenin from the Nucleus in Mammalian Cells. Next, we examined whether and how JNK signaling interacts with the canonical Wnt/β-catenin pathway in mammalian cells. We found that cotransfection of the constitutive-active JNK plasmid reduced WT β-catenin, ΔN-β-catenin, or Wnt-3A-induced gene expression from the TOPFLASH plasmid in HEK293T cells (Fig. 8, which is published as supporting information on the PNAS web site). In another experimental setting of L cells transfected with a superTOPFLASH plasmid, Wnt-3A conditional medium induced both β-catenin expression and β-catenin/TCF-mediated luciferase activity (Fig. 4*A*). In this system, the activation of JNK signaling by low-dose anisomycin (67 ng/ml) induced c-Jun phosphorylation and decreased superTOPFLASH reporter-gene expression, but did not prevent Wnt-3A-induced β-catenin synthesis and accumulation. The JNK inhibitor SP600125 alleviated the anisomycin-mediated inhibition of superTOPFLASH reporter-gene expression in a dose-dependent manner (Fig. 4*A*). These results suggest that JNK also inhibits canonical Wnt/β-catenin signaling in mammalian cells. Moreover, it appears that JNK signaling does not solely accelerate the cytoplasmic degradation of β-catenin to antagonize canonical Wnt signaling. We next performed a series of biochemical assays to determine the mechanism by which JNK inhibits canonical Wnt/β-catenin signaling. These experiments showed that JNK does not phosphorylate β-catenin, does not inhibit β-catenin binding to TCF3 in cells, and does not interfere with the formation of β-catenin/TCF3/DNA complex (Fig. 9, which is published as supporting information on the PNAS web site).

Another mechanism by which JNK may regulate canonical Wnt signaling is the nuclear translocation of β-catenin, which is related to but does not solely depend on its cytoplasmic level (4, 26, 27). We considered this possibility because JNK has been shown to export the transcription factor PDX-1 and the proapo-

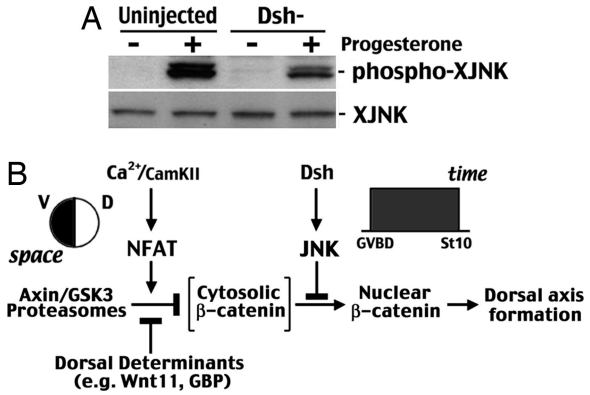

Fig. 5. Maternal JNK is activated through Dsh and provides temporal regulation of canonical Wnt/β-catenin signaling for axis formation in *Xenopus*. (*A*) Oocytes injected with antisense oligos against Dsh (Dsh−) still undergo germinal vesicle breakdown, but have a reduced level of JNK phosphorylation and activity as shown in this representative blot from three independent experiments. XJNK, *Xenopus* JNK. (*B*) Summary of the results from the present study and the literature to illustrate how Dsh/JNK and Ca^{2+}/CamKII/NFAT provide temporal and spatial restriction of the canonical Wnt/β-catenin pathway, respectively, for axis formation in early *Xenopus* development. CamKII, camodulin kinase II; GVBD, germinal vesicle breakdown.

ptotic protein Nur77 from the nucleus (28, 29). Consistent with this possibility, JNK protein is concentrated in the nucleus of *Xenopus* embryos (Fig. 1*E*), and JNK knockdown leads to increased nuclear β-catenin accumulation (Fig. 2*I*). To test this possibility, COS-1 cells were transfected with a β-catenin-EGFP plasmid to monitor the nucleocytoplasmic shuttling of β-catenin. At 24 h after transient transfection, bright fluorescent aggregates were found inside the nucleus in 79.3% of the cells that express β-catenin-EGFP (Fig. 4 *B* and *E*). UV irradiation (100 J/m^2) rapidly induced the JNK activity and reduced the percentage of cells with nuclear aggregates of β-catenin-EGFP (31.8%; Fig. 4 *C* and *E*). The addition of 10 μM JNK inhibitor SP600125 partially prevented the disappearance of nuclear β-catenin-EGFP aggregates after UV irradiation (66%; Fig. 4 *D* and *E*). This result suggests that JNK activation induces nuclear export of β-catenin.

To further test this idea, immunoblot against β-catenin was performed by using the nuclear and cytosol fractions of β-catenin-EGFP-transfected HEK293T cells after UV irradiation in the presence or absence of the JNK inhibitor SP600125 (10 μM) (Fig. 4 *F* and *G*). This analysis revealed a substantial level of basal β-catenin in the nucleus of HEK293T cells, which is replaced by exogenous β-catenin-EGFP after transfection. UV irradiation (100 J/m^2) induced JNK activity and expelled nuclear β-catenin-EGFP without affecting the cytoplasmic level of β-catenin. Furthermore, the application of JNK inhibitor retained nuclear β-catenin-EGFP after UV irradation. Together, these results suggest that JNK activation expels nucleus β-catenin.

