# Custos controls β-catenin to regulate head development during vertebrate embryogenesis

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**Precise control of the canonical Wnt pathway is crucial in embryogenesis and all stages of life, and dysregulation of this pathway is implicated in many human diseases including cancers and birth defect disorders. A key aspect of canonical Wnt signaling is the cytoplasmic to nuclear translocation of β-catenin, a process that remains incompletely understood. Here we report the identification of a previously undescribed component of the canonical Wnt signaling pathway termed Custos, originally isolated as a Dishevelled–interacting protein. Custos contains casein kinase phosphorylation sites and nuclear localization sequences. In *Xenopus*, *custos* mRNA is expressed maternally and then widely throughout embryogenesis. Depletion or overexpression of Custos produced defective anterior head structures by inhibiting the formation of the Spemann-Mangold organizer. In addition, Custos expression blocked secondary axis induction by positive signaling components of the canonical Wnt pathway and inhibited β-catenin/TCF-dependent transcription. Custos binds to β-catenin in a Wnt responsive manner without affecting its stability, but rather modulates the cytoplasmic to nuclear translocation of β-catenin. This effect on nuclear import appears to be the mechanism by which Custos inhibits canonical Wnt signaling. The function of Custos is conserved as loss-of-function and gain-of-function studies in zebrafish also demonstrate a role for Custos in anterior head development. Our studies suggest a role for Custos in fine-tuning canonical Wnt signal transduction during embryogenesis, adding an additional layer of regulatory control in the Wnt-β-catenin signal transduction cascade.**

Understanding the molecular mechanisms of pattern formation during embryogenesis remains a challenge for biologists. One key family of signaling molecules that have been shown to play crucial roles in this process is the Wnt family. Wnt proteins are conserved secreted glycoproteins that govern major developmental processes including cell fate determination, cell proliferation, cell motility and establishment of the primary axis and head formation during vertebrate development (1, 2). In addition to regulating embryonic development, defects in Wnt signaling have also been implicated in tumorigenesis and birth defect disorders (1). The Wnt ligands bind to their cognate receptors and coreceptors, which are encoded in the Frizzled (Fz) and Lipoprotein Related Protein 5/6 (LRP5/6) gene families (2, 3). Through intensive studies, a molecular signaling pathway has emerged. Upon the binding of Wnt to a receptor complex, a signal is transduced to the cytoplasmic phosphoprotein Dishevelled (Dvl); at the level of Dvl and using distinct domains within Dvl, the Wnt signal branches into two signaling pathways, a "canonical" and a "noncanonical" pathway (3). A large number of Dvl-interacting proteins have been identified that function to link Dvl to the downstream pathway or influence its ability to signal, including Casein Kinase 1 (CK1) (4) for the canonical signaling and Daam1 (5) for the noncanonical Wnt signaling pathway (3). For canonical signaling, which acts in axis formation, Wnt signaling through Dvl induces the stabilization of cytosolic β-catenin (6). In the absence of Wnt signaling, β-catenin is phosphorylated by CK1 and GSK3β, and targeted by a destruction complex for ubiquitination and degradation by β-TrCP and the proteasome (7). In the presence of Wnt signaling, Dvl blocks this phosphorylation of β-catenin, and the inhibition of degradation of β-catenin allows for its cytoplasmic accumulation and subsequent nuclear translocation. In the nucleus, β-catenin complexes with the Lef/Tcf family of transcription factors and regulates transcription of Wnt-target genes (1). β-catenin binding proteins are known, and they regulate its ability to interact with Tcf or influence its transcriptional activity (8). The cytoplasmic–nuclear translocation of β-catenin remains poorly understood, as β-catenin has no identified nuclear localization sequences (9–11). It has been proposed that β-catenin may "piggy-back" into the nucleus by interacting with factors that traffic this protein across the nuclear envelope (11, 12). Specifically, β-catenin was proposed to interact with importin-β for nuclear import (13), but it remains unclear if β-catenin docks with any proteins at the nuclear pore for its entry and how this entry is regulated (11). For nuclear export of β-catenin there is evidence that β-catenin may exit via binding to proteins such as Axin (12) and APC (10) using a Ran and CRM1-independent mechanism (11). Alternatively, β-catenin can interact with RanBP3 to be exported from the nucleus (14). In the present work we show that Custos inhibits canonical Wnt signaling in frog embryos and cultured cells by antagonizing nuclear import of β−catenin.

