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The Postsynaptic Density 95/Disc-Large/Zona Occludens Protein Syntenin Directly Interacts with Frizzled 7 and Supports Noncanonical Wnt Signaling

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Wnt signaling pathways are essential for embryonic patterning, and they are disturbed in a wide spectrum of diseases, including cancer. An unresolved question is how the different Wnt pathways are supported and regulated. We previously established that the postsynaptic density 95/disc-large/zona occludens (PDZ) protein syntenin binds to syndecans, Wnt coreceptors, and known stimulators of protein kinase C (PKC)α and CDC42 activity. Here, we show that syntenin also interacts with the C-terminal PDZ binding motif of several Frizzled Wnt receptors, without compromising the recruitment of Dishevelled, a key downstream Wnt-signaling component. Syntenin is coexpressed with cognate Frizzled during early development in *Xenopus*. Overexpression and down-regulation of syntenin disrupt convergent extension movements, supporting a role for syntenin in noncanonical Wnt signaling. Syntenin stimulates c-jun phosphorylation and modulates Frizzled 7 signaling, in particular the PKCα/CDC42 noncanonical Wnt signaling cascade. The syntenin–Frizzled 7 binding mode indicates syntenin can accommodate Frizzled 7–syndecan complexes. We propose that syntenin is a novel component of the Wnt signal transduction cascade and that it might function as a direct intracellular link between Frizzled and syndecans.

INTRODUCTION

Wnt proteins are involved in cell proliferation, differentiation, polarity, migration, and apoptosis, controlling a variety of processes during embryonic development and adult homeostasis (Logan and Nusse, 2004). Various inborn and acquired diseases are based on aberrant Wnt signaling (Johnson and Rajamannan, 2006). Wnt signaling requires the interplay of multiple proteins (http://www.stanford.edu/~rnusse/wntwindow.html), but the reception and transduction of Wnt signals are predominantly based on the binding of Wnt proteins to Frizzled (Fz) cell surface receptors (Yang-Snyder *et al*., 1996). The various Fz receptors differ in their spatial and temporal expression patterns and in their relative affinities for ligand. Receptor activation by Wnt somehow activates Dishevelled (Dsh), the most upstream component of the cytosolic signal transduction cascade. The role of Dsh is intricate because downstream of Dsh, the signaling branches into either the canonical Wnt/β-catenin or the noncanonical (β-catenin–independent) pathway (Boutros and Mlodzik, 1999). Activation of the canonical pathway drives β-catenin–dependent transcription of target genes, controls tissue-specific cell fate decisions during embryogenesis, and regulates cell proliferation in adult tissues (Logan and Nusse, 2004). The noncanonical pathway controls reorganization of the actin cytoskeleton, tissue polarity, and cell movement (Strutt, 2003). Several molecular cascades, which may overlap, seem to function in noncanonical Wnt signaling (Veeman *et al*., 2003; Kohn and Moon, 2005). One is similar to the planar cell polarity or PCP pathway in *Drosophila*, and it activates small GTPases of the Rho family and the c-Jun-NH_2-terminal kinase (JNK). Another, triggered by Wnt4, Wnt5a, and Wnt11, involves activation of calcium/calmodulin-dependent kinase II and protein kinase C (PKC), and it exerts antagonistic effects on the canonical pathway (Kuhl *et al*., 2001; Sheldahl *et al*., 2003).

One major question is how Wnt signaling activates different downstream pathways. Clearly, the Wnt–Fz combination and the presence of coreceptors for Wnts, like the members of the low-density lipoprotein receptor-related proteins (Wehrli *et al*., 2000) or heparan sulfate proteoglycans (Reichsman *et al*., 1996), are important. Wnt signaling also relies on the subcellular localization of the Fz (Wu *et al*., 2004) and the nature of the intracellular components adapting to Fz. The Fz family members are serpentine receptors with an extracellular cysteine-rich domain important for Wnt binding, and seven membrane-spanning domains (Vinson *et al*., 1989; Wang *et al*., 2006). Fz display a C-terminal cytosolic tail with two postsynaptic density 95/disc-large/zona occludens (PDZ) binding motifs, or PDZBMs. So far, proteins containing PDZ domains are the only well established intracellular direct ligands for Fz (Tan *et al*., 2001;

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Wong *et al*., 2003; Yao *et al*., 2004; Djiane *et al*., 2005; Ataman *et al*., 2006).

Proteins containing PDZ domains (PDZ proteins) are scaffold proteins particularly abundant in multicellular organisms and probably evolved in response to the increased signaling needs of multicellularity. PDZ proteins have been implicated in the establishment and maintenance of cell polarity, in the formation and the stability of adhesion structures and in the targeting and organization of large signaling complexes at the membrane (Nourry *et al*., 2003; Margolis and Borg, 2005). PDZ interactions are reversible and particularly versatile.

We identified the PDZ protein syntenin as an intracellular ligand of the syndecans (Grootjans *et al*., 1997) and as an important regulator of cell shape (Zimmermann *et al*., 2001, 2005). Syndecans are abundant and ubiquitous type I transmembrane heparan sulfate proteoglycans that emerge as essential for the reception, dissemination and readout of morphogen signals during development and in pathology. The heparan sulfate chains of their extracellular domain bind numerous growth factors, including Wnts (Couchman, 2003; Bishop *et al*., 2007). Here, we tested for a direct interaction between syntenin and Fz, and for a role for syntenin in Fz signaling.