Maternal JNK Activity in *Xenopus* Requires Dsh. JNK can be activated through a sequential MAP kinase cascade or by Dsh in a noncanonical Wnt signaling pathway (6, 12–14). To test whether maternal JNK siganling in *Xenopus* requires Dsh, we used antisense oligos to reduce the protein level of Dsh in *Xenopus* oocytes (Fig. 10, which is published as supporting information on the PNAS web site) and applied progesterone to induce oocyte maturation. This analysis revealed that the maternal JNK activity is diminished in the Dsh-reduced oocytes (Fig. 5*A*).

Discussion

The present study aims to elucidate the functions of maternal JNK signaling in early *Xenopus* development that spans from oocyte maturation until the onset of gastrulation (Fig. 1). Here, we show that knockdown of maternal JNK hyperdorsalizes *Xenopus* embryos and hyperactivates the Wnt/β-catenin target gene expression (Fig. 2). Conversely, dorsal expression of constitutive-active JNK decreases the Wnt/β-catenin-mediated transcription, and ventral injection of active JNK inhibits β-catenin-induced axis duplication (Fig. 3). Results of these gain- and loss-of-function analyses strongly suggest that maternal JNK inhibits canonical Wnt/β-catenin signaling and axis formation in *Xenopus* embryos. In addition, we show that JNK inhibits Wnt/β-catenin-mediated transcription and the nuclear accumulation of β-catenin in mammalian cells (Fig. 4). Together, these results demonstrate that JNK negatively regulates canonical Wnt/β-catenin signaling.

The present study also adds to the increasing evidence that JNK signaling has a role in nucelocytoplasmic shuttling of signal transducers (30). It has been shown that JNK induces nuclear export of PDX-1 and Nur77 (28, 29). A recent study also showed that JNK phosphorylates Nup214, a subunit of the nuclear pore complex (31). Here, we show that knockdown of maternal JNK increases nuclear β-catenin in *Xenopus* embryos, and that JNK activation induces nuclear export of β-catenin in mammalian cells (Figs. 2 and 4). Thus, the high level of widespread JNK activity in early *Xenopus* embryos may raise the threshold for nuclear β-catenin accumulation and restrict the formation of future dorsal axis structures. The mechanism by which JNK prevents nuclear β-catenin accumulation, however, remains unclear. Previous studies showed that exogenously injected β-catenin protein accumulates in the outer nuclear ring without entering the nucleus in *Xenopus* embryos (32). Thus, *Xenopus* JNK may modify the nuclear pore complex to block the nuclear entry of β-catenin. Alternatively, JNK may phosphorylate an undefined β-catenin-associated protein inside the nucleus to reduce the retention of β-catenin, similar to the actions of related MAP kinases NLK and LIT-1 (33, 34). Additional studies are needed to define the underlying mechanism.

Importantly, here we show that reduction of Dsh diminishes the JNK activity in *Xenopus* oocytes (Fig. 5*A*), suggesting that maternal JNK signaling may be activated through a Dsh-mediated noncanonical Wnt pathway. Hence, our results support the notion that noncanonical Wnt signaling may inhibit the canonical Wnt/β-catenin pathways for axis formation in *Xenopus* embryos (refs. 7–11 and Fig. 5*B*). Several lines of evidence suggest that maternal determinants of the future dorsal axis in *Xenopus* embryos are composed of Wnt or subcellular components of the Wnt/β-catenin pathway, which inhibit the Axin/glycogen synthase kinase-3-mediated degradation of β-catenin in the cytoplasm (35, 36). Although these maternal determinants are dorsally enriched in early *Xenopus* embryos, there are additional mechanisms to further confine the axis formation. For example, recent studies showed that NFAT, as a downstream effector of the Wnt/Ca^{2+} and Ca^{2+}/camodulin kinase II (CamKII) pathways, destabilizes cytosolic β-catenin on the ventral side of *Xenopus* embryos, thus providing a spatial restriction of axis formation (7, 9). Our results show that JNK inhibits canonical Wnt/β-catenin signaling like NFAT, but it targets a different mechanism (i.e., nucleocytoplasmic transport) and shows no obvious spatial differences in *Xenopus* embryos. Instead, maternal JNK has an unusual pattern of temporal activity that starts from oocyte maturation and ends at early gastrulation, coinciding with the formation of dorsal structures. Thus, although a confined and low level of nuclear β-catenin specifies the future dorsal axis in early *Xenopus* embryos (37), the sustained high level of maternal JNK activity may act as a gatekeeper to prevent promiscuous nuclear β-catenin accumulation that could lead to hyperdorsalization. Taken together, these results suggest that Ca^{2+}/CamKII/NFAT and Dsh/JNK provide spatial and temporal regulation of the

canonical Wnt signaling, respectively, for axis formation in *Xenopus* embryos.

Materials and Methods

The sequence of the MO antisense oligo complementary to *Xenopus* JNK is 5′- TGCTGTCACGCTTGCTTCGGCTCAT-3′, which has been characterized (16). The sequence of the phosphorothioate-modified antisense oligo against Dsh is 5′-G*G*T*CTCTGCTTGCGG*C*C*G-3′. Asterisks indicate a phosphorothioate linkage. The constitutive-active JNK plasmid (Flag-MKK7-hJNK1) has been characterized (21). Oocyte injection and host transfer were performed as described (23, 24). For detailed description of materials and methods, see *Supporting Text*, which is published as supporting information on the PNAS web site.

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