## Results

In our efforts to understand how Dvl branches the extracellular Wnt signal into different intracellular signaling cascades, we

### Significance

**Canonical Wnt pathway is essential for primary axis formation and establishment of basic body pattern during embryogenesis. Defects in Wnt signaling have also been implicated in tumorigenesis and birth defect disorders. Here we characterize a novel component of canonical Wnt signaling termed Custos and show that this protein binds to and modulates β-catenin nuclear translocation in the canonical Wnt signal transduction cascade. Our functional characterization of Custos further shows that this protein has a conserved role in development, being essential for organizer formation and subsequent anterior development in the *Xenopus* and zebrafish embryo. These studies unravel a new layer of regulation of canonical Wnt signaling that might provide insights into mechanisms by which deregulated Wnt signaling results in pathological disorders.**

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The authors declare no conflict of interest.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. KM235953).

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screened for binding partners of Dvl using a PDZ containing Dvl fragment as bait in a yeast two-hybrid assay under conditions previously described (5). This screen uncovered a previously uncharacterized protein that we have named Custos, for the Latin term for "guard" or "keeper." Here we report on the biological function of Custos during *Xenopus* and zebrafish embryogenesis. *Xenopus* Custos (KM235953) encodes a 237-aa protein that contains two putative CK1 phosphorylation sites as well as two clusters of dual putative nuclear localization signals (NLS) (Fig. 1*A*). *Xenopus* Custos shares 45%, 46%, 48%, and 50% amino acid sequence identity to zebrafish (NP_001008602), rat (NP_001009630), mouse (NP_082487), and human (NP_075046) Custos, respectively, indicating that Custos is conserved among vertebrates; however, no ortholog based on sequence comparison was identified in invertebrates (Fig. S1).

We observed that Custos binds to Dvl in coimmunoprecipitation studies and this interaction was negatively regulated by Wnt stimulation (Fig. S2 *A* and *B*). The interacting domains were mapped to between the PDZ and DEP domains of Dvl, and in the area of the NLS1 and NLS2 motifs of Custos (Figs. S2 *C-E* and S3 *A* and *B*). The expression of Dvl with Custos resulted in a downshift in the migration of Custos on SDS/PAGE gels due to a dephosphorylation of this protein (Fig. S3*C*), and we found that Custos interacted with CK1 and was a substrate for CK1 (Fig. S2 *F* and *G*). These results suggest Custos is a potential novel regulator of Wnt signaling pathway.

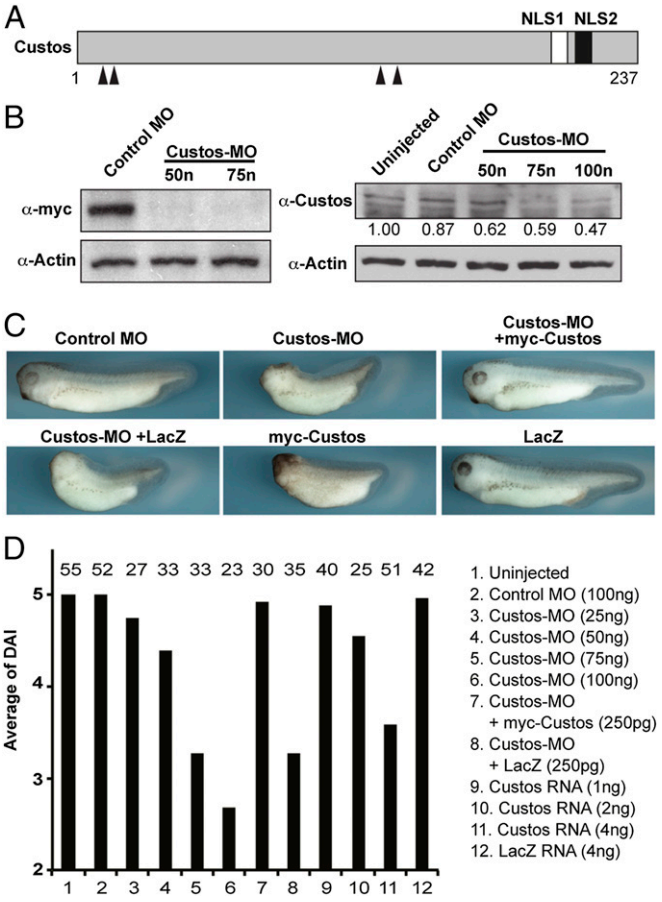


**Fig. 1.** Custos is required for anterior development. (*A*) Schematic representation of Custos and its domains. Arrowheads indicate the two clusters of dual CKI phosphorylation sites. (*B*) Custos-MO blocks translation of tagged-Custos protein and depletes endogenous *Xenopus* Custos protein levels. Myc-tagged Custos RNA containing the MO binding site was coinjected with control MO or Custos-MO, and tagged-protein was visualized by SDS/PAGE and Western blotting (*Left*). Endogenous Custos protein was assessed using anti-Custos antibody (*Right*). (*C*) Depletion and overexpression of Custos induce anterior head defects. The indicated RNA or MO was injected into the two dorsal blastomeres of 4-cell embryos. (*D*) Suppression of head development was scored by the dorso-anterior index (DAI). The numbers above each bar indicate the number of injected embryos.