MATERIALS AND METHODS

Molecular Biology

Human syntenin, syntenin-2, and syndecan cytoplasmic domain constructs were described previously (Grootjans *et al*., 1997, 2000; Grootjans *et al*., 2000). Fz cytoplasmic domains were cloned in pGEX-5X-1 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Ligand overlays were performed with purified glutathione transferase (GST)-fusion proteins as described previously (Grootjans *et al*., 2000). Human Fz 7 cytoplasmic domain was cloned in peYFP-C1 for immunoprecipitation experiments. The human Fz 7 full cDNA clone (IMAGp958K221240Q) was purchased from RZPD Deutsches Ressourcenzentrum fur Genomforschung (Berlin, Germany), and the open reading frame was introduced into pCDNA3.1/zeo(+) (Invitrogen). Quickchange mutagenesis (Stratagene, La Jolla, CA) was used to generate mutant Fz 7 constructs. The Xsyntenin-a clone (IMAGp998K0110804Q), Xsyntenin-a′ clone (IRBHp990H0541D2), and Xsyntenin-b clone (RZPDp988D1265D) were purchased from RZPD Deutsches Ressourcenzentrum fur Genomforschung, and sequence analysis confirmed the sequences present in the database. To produce the Xsyntenin-a, a′, and b probes for whole-mount in situ hybridization (WISH), the full cDNA was sublconed into pGEM-T. To produce RNA for injection, Xsyntenin-a was cloned in pCS2+, flanked or not with myc or hemagglutinin (HA) tags. All constructs were verified by sequencing. The morpholino (MO) oligonucleotides (Gene Tools, Philomath, OR) used were for Xsyntenin-a, 5′-ACAGACCTGCCGACAGTCGAATAAG-3′; for Xsyntenin-a′, 5′-GAGGGATATAGAGACATTTTCACAG-3′; and for Xsyntenin-b, 5′-GGATAGATAGACATGGTGTAAGACC-3′. All three MOs efficiently block in vitro transcription/translation of the respective Xsyntenins. The mismatch MO used was 5′-GACGCATAGAGAGAGATATT-3′. The MO for XFz 7 was 5′-GTGAGCAGAAATCGGCTGATACTGG-3′, as described by Sumanas *et al*. (2001). For reverse transcription-polymerase chain reaction (RT-PCR), total RNA extracted from embryos was reverse transcribed using RTase MuLv (20 U/μl) (Promega, Madison, WI). Histone H4 was used as an internal standard. The following PCR conditions were applied: 26 cycles for H4 and 32 cycles for Xsyntenin-a. The primers used for Xsyntenin-a, forward 5′-CCTTTGAACGCACCATCACCATG-3′ and reverse 5′-GAATTCTTAAACCTCAGGGACAGAATG-3′, generate fragments of 300 base pairs. H4 primers were as described in http://www.hhmi.ucla.edu/derobertis/index.html. Reactions were carried out using a Gene Amp TM PCR system 9600 (PerkinElmer Life and Analytical Sciences, Boston, MA).

Surface Plasmon Resonance Experiments

Surface plasmon resonance was measured using a Biacore 2000 instrument. N-terminal biotinylated Fz 7 synthetic peptides, corresponding to the CD of Fz 7, were immobilized on a streptavidin-sensor chip. Analytes (GST fusion proteins) were perfused at 10 μl/min in running buffer (100 mM NaCl/10 mM HEPES/0.005% Tween 20, pH 7.4). The surface was regenerated through 1-min pulses of 1 M NaCl/0.05 M NaOH. For the determination of the apparent K_D, signals obtained at equilibrium (Req with different concentrations from 300 to 1500 nM GST-syntenin) were plotted as a function of protein concentration. Apparent K_D values were calculated from these plots, as the concentration corresponding to Req-max/2.

Cells, Transfections, Extractions, Coimmunoprecipitation, and Fluorescence Microscopy

Cells originated from American Type Culture Collection (Manassas, VA), and they were routinely grown in DMEM/F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). For microscopic analysis, cells were plated on eight-well chamber slides (Nalge Nunc International, Rochester, NY), transfected using the FuGENE transfection reagent (Roche Diagnostics, Basel, Switzerland), and then they were fixed and stained as described previously (Zimmermann *et al*., 2001) by using 10 μg/ml anti Fz 7 antibody (R & D Systems, Minneapolis, MN) and Alexa 568-conjugated donkey anti-goat secondary antibodies (Invitrogen). The enrichment of enhanced green fluorescent protein (eGFP)-syntenin at the plasma membrane was scored by confocal microscopy, in three independent experiments, looking at 30 cells per transfection. Coimmunoprecipitations were performed as described previously (Djiane *et al*., 2005). Enhanced yellow fluorescent protein (eYFP)-Fz 7 was immunoprecipitated with goat anti-GFP antibodies (ab5449; Abcam, Cambridge, United Kingdom). Detection of eYFP-Fz 7 in Western blot was with mouse anti-GFP antibodies (G1546, 1/1000; Sigma Chemical, Pool, Dorset, United Kingdom). Endogenous syntenin was detected with purified rabbit polyclonal antibodies (Zimmermann *et al*., 2001). For Jun-phosphorylation assays, human embryonic kidney (HEK)293T cells were plated at a density of 150,000 cells/well in six-well dishes. Extracts were prepared 48 h after transfection in the presence of detergent and phosphatase- and protease-inhibitors: 0.1 mM sodium vanadate, 0.1 mM aprotinin, 0.1 mM leupeptin, and 0.5 mM pepstatin A in 50 mM β-glycerophosphate, 1 mM EDTA, 1 mM benzamidine, 1.5 mM EGTA, and 60 mM octylglucoside. Protein samples (10 μg) were separated on 10% SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose filter, and incubated with 1/1000 anti-phospho c-jun (ser-63), 1/1000 anti-c-jun (Cell Signaling Technologies, Danver, MA) or 1/500 anti-actin (Sigma Chemical) antibodies.

Xenopus Embryos, In Situ Hybridization, Microinjections, and Membrane Translocation Assays

Xenopus embryos were obtained from adult frogs by hormone induced egg-laying and in vitro fertilization by using standard methods (Sive *et al*., 2000), and they were staged according to Nieuwkoop and Faber (1967). Synthesis of capped RNA was performed with a Message Machine kit (Ambion, Austin, TX), and injection was carried out as described previously (Bellefroid *et al*., 1996). For whole-mount in situ hybridization, embryos were fixed in MEMFA (0.1 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde), and they were processed using dioxygenin-labeled antisense RNA probes (Sive *et al*., 2000). Membrane translocation assays were carried out as described for XDsh-myc (Umbhauer *et al*., 2000). *Xenopus* embryos were injected into the animal pole with either Xsyntenin-a-myc (200 pg) or XDsh-myc (200 pg) in the presence or absence of XFz 7 mRNA (500 pg). For the competition experiments, 100 pg of XDsh-myc, 100 pg of Xkermit-myc, 100 pg of XPKCα-GFP, 250 pg of XFz 7, 500 pg of Xsyntenin-a-HA, and 5 ng of each Xsyntenin MO or 15 ng Mismatch MO were used. Animal caps were dissected and fixed at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h. After washing with PBT-10% goat serum (PBS + 2 mg/ml bovine serum albumin + 0.1% Triton X-100), the caps were incubated overnight at 4°C with 1.5 μg/ml anti-myc antibodies (9E10; Santa Cruz Biotechnologies) and when relevant with 1 μg/ml anti-HA antibodies (3F10; Roche Diagnostics) or anti-GFP antibodies (G1546, 1/1000; Sigma Chemical). Alexa 488-conjugated goat anti-mouse secondary antibodies and when required Alexa 594-conjugated goat anti-rabbit secondary antibodies (Invitrogen), were incubated overnight at 4°C. Secondary antibodies did not show species cross-reactivity in our experimental settings. The caps were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were obtained with the MRC-1024 laser scanning confocal imaging system (Bio-Rad, Hemel Hempstead, United Kingdom) or the FluoView FV1000 (Olympus, Tokyo, Japan).