To uncover Custos' potential role during embryogenesis, we first examined the temporal and spatial expression pattern of *custos* RNA in the *Xenopus* embryo. RT-PCR analysis revealed stable expression of *custos* maternally to the tadpole stage (Fig. S4*A*). In situ hybridization demonstrated broad expression of *custos* from egg to cleavage-stage embryos (Fig. S4*B*). During the neurula stage, *custos* was highly expressed in the neural plate and neural fold. Strong expression of *custos* was also detected in brain, eyes, and spinal cord at the tadpole stage.

To delineate the biological function of Custos during *Xenopus* embryogenesis, we conducted gain-of-function (RNA expression) and loss-of-function studies (antisense Morpholino oligonucleotide, Custos-MO) (Fig. S4*D*). It is well established that the canonical Wnt pathway regulates primary axis formation and signaling occurs in two distinct phases: the early canonical and late canonical Wnt pathway (15). The activation of the early canonical Wnt pathway is essential for induction of the Spemann–Mangold organizer so that defects in this pathway produce a ventralized phenotype, including small eyes, cement gland, and brain, due to defects in Spemann–Mangold organizer formation. During gastrulation, organizer tissue secretes several Wnt antagonists, which function to establish a gradient of the canonical Wnt activity along the anterior–posterior axis that is important for patterning the neural plate and brain. Disruption of the gradient by ectopic activation of the canonical Wnt pathway following the Spemann–Mangold organizer formation produces defects in anterior development (16).

Custos-MO injection efficiently blocked translation of myc-tagged 5′ UTR Custos and reduced the levels of endogenous Custos protein in *Xenopus* lysates, whereas the control MO did not affect protein levels (Fig. 1*B*). Custos-MO morphants displayed reduced head structures, including cement gland and eyes, at the tadpole stage (Fig. 1*C*). In the most severely affected embryos, the head structures were missing entirely. The phenotype was scored using the dorso-anterior index (DAI) (17), showing that the DAI averages of Custos-MO injected embryos were reduced maximally to 2.68, dose-dependently (Fig. 1 *C* and *D*). Importantly, the Custos-MO–induced phenotypic abnormalities could be rescued by coinjection of the Custos-MO with myc-Custos RNA lacking the MO-binding site, but not by coinjection of LacZ RNA. We next tested the effect of overexpression of Custos on embryogenesis by injection of capped-Custos RNA. Injection of Custos RNA, but not LacZ RNA, into the dorsal marginal zone of the four cell stage embryo also resulted in a smaller cement gland, eyes, and head (Fig. 1 *C* and *D*), which phenocopies the effect produced by the Custos-MO. Injection of Custos-MO or Custos RNA into ventral blastomeres had no effect on *Xenopus* development. The abnormalities caused by Custos-MO and Custos RNA injection support a role for Custos in the canonical Wnt pathway in the regulation of primary axis formation. Expression of Custos in mammalian cells did not affect levels of activated RhoA, a marker of noncanonical Wnt signaling (Fig. S4*C*), further supporting an exclusive role for Custos in the canonical Wnt pathway.

As a further test for Custos function in the embryo, we examined organizer gene expression at stage 10.5 by in situ hybridization. Custos-MO or RNA was injected into a single dorsal blastomere with LacZ RNA, and the expression of organizer genes Xnr3 and Goosecoid were examined. Although the effect of the Custos-MO on organizer gene expression was mild, likely due to maternal Custos protein, ~20% of Custos-MO injected embryos exhibited reduced expression of Xnr3 and Goosecoid (Fig. 2*A*). Overexpression of Custos had a more marked effect, with 52% and 45% of Custos-RNA injected embryos exhibiting