RESULTS

Syntenin Interacts with the Fz Cytoplasmic Domain in a PDZ-dependent Mode

We tested for direct interaction of Fz with syntenin in ligand overlay. Therefore, we designed primers for the PCR amplification of sequences encoding the last C-terminal 25 cytosolic amino acids of all Fz (Figure 1A) of a human brain cDNA library. The amplification was successful for Fz 1, 2, 3, 5, 6, 7, and 8. We produced and purified GST fusion constructs of these peptides and overlayed these with GST-myc-syntenin. A weak signal was observed for Fz 1; no signal was observed for Fz 2, 5, and 6; and strong signals were observed for Fz 3, 7, and 8 (Figure 1B). Overlay using a

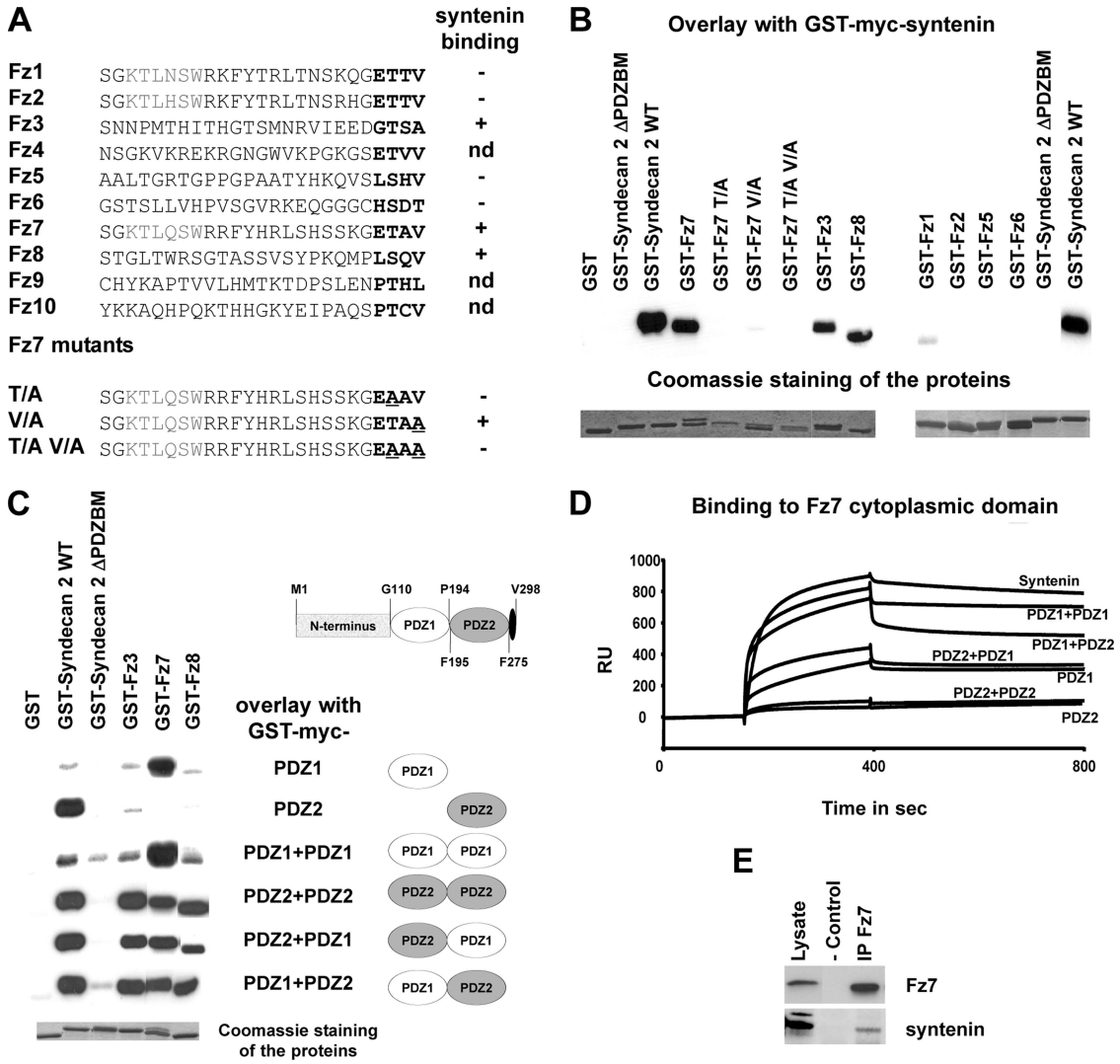

Figure 1. Syntenin interacts with Fz in a PDZ-dependent mode. (A) Sequences of the last 25 cytosolic amino acids of all human Fz and Fz 7 mutants; overview of syntenin binding to these peptides as detected in overlay; nd, not determined. C-terminal PDZBMs are in bold. For Fz 1, 2, and 7, the membrane proximal PDZBM for Dsh is also present in the 25 last amino acids, and it is indicated in gray. (B) Overlays illustrating syntenin interaction with Fz. GST-Syndecan-2 cytoplasmic domain (WT) was used as a positive control, and GST or GST-Syndecan-2 ΔPDZBM (cytoplasmic domain deleted for the last 2 amino acids) were used as negative controls. Note the interaction of syntenin with Fz 7, 3, and 8 last 25 amino acids, and the lack of interaction with Fz 7 T/A and Fz 7 T/A V/A mutants. The quality and concentration of the fusion proteins were controlled in Coomassie as shown at the bottom. (C) Respective roles of the PDZ domains of syntenin in Fz interaction. Structure of syntenin and coordinates of the amino acids that define the different domains are shown on the right. The relevant GST fusions were overlayed with recombinant proteins containing different combinations of the PDZ domains of syntenin as indicated. Note that Fz 7 interacts preferentially with the PDZ1 domain, whereas syndecan 2, Fz 3, and 8 interact preferentially with the PDZ2 domain of syntenin. The quality and concentration of the fusion proteins were controlled in Coomassie as shown at the bottom. (D) Interaction of syntenin and syntenin PDZ domains with Fz 7 cytoplasmic domain in surface plasmon resonance. RU, response units. Note that the binding relies primarily on the PDZ1 domain of syntenin. (E) Coimmunoprecipitation of endogenous syntenin with eYFP-tagged Fz 7 cytoplasmic domain. MCF-7 cells overexpressing the Fz 7 cytoplasmic domain fused N-terminally to eYFP were extracted with detergent. The cell lysate was immunoprecipitated with anti-eYFP antibodies and protein G beads before immunoblotting (right lanes, IP Fz7) with anti-eYFP to detect the Fz 7 fusion (top) or anti-syntenin antibodies (bottom). The cell lysate was used as positive control (left), in the negative control the anti-eYFP antibodies were omitted (middle). Note that endogenous syntenin coimmunoprecipitates with the eYFP-Fz 7 fusion (right bottom lane).

syntenin construct containing solely the PDZ domains yielded similar results, whereas overlay with GST-myc or GST-myc fused to the N-terminal domain of syntenin showed no signal (data not shown). We concluded for specific interaction between the PDZ domains of syntenin and Fz 3, 7, and 8. The primary structure of the Fz C-terminal region provides no direct explanation why Fz 2, 5, and 6 do not interact with syntenin, whereas Fz 3, 7, and 8 do.

The last 25 amino acids of Fz 3 and 8 solely contain the C-terminal PDZBM of the receptor, whereas those of Fz 7 also contain the membrane proximal or Dsh PDZBM (Figure 1A). Because all three Fz bind syntenin, we assumed syntenin would interact with the C-terminal PDZBM. To test this hypothesis, we investigated whether point mutations in the C-terminal PDZBM of Fz 7 (Figure 1A) affect the interaction with syntenin. In ligand overlay, a weak signal was still

observed for the GST-Fz 7 V/A mutant, but the interaction was abolished for the GST-Fz 7 T/A and GST-Fz 7 T/A V/A mutants (Figure 1B). The Fz 7-syntenin interaction was also confirmed by surface plasmon resonance (Figure 1D), and the apparent K_D for syntenin-Fz 7 interaction was determined to be 10^{-7} M, similar to that of syntenin-syndecan-2 interaction (data not shown). We concluded that syntenin interacts with the C-terminal PDZBM and probably not with the membrane-proximal PDZBM.

The Relative Preference for the First or the Second PDZ Domain of Syntenin Varies, Depending on the Fz

We also investigated the respective roles of the two PDZ domains of syntenin in binding Fz, and we compared this binding to the syndecan-2 interaction. This was tested in ligand overlay, by using GST-fusion constructs of single PDZ domains (GST-myc-PDZ1 and GST-myc-PDZ2) or homodimeric and heterodimeric tandem PDZ domains (GST-myc-PDZ1-PDZ1, GST-myc-PDZ2-PDZ2, GST-myc-PDZ1-PDZ2, and GST-myc-PDZ2-PDZ1). In overlay assays, PDZ2 was sufficient for syndecan-2 binding, PDZ1 was sufficient for the interaction with Fz 7, whereas Fz 3 and 8 interaction required two PDZ domains and at least one PDZ2 domain (Figure 1C). Surface plasmon resonance confirmed the interaction of Fz 7 with the PDZ1-PDZ1, PDZ1-PDZ2 and PDZ2-PDZ1 tandem, and it showed that PDZ1 is sufficient for Fz 7 binding. Contrary to overlay results, the PDZ2-tandem did not display strong interaction with Fz 7 in surface plasmon resonance (Figure 1D). Syntenin-2, a close homologue of syntenin (Mortier *et al*., 2005), did not bind to Fz 7 or syndecan-2 (data not shown), underscoring the specificity of the interactions. We concluded that syntenin can interact with Fz 3 and 8 via its two PDZ domains, but preferentially via the PDZ2 domain, reminiscent of the syndecan-2 mode of interaction (Grootjans *et al*., 2000), whereas the interaction with Fz 7 relies on the PDZ1 domain.

Syntenin Is Recruited by Fz 7 and Stimulates c-Jun Phosphorylation in Cultured Cells

To test for syntenin-Fz 7 interaction in a physiological context, we performed coimmunoprecipitation experiments. The available anti-Fz 7 antibodies were unable to detect the endogenous protein. Therefore, we performed coimmunoprecipitation experiments on cells overexpressing the cytoplasmic domain of Fz 7 tagged N-terminally with eYFP, a strategy used successfully by others (Djiane *et al*., 2005). Endogenous syntenin could be coimmunoprecipitated with Fz 7 in these experimental settings (Figure 1E). To further address the biology of the interaction, we transiently transfected MCF-7 cells with plasmids encoding an eGFP-syntenin fusion protein, together or without expression plasmids encoding full-length human Fz 7. The subcellular localization of these proteins was examined by fluorescence microscopy. Fz 7, as detected by antibodies, showed predominant plasma membrane localization, and its distribution was not affected by eGFP–syntenin coexpression. eGFP-syntenin expressed in isolation was distributed all over the cell (Figure 2A), with only 20% of the cells showing some concentration at the plasma membrane (Figure 2D, left column). Coexpression of wild-type Fz 7 and eGFP-syntenin in the same cells resulted in a significant enrichment of eGFP-syntenin at the plasma membrane (Figure 2, B and D, compare middle and left columns). Coexpression of eGFP-syntenin with mutant Fz 7 T/A did not result in similar translocation of eGFP-syntenin to the plasma membrane (Figure 2, C and D, compare right and middle columns), consistent with the binding data represented in Figure 1B. Similar results were obtained