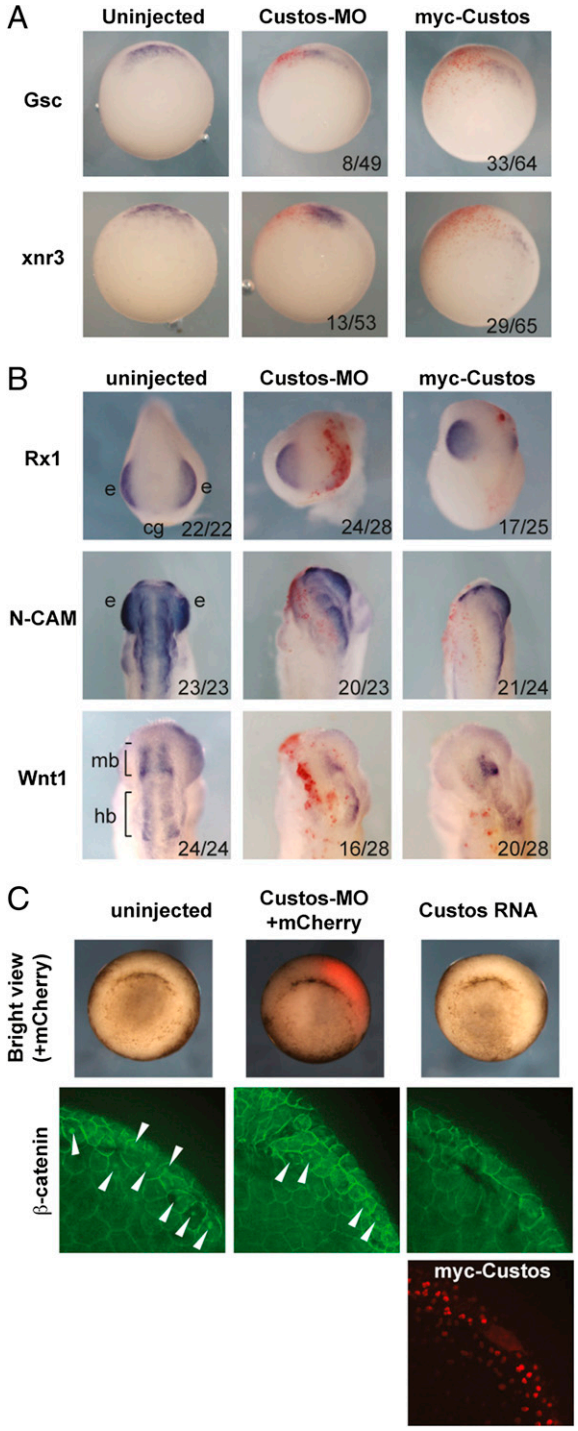


**Fig. 2.** Dysregulation of Custos inhibits the canonical Wnt pathway. (*A* and *B*) Overexpression or depletion of Custos reduced organizer gene expression (*A*) and anterior markers (*B*). Custos RNA (1.5 ng) or Custos-MO (50 ng) was injected with LacZ (250 pg) RNA into one dorsal cell of four-cell embryos. At stage 10.5, the expression level of organizer genes Xnr3 and Goosecoid (Gsc), or at stage 26, the expression of anterior markers Rx1, *N*-CAM and Wnt1, was examined by in situ hybridization. Red staining indicates the injected side. The number of embryos scored is shown on each panel. (*C*) Custos regulates nuclear accumulation of β-catenin at stage 10.5. Custos-MO (50 ng) or myc-Custos RNA (1 ng) was injected into one dorsal blastomere of four-cell embryos. mCherry RNA (250pg) was coinjected and red fluorescence was used as tracer for the injected side. Embryos were fixed at stage 10.5, and immunohistochemistry was performed with anti–β-catenin and anti-myc antibodies. Arrowheads indicate cells with nuclear accumulation of β-catenin.

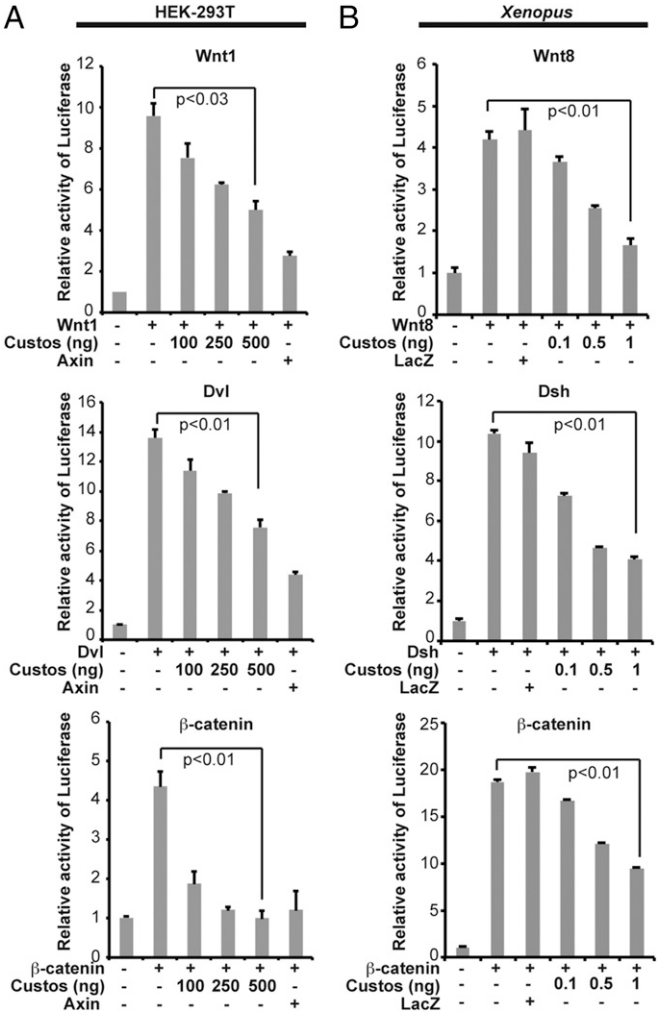


**Fig. 3.** Custos inhibits Wnt signal transduction. (*A*) Topflash reporters were cotransfected into HEK-293T cells with expression plasmids for Wnt1, Dvl or β-catenin and Custos, as indicated. (*B*) Topflash reporter was coinjected with Wnt8, Dsh, or β-catenin and Custos RNAs into all cells of two-cell embryos. Cell or embryo lysates (stage 10.5) were subjected to luciferase assay and normalized to reporter-only samples.

reduced or absent expression of Goosecoid and Xnr3, respectively (Fig. 2*A*). We next performed in situ analysis of stage 25 embryos injected with Custos-MO or RNA for the pan-neural marker *N*-CAM and a battery of eye (Rx1 and Six3), forebrain (FoxG1), midbrain (Wnt1, Pax2, and En2) and hindbrain markers (Krox20). These studies demonstrated that both depletion and overexpression of Custos reduced expression of these genes (Fig. 2*B* and Fig. S5).

The most immediate consequence of canonical Wnt signaling is nuclear translocation of β-catenin. We examined whether Custos mRNA or MO affects this process in stage 10.5 embryos using immunocytochemistry. Nuclear accumulation of β-catenin was observed in the organizer of uninjected embryos (Fig. 2*C*, arrowheads), whereas injection of myc-Custos RNA prevented nuclear accumulation of β-catenin in nearly all cells. The effect of injection of the Custos-MO was less pronounced, with ∼75% of cells showing reduced accumulation of nuclear β-catenin (Fig. 2*C*).

Thus far, our data indicated a role in canonical Wnt signaling, and we next sought delineate the epistatic position that Custos occupies in this pathway. We performed Topflash assays in HEK-293T cells transfected with Wnt1, Dvl2, and β-catenin along with

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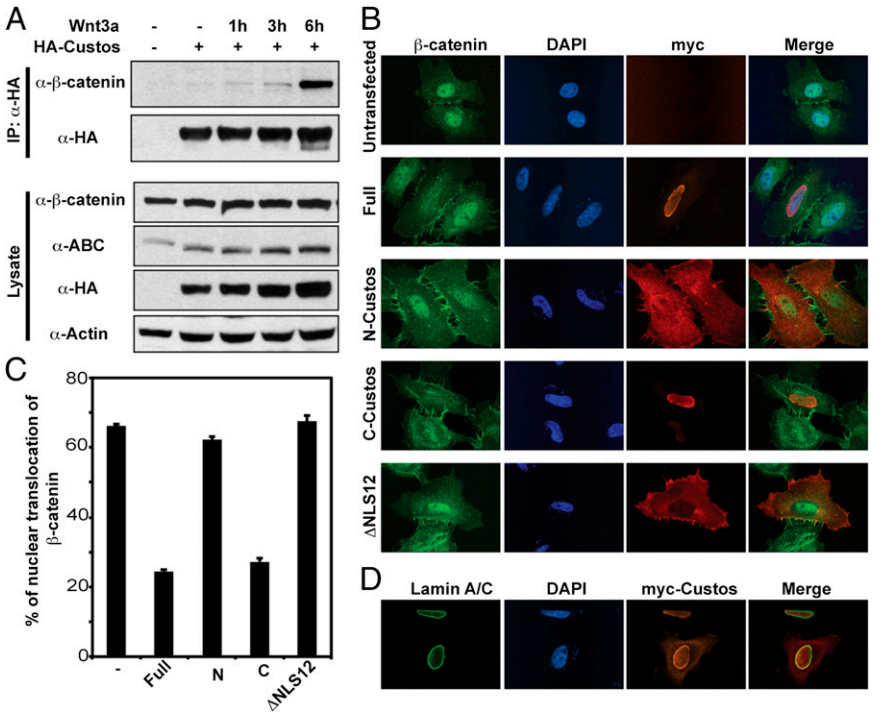


**Fig. 4.** Custos binds to β-catenin and regulates its nuclear translocation. (*A*) HA-Custos was transfected into HEK-293T cells, the cells were treated with 250 ng/mL recombinant mouse Wnt3a, and lysed at different time points. HA-Custos was immunoprecipitated from cell lysates, and coimmunoprecipitated β-catenin was detected by SDS/PAGE and Western Blotting. The α-ABC antibody detects the active form of β-catenin. (*B*) Overexpression of Custos and truncated forms of Custos inhibits nuclear accumulation of β-catenin in HeLa cells. Myc-Custos transfected cells were treated with 250 ng/mL recombinant mouse Wnt3a for 3 h and then subjected to immunocytochemistry with the indicated antibodies. DAPI staining was used to show the cell's nucleus. (*C*) Quantification of the studies in *B*; nuclei positive for β-catenin staining (as in the first panel of *B*) were counted. (*D*) Colocalization of myc-Custos with laminA/C. HeLa cells were transfected with myc-Custos and then subjected to immunocytochemistry with the indicated antibodies.