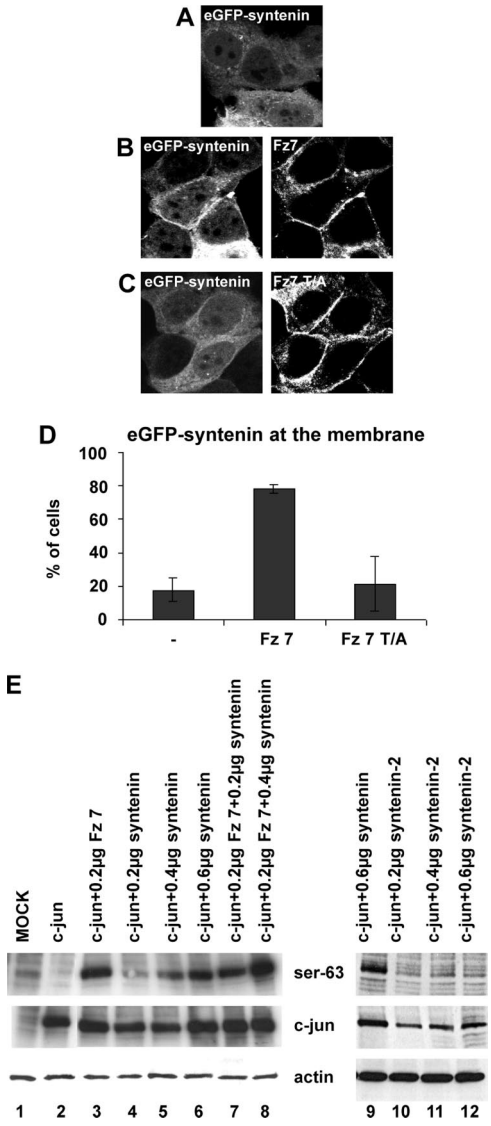

Figure 2. Syntenin is recruited by Fz 7 and stimulates c-jun phosphorylation. Confocal micrographs of MCF-7 cells overexpressing eGFP-syntenin alone (A), overexpressing eGFP-syntenin together with Fz 7 (B), or overexpressing eGFP-syntenin together with an Fz 7 carrying a mutated C-terminal PDZBM (T/A) (C). (D) Results are expressed as the mean percentage of cells where the syntenin fluorescence was concentrated at the plasma membrane; bars represent standard deviations. Note that the recruitment of eGFP-syntenin to the plasma membrane relies on Fz 7 and on the integrity of its C-terminal PDZBM. (E) Cell lysates originating from HEK293T cells transfected as indicated on top were tested for c-jun expression and c-jun phosphorylation (ser-63). Actin was used as a loading control. Note that syntenin addition stimulates c-jun phosphorylation in a concentration dependent manner.

in HUH-7 cells (data not shown). We concluded that syntenin–Fz 7 interaction can take place at the membrane, depending on the integrity of the Fz 7 C-terminal PDZBM.

To test for a role for syntenin in Wnt signaling, we used standard in vitro reporter assays (Billin *et al*., 2000; Yao *et al*., 2004). Overexpression of syntenin had no significant stimulatory or inhibitory effect in TOP/FOP flash experiments, neither in MCF-7 nor in Chinese hamster ovary-K1 cells (data not shown), suggesting syntenin has no role in the canonical Wnt pathway. To further investigate a potential

A

N-terminal domain

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Xsyntenin-a' 1  MSLYPSLEDLKVDKVIQAQTTFSANPVNPAILPAASAATAQYGGMYPKLYPELEQYMGLSLSEDEVHRNMSMVPSA...GNQVAVPTALNN.MVAPVSGNDBGIRRAEIK
Xsyntenin-a  1  MSLYPSLEDLKVDKVIQAQTTFSANPVNAAILPSASVSTAQYGGMYPKLYPELEQYMGLSLSEDEIHRNMSVVPSA...GNQVAVPTALNN.MVAPVSGSDVGIRRAEIK
Husyntenin   1  MSLYPSLEDLKVDKVIQAQTAFSANPANPAILSEASAPIPHDGNLYPRLYPELSQYMGLSLNEEEIRASVAVVSGAPLQGQLVARPSSINY.MVAPVTGNDVGIRRAEIK
Xsyntenin-b  1  MSIYPSLEDLKVDRVMQAQSTQMAQP..AAIMPSVPQSNP..G LYPNLS.ELSNYMGLSLTDEEIQLNMSLVPTG...GSEIAVPQALSGGLVAPVTGNDIGLRRAEIK
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PDZ1

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Xsyntenin-a' 108 QGIREVILCKDQDGKIGLRLKSIDNGIFVQLVQGNSPASLAGLKFGDQILQINGENCAGWSSDKSHKVLKQVSGERISMVVRDR
Xsyntenin-a  108 QGIREVILCKDQDGKIGLRLKSIDNGIFVQLVQVNSPASLAGLKFGDQILQINGENCAGWSSDKSHKMLKQVSGERISMVVRDR
Husyntenin   111 QGIREVILCKDQDGKIGLRLKSIDNGIFVQLVQANSPASLVGLRFGDQVLQINGENCAGWSSDKAHKVLKQAFGEKITMTIRDR
Xsyntenin-b  103 NGVREVILCKDQHGKVGLRLRAVDKGIFLQLVQANSPASLVGLRFGDQVLQIDGDSCAGWSTDRAHKALKKASQDRISLIVRDR
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PDZ2

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Xsyntenin-a' 192 PFERTITMHKDSTGHVGFIFKNGKITSIVKDSSAARNGLLTEHNLCEINGQNVIGLKDSQVAEILATSVNVVTLTVMPSYI
Xsyntenin-a  192 PFERTITMHKDSTGHVGFIFKNGKITSIVKDSSAARNGLLTDHNLCEINGQNVIGLKDSQVAEILATSANVVTLTVMPSFI
Husyntenin   195 PFERTITMHKDSTGHVGFIFKNGKITSIVKDSSAARNGLLTEHNICEINGQNVIGLKDSQIADILSTSGTVVTITIMPAFI
Xsyntenin-b  187 PFQRTITLQKDSTGHVGFIYKKGLITSLVKDGSAARNGLLTNHYLCEVNGQNVIGLKDHQVGDILASCGRTVTVTVIPNKI
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C-terminal domain