increasing doses of Custos cDNA. We observed that Custos dose-dependently suppressed reporter induction by all cases (Fig. 3*A*). We verified these findings by Topflash assays in the *Xenopus* embryo and observed that, similar to the mammalian cells, coinjection of Custos with XWnt8, XDsh, or β-catenin reduced the induction of luciferase activity (Fig. 3*B*). Additionally, using an in vivo assay in *Xenopus* embryos, we found that Custos dose-dependently suppressed secondary axis induction by XWnt8, XDsh, and β-catenin (Fig. S6). These studies indicate that Custos functions at the same level or downstream of β-catenin in the canonical Wnt pathway.

Given that Custos may be functioning at the level of β-catenin, we next tested whether Custos interacts with β-catenin in a Wnt-dependent manner. We conducted coimmunoprecipitation assays using HA-Custos cDNA transfected into HEK-293T cells. In the absence of Wnt3a treatment, coimmunoprecipitation showed a weak interaction between Custos and endogenous β-catenin, but this interaction was strengthened substantially by Wnt3a stimulation over a 6-h time course (Fig. 4*A*) (18, 19).

There are two critical control points by which β-catenin's activity in canonical Wnt signaling is controlled. The first is by regulation of β-catenin cytoplasmic protein levels by the destruction complex, and the second is by regulating nuclear accumulation of β-catenin. We note that injection of the Custos-MO and Custos RNA interferes with β-catenin nuclear translocation in the embryo at stage 10.5 (Fig. 2*C*). Overexpression of Custos in HEK-293T cells does not change β-catenin protein levels (Fig. 4*A*). We investigated the effect of Custos on the nuclear accumulation of β-catenin using HeLa cells. Overexpression of myc-Custos significantly reduced nuclear accumulation of β-catenin in HeLa cells, and this effect required the C-terminal NLS domains within Custos (Fig. 4 *B* and *C*). Strikingly, Custos colocalizes with lamin A/C at the nuclear envelope (Fig. 4*D*). Deletion mutants of Custos that are unable to localize to the nuclear membrane did not inhibit β-catenin accumulation (Fig. 4 *B* and *C*), indicating that localization of Custos to the nuclear envelope is necessary for Custos function. Further, we note that function of Custos appears to be specific to the canonical Wnt pathway as Custos expression did not inhibit Activin signaling in a luciferase reporter assay (Fig. S7*A*). These results strongly suggest that Custos modulates canonical Wnt signaling by controlling the translocation of β-catenin at the nuclear envelope.

To determine if the function of Custos is conserved in another vertebrate model, we examined the role of Custos during zebrafish development using gain-of-function (RNA expression) and loss-of-function studies [antisense Morpholino oligonucleotides (Custos-MO1 or Custos-MO2) (Fig. S7*B*)]. Injection of Custos RNA into the yolk mass of one-cell zebrafish embryos dose-dependently produced embryos with reduced head structures and small or absent eyes (Fig. 5 *A* and *B*). We scored the embryos using the established V1–V4 scoring method (20), and observed 48% of injected embryos having a V2 and V3 classification at the 100-pg RNA injection dose. We next used two independent Custos-MOs, and found that injection of either MO resulted in embryos with small heads and reduced or absent eye structures in a dose-dependent manner (Fig. 5 *C*–*F*). Coexpression of either Custos-MO1 or Custos-MO2 with a rescue construct lacking the complete recognition sites of both MOs suppressed these effects, demonstrating specificity of the MOs (Fig. 5 *C*–*F*). These studies show that the function of Custos is conserved between *Xenopus* and zebrafish such that overexpression or depletion of Custos results in defects in anterior body formation in both species.

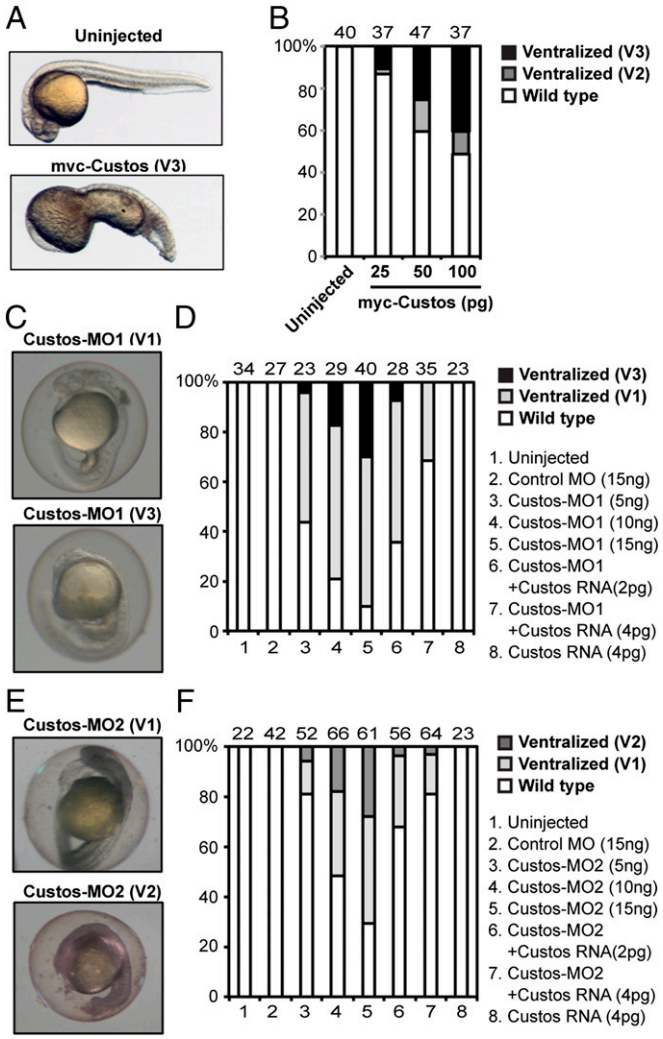


**Fig. 5.** Custos is required for anterior development in zebrafish. (*A* and *B*) 25–100 pg of Myc-tagged Custos RNA was injected into the one-cell embryo and scored at 28 h postfertilization (hpf) for suppression of head development by the V1–V4 index. The numbers above each bar indicate the number of injected embryos. (*C* and *E*) Injection of Custos MO1 (*C*) or Custos MO2 (*E*) suppressed head and eye development which was rescued by coinjection of Custos Rescue RNA (2–4 pg). MOs (5–15 ng) were injected at the one-cell stage and embryos were scored at 24 hpf. (*D* and *F*) Quantitation of the results in *C* and *E*, respectively. The numbers above each bar indicate the number of injected embryos.

## Discussion

In this study, we identified Custos as a component of the canonical Wnt pathway during *Xenopus* and zebrafish embryogenesis. Our data reveal that Custos binds to Dvl and β-catenin, and that depletion or overexpression of Custos interferes with nuclear accumulation of β-catenin. The translocation of β-catenin to the nucleus is the key event in canonical Wnt activation although this process is not well understood. It is known that β-catenin directly binds to nuclear pore proteins and translocates into the nucleus in an NLS-importin-independent manner (11, 21). Recent studies have also uncovered the importance of nuclear envelope associated proteins for the cytoplasmic to nuclear shuttling of β-catenin. For example, Nesprin-2, a giant spectrin repeat protein, which localizes at the nuclear envelope, regulates nuclear translocation of β-catenin (22). Emerin and lamin A/C are also nuclear envelope proteins that regulate cytoplasmic-nuclear shuttling of β-catenin (23, 24). Custos localizes on the nuclear envelope in HeLa cells as well as *Xenopus* embryos, and this localization appears to be required for inhibition of nuclear accumulation of β-catenin. Although the mechanism by which Custos regulates β-catenin nuclear localization has not been elucidated, one possibility is that Custos modulates the accessibility of β-catenin to the nuclear pore complex. In this scenario, Custos binds β-catenin and functions as a docking protein at the nuclear envelope to establish the directionality of shuttling into and out of the nucleus to modulate canonical Wnt signaling.

Interestingly, injection of the Custos-MO in *Xenopus* generates similar defects as injection of Custos RNA, including embryos with small eyes, head, and brain structures. We suggest that these defects arise by inhibition of the formation of the Spemann-Mangold organizer by nonoptimal levels of Custos. Indeed, marker genes for the organizer including Xnr3 and Goosecoid are reduced by overexpression of Custos RNA and less so by Custos-MO, the latter likely being due to maternal Custos. The defects in organizer formation lead to reduced expression of marker genes of eye and brain development at later stages. These defects produced by the Custos-MO are likely specific as they can be rescued by coexpression of MO-insensitive Custos mRNA. Similar inhibition of anterior development by Custos overexpression or inhibition using two independent MOs was seen in zebrafish embryos, further supporting the view that optimal levels of Custos are required for canonical Wnt signaling in early vertebrate development.