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Xsyntenin-a' 273 FDHMVKRMASSVLKSLMDHSVPEV*
Xsyntenin-a  273 FDHMVKRMASSVLKSLMDHSVPEV*
Husyntenin   276 FEHIIKRMAPSIMKSLMDHTIPEV*
Xsyntenin-b  268 YEHMVKRLSSGLLKNSMDHSVPEV*
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B

	N-terminal domain				PDZ1				PDZ2				C-terminal domain			
	Xa'	Xa	Hu	Xb	Xa'	Xa	Hu	Xb	Xa'	Xa	Hu	Xb	Xa'	Xa	Hu	Xb
Xsyntenin-a'	100	93	67	59	100	97	86	69	100	96	88	72	100	100	67	67
Xsyntenin-a		100	68	56		100	87	69		100	88	72		100	67	67
Husyntenin			100	51			100	71			100	69			100	46
Xsyntenin-b				100				100				100				100

Figure 3. (A) Sequence alignment of the human syntenin (Husyntenin) with the three orthologues found in *X. laevis* (Xsyntenin-a, -a′, and -b). (B) Percentage of identity between the different domains of the various syntenins. Note the extensive conservation of the PDZ domains.

role for syntenin–Fz interaction in Wnt signaling, we tested for JNK activation, because it has been established that this kinase is downstream of a noncanonical Wnt signaling cascade (Boutros *et al*., 1998). We therefore measured the phosphorylation of the JNK target c-jun upon syntenin overexpression. In HEK293T cells, syntenin stimulates c-jun phosphorylation, in a concentration dependent manner (Figure 2E, compare lanes 4–6 with lanes 1–2). Addition of exogenous Wnt was not necessary for the stimulatory effect of syntenin, possibly because sufficient endogenous Wnt was provided or indicating syntenin functions downstream of Wnt/Fz. Similarly, Dsh overexpression stimulated c-jun phosphorylation without exogenous Wnt addition (data not shown). As expected, overexpression of Fz 7 was also stimulatory (Figure 2E, compare lane 3 with lanes 1–2). Combination of Fz 7 and syntenin overexpression was slightly more potent than either of these alone (Figure 2E, lanes 7–8). When c-jun was phosphorylated, we also observed a slight increase in total c-jun signal, consistent with the increased stability of the phosphorylated form of the protein (Yao *et al*., 2004). As JNK activation is not specific for Fz-mediated signaling, we tested whether syntenin-2, which does not interact with Fz 7, would also be able to stimulate c-jun phosphorylation. This was not the case (Figure 2E, lanes 10–12). Based on these results, we hypothesized that syntenin-Fz 7 interaction might play a role in noncanonical Wnt signaling. We further investigated this hypothesis in *Xenopus laevis*.

Syntenin and Cognate Fz Are Coexpressed during Xenopus Early Development

We first examined when and where syntenin and Fz are coexpressed during *X. laevis* embryonic development. By in silico searches, we found three orthologues of syntenin in this organism (Figure 3). All three show higher homology to human syntenin than to human syntenin-2. These were designed as Xsyntenin-a, -a′, and -b. Xsyntenin-a and -a′ are 95.6% identical to one another, and they are more closely related to human syntenin than Xsyntenin-b. RT-PCR analysis, on RNA samples originating from embryos collected at different stages of development, identified a signal for Xsyntenin-a at all stages, albeit lower at stages 9–13 (Figure 4A). WISH for Xsyntenin-a, a′, and b showed that maternal transcripts localized to the animal pole at stage 3 (four-cell). Signals observed for Xsyntenin-a (Figure 4B) were stronger than those obtained for Xsyntenin-a′ and b (data not shown).

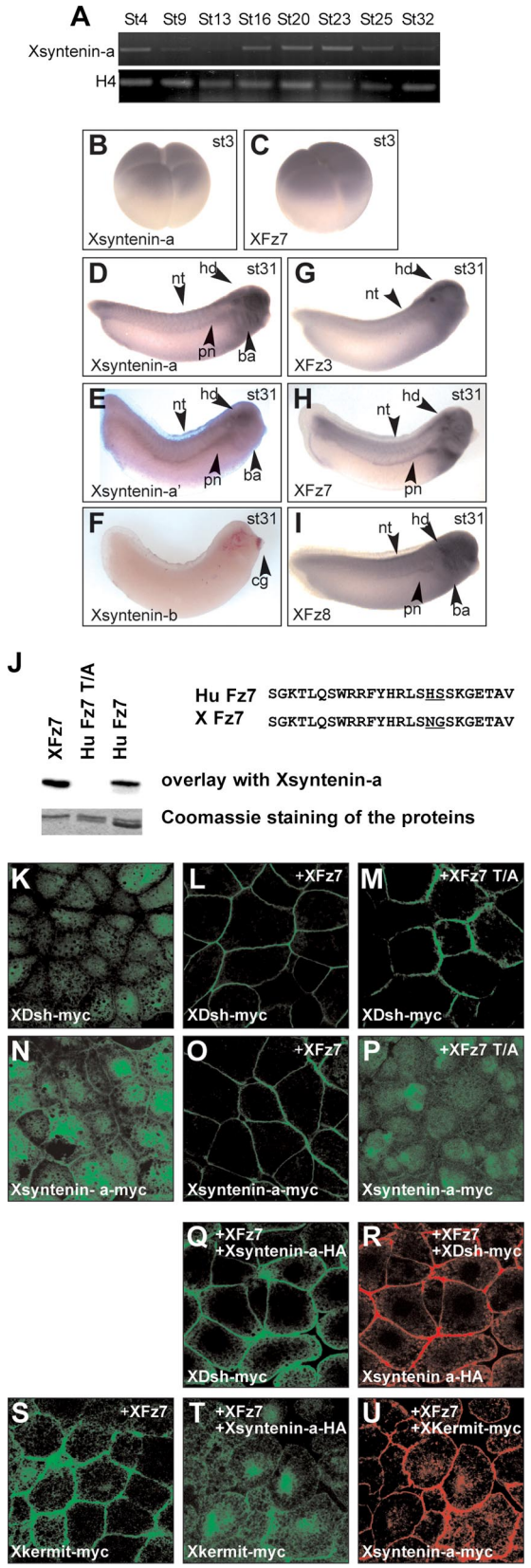

Figure 4. Xsyntenin expressions during *X. laevis* early development and Xsyntenin-a corecruitment with XDsh, by XFz 7, to the plasma membrane of animal caps. (A) RNA extracts from embryos at different stages were used to analyze Xsyntenin-a expression by RT-PCR. Histone H4 amplification was used as an internal control. (B–I) Xsyntenin-a, -a', and b and XFz 3, 7, and 8 mRNA distributions (purple) at different stages (st) of development. Hd, head; nt, neural tube; pn, pronephros; ba; branchial arches; cg, cement gland. (J) Syntenin-Fz 7 interaction is conserved in *Xenopus*. Hu Fz7 and XFz7 cytoplasmic domain sequences are shown on the right. The sequences vary for two amino acids (underlined). Fz 7 cytoplasmic domain from *Xenopus* or human, mutated (T/A) or not mutated in the PDZBM, was overlayed with Xsyntenin-a (top). The quality and concentration of the fusion proteins were controlled in Coomassie (bottom). (K–U) Confocal micrographs of *Xenopus* animal caps at stage 9 showing the subcellular distribution of XDsh-myc (K–M and Q), Xkermit-myc (S and T), Xsyntenin-a-myc (N–P) or Xsyntenin-a-HA (R and U) as indicated at the bottom. The caps originate from embryos injected at two-cell stage with different combinations of mRNAs encoding proteins indicated on each micrograph. Note the translocation of XDsh to the plasma membrane upon XFz 7 and XFz 7 T/A expression (compare M and L with K) and the translocation of Xsyntenin-a upon XFz 7 but not XFz7 T/A expression (compare O and P with N). Note also that although Xsyntenin-a impairs Xkermit translocation (T), XDsh translocation is maintained (Q).

During neurula to early tadpole stages, Xsyntenin-a transcripts were found in the neural tube, head, neural crest, and the pronephros region (Figure 4D). A similar distribution was observed for Xsyntenin-a' (Figure 4E). Xsyntenin-b expression was restricted to the cement gland and the epidermis (Figure 4F).

XFz 7 mRNA is also provided maternally, and it localizes in the animal region of early cleavage stage embryos (Medina *et al*., 2000) like Xsyntenin transcripts (Figure 4, compare B and C). During neurula to early tadpole stages, Xsyntenin-a and a' overlap with XFz 7 (Medina *et al*., 2000) in the neural tube, head, neural crest, and the pronephros region (Figure 4H). Xsyntenin-a and a' expression patterns also overlap with that of XFz 3 in developing nervous system and XFz 8 in the pronephros (Figure 4, G and I). Thus, overlapping RNA expression patterns suggested multiple opportunities for syntenin-Fz interaction during development.

XFz 7 Corecruits Xsyntenin-a and XDsh at the Membrane in X. laevis Animal Caps

After controlling that the Fz 7–syntenin interaction is conserved in *Xenopus* (Figure 4J), we tested for a functional relationship between XFz 7, Xsyntenin-a and XDsh in animal cap recruitment assays. These assays were previously implemented by other groups, studying Wnt signaling components that directly interact with Fz, such as XDsh and Xkermit (Umbhauer *et al*., 2000; Tan *et al*., 2001). Overexpressed XDsh distributes diffusely in animal cap cells in the absence of exogenous XFz 7 (Figure 4K), but it translocates to the plasma membrane upon simultaneous XFz 7 overexpression (Figure 4L). We found that overexpression of XFz 7 mutant for the C-terminal PDZBM induces similar XDsh translocation (Figure 4M). Overexpressed Xsyntenin-a, by itself, mainly distributes to both the cell interior and the nucleus, with a small proportion at the plasma membrane (Figure 4N). On XFz 7 overexpression, most Xsyntenin-a translocates to the plasma membrane (Figure 4O). Syntenin translocation was not observed with an XFz 7 mutant for its C-terminal PDZBM (Figure 4, compare P and O), contrary to Dsh and consistent with the in vitro mode of binding. Next, we found that the membrane translocation of XDsh is largely unaffected by Xsyntenin-a, even after injection of a fivefold excess of RNA encoding Xsyntenin-a (Figure 4, compare Q and L). Xsyntenin-a is also not displaced from the

membrane upon XDsh expression (Figure 4R). In contrast, Xsyntenin-a, when used in excess, markedly influenced the XFz 7-mediated membrane recruitment of Xkermit (Figure 4, S and T), an alternative C-terminal PDZ partner of XFz 7 (Tan *et al*., 2001). We concluded that Fz 7 overexpression can induce the translocation of syntenin at the membrane of *Xenopus* animal caps without compromising the Dsh translocation.

Xsyntenin Overexpression or Down-Regulation Disrupts Convergent Extension Movements

Functional studies in *Xenopus* have revealed that noncanonical Wnt signaling and XFz 7 are required for convergence extension movements (CEs) during gastrulation (Djiane *et al*., 2000; Sumanas and Ekker, 2001). During CE, mesoderm and ectoderm cells intercalate along the medio-lateral axis, narrowing the tissues (convergence) and extending these along the anterior-posterior axis (extension). Because of the evidence for a role of syntenin in noncanonical (Figure 2G), rather than in canonical Wnt signaling, we tested for a role of syntenin in CE. For that, we first used activin-induced animal caps (Figure 5, A–D, compare left and right), a common ex vivo model for studying CE (Tada and Smith, 2000). It has previously been established that the overexpression and/or down-regulation of noncanonical Wnt signaling

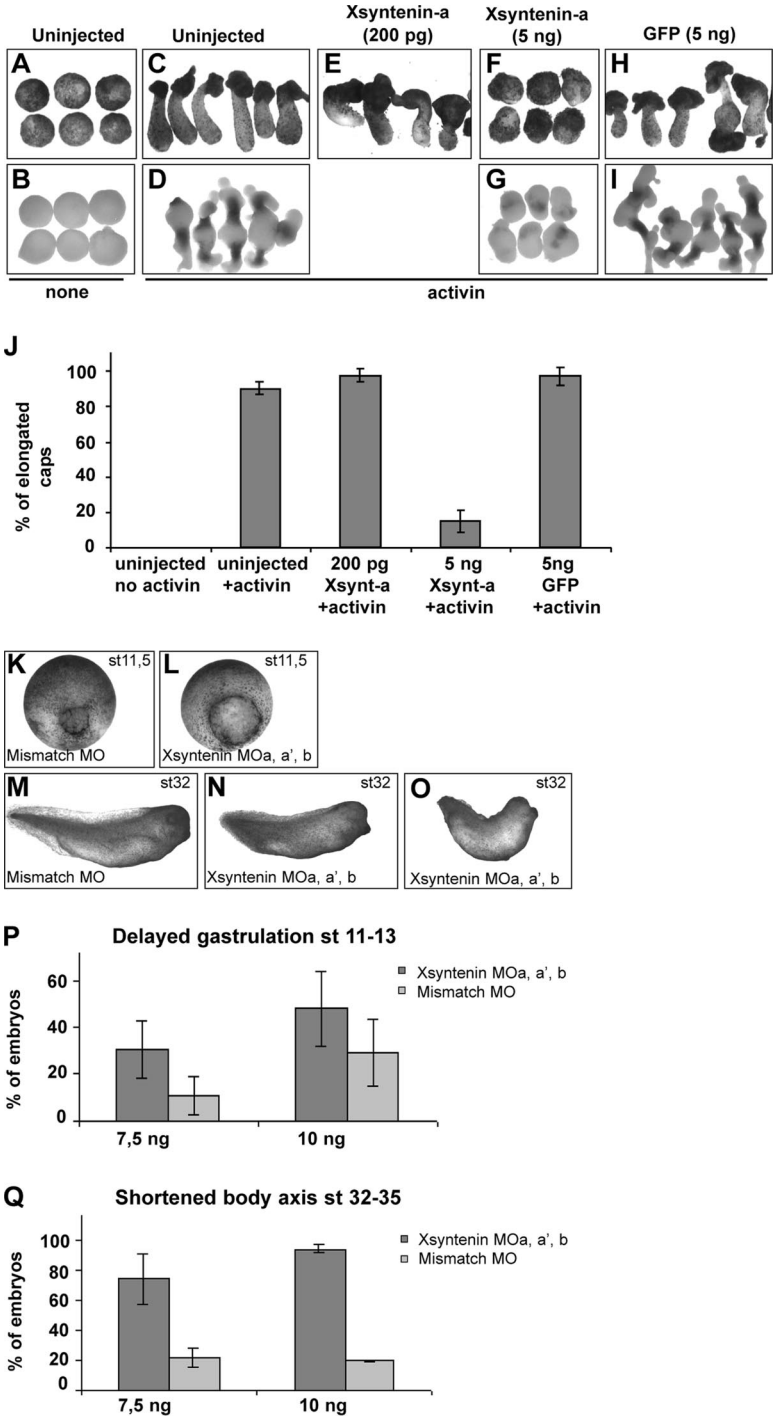

Figure 5. Xsyntenin overexpression and down-regulation block the elongation of activin-treated animal caps and of whole embryos. (A–I) Pictures of stage 19 animal cap explants, originating from embryos injected at two cell stage with different mRNAs as indicated on top, dissected at stage 9, and cultured in the absence or in the presence of 50 ng/ml activin (as indicated at the bottom). In situ hybridization with the mesodermal marker cardiac actin (B, D, G, and I) shows that the mesodermal differentiation is sustained in all activin-treated caps. Note the specific block of elongation induced by high dose of X-syntenin-a mRNA (F–G). (J) Results are expressed as the mean percentage of elongated caps per condition, bars represent standard deviations. (K–Q) Illustration of selected anomalies in *X. laevis* embryos depleted for Xsyntenin-a, a', and b. Embryos injected with Xsyntenin MOs show a delayed gastrulation (compare L with K), and they have a shorter body-axis (compare N–O with M). Injections were performed at the four-cell stage with 7, 5, or 10 ng of each Xsyntenin MO or with 22 or 30 ng mismatch MO in each blastomere. Embryos with body-axis length <60% (O) and embryos with body-axis between 60 and 80% (N) of the average length of noninjected controls were pooled for quantitative analysis (Q). The percentage of embryos showing delayed gastrulation (P) and shortened body-axis (Q) was scored in three independent experiments, with at least 80 embryos. Results are expressed as the mean percentage of embryos showing the defects at the stages indicated; bars represent standard deviations.

components such as XFz 7, XWnt 11, Strabismus, Crescent, and Syndecan-4 block CE in this assay (Djiane *et al*., 2000; Sumanas and Ekker, 2001; Darken *et al*., 2002; Shibata *et al*., 2005; Munoz *et al*., 2006). Animal hemisphere injection of a low dose (200 pg) of Xsyntenin-a mRNA did not affect cap elongation (Figure 5, E and J). However, a high dose (5 ng) of Xsyntenin-a mRNA efficiently blocked this elongation (Figure 5, F and J). To exclude that the block of elongation was due to a to a lack of mesoderm induction, we tested for the expression of the mesodermal marker cardiac actin. The expression of this marker was preserved (Figure 5G), indicating CE defects. The block of elongation was not observed when injecting a similar, high dose of GFP mRNA (Figure 5, H–J), confirming the specificity of the effect. Injection of a high dose of Xsyntenin-a mRNA also severely blocked the elongation of the body-axis in total embryos, further establishing a role for syntenin in CE (data not shown). MOs directed against Xsyntenin-a, Xsyntenin-a′, or Xsyntenin-b, injected together or in isolation, did not affect the elongation of activin-treated animal caps (data not shown). Yet, when testing these MOs together on total embryo development, we observed delayed gastrulation and shortening of the trunk (Figure 5, K–Q), hallmarks of CE problems. Together, these observations indicate a role for syntenin in CE and thereby support a role for syntenin in noncanonical Wnt signaling.

Xsyntenin Acts as an Activator of XFz 7 Signaling to XCDC42 and Stimulates XPKCα Membrane Recruitment

To test for the role of syntenin–Fz 7 interaction in CE, we analyzed the effect of injecting Xsyntenin RNA, together with XFz 7 MOs, on activin-induced elongation of *Xenopus* animal caps. Down-regulation of XFz