Observing similar outcomes following overexpression or depletion of a signaling component is unusual for the canonical Wnt pathway, although it is common for the noncanonical pathway (25); nevertheless, such effects have been reported for the WISE protein (26). WISE is a secreted modulator of canonical Wnt signaling and is thought to work by modulation of the Wnt ligand and coreceptor LRP5/6 interaction (27). The mechanism of WISE action is not fully understood, but it has been shown that optimal levels of the protein are critical for effective modulation of canonical Wnt signaling. Thus, Custos is the second protein that requires an optimal level of protein for function in this pathway. One possible mechanism is that excess Custos could act as a dominant negative effector, for example by sequestering other critical factors. It is important to note that Custos is not a global modulator of signaling pathways within the embryo that involve cytoplasmic to nuclear transport of signal transduction molecules, as Custos did not interfere with Activin signaling that requires Smad nuclear translocation.

In summary, we have identified a previously undescribed component of canonical Wnt signaling that is required for β-catenin translocation into the nucleus during organizer formation. Custos orthologs are observed in vertebrates only. Custos therefore imparts a level of regulation of canonical Wnt signaling at the level of β-catenin that was hitherto undefined, and its study can provide new insights into β-catenin nuclear translocation.

## Materials and Methods

**Antibodies.** Mouse monoclonal antibodies against Myc (9E10), actin (sc-8432), anti-HA (F-7), and anti–Dvl-2 (10B5) were obtained from Santa Cruz Biotechnology; anti–β-catenin was from Transduction Laboratories; and anti–active-β-catenin (8E7) was from Millipore. Rat monoclonal antibody against HA (3F10) was from Roche. Mouse monoclonal anti-Flag (F1804) was from Sigma. Anti-laminA/C antibody was obtained from ABcam.

**Plasmids and Morpholino Oligonucleotides.** *Xenopus* Custos (XCustos) and deletion fragments generated by restriction digestion or PCR amplification were subcloned into pCS2+MT and pCS2-HA vectors. XCustos morpholino oligonucleotide (MO) complementary to the ATG start codon region (5′-TGACCAACAAGTAAGATGGCGGCG-3′) was synthesized by Gene Tools, Inc. The Control MO was obtained from Gene Tools. Zebrafish Custos (zCustos) was amplified by RT-PCR and cloned into pCS2+MT vector. Target sequences of zCustos-MO1 and 2 are followings: MO1: 5′-CCTCAAATATGTCTGAAAGGAGCAG-3′, MO2; 5′- AATATGTCTGAAAGCAGCAGTGAAG-3′.

**Yeast Two-Hybrid Screen.** A rat brain cDNA library (Clontech) was screened using a Dvl2 fragment containing the PDZ domain as bait. We screened 3.8 million independent clones, and 24 positives were obtained, of which 22 were overlapping fragments of Custos.

**Antibody Generation.** Anti-Custos polyclonal antibody was generated against a peptide (amino acid 127–147) of Custos (Convance). The Custos specific antibody was affinity purified using an antigen peptide column.

**Cell Culture.** Mammalian cell culture studies were performed using HEK-293T cells or HeLa cells. Cells in 60-mm dishes were transfected using Polyfect reagent (Qiagen) with 1–2 μg of the indicated plasmids. The total amount of transfected DNA was equalized by supplementation with vector DNA. For Wnt stimulation, transfected cells were serum starved for 24 h and treated with recombinant mouse Wnt3a (R&D systems) at 250 ng/mL for 1–6 h. Wnt3a protein was diluted with serum free medium containing 1% BSA. Cells were lysed after Wnt treatment, and immunoprecipitation was performed as described (5). Immunocytochemistry was performed as described (28). Images were obtained using a Zeiss Axiovert 100 confocal microscope. Rho activation assays were performed as described (29).

**Embryo Manipulations.** *Xenopus laevis* embryos were obtained, microinjected and subjected to whole-mount in situ hybridization as described (28). Probes of anterior marker genes were kind gifts from Heithem M. El-Hodiri (National Children's Hospital, Columbus, OH) (30). Embryo injections were performed with in vitro transcribed capped-RNAs. For the Topflash assay, embryos were injected with Topflash reporter (Upstate) and pSV40-RL (Promega) plasmids, and subjected to luciferase assay using Dual Luciferase Reporter Assay system (Promega), according to the manufacturer's protocol. All experiments were repeated at least three times. ARE reporter construct was a kind gift from Ali H. Brivanlou (Rockefeller University, New York). Zebrafish embryos were obtained from natural crosses for microinjection. RNA and MO were injected into the yolk mass of one-cell stage embryos. Four hours after injection, damaged embryos were removed; the rest were grown at 28 °C in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$).

**Whole Mount Immunocytochemistry.** LacZ RNA, myc-Custos RNA, or Custos MO was injected into one dorsal blastomere of four-cell stage embryos. Embryos were fixed at stage 10.5 or stage 22. Whole mount immunocytochemistry was performed as described (31, 32).

**Statistics.** All experiments were performed at least three times. Error bars indicate SE.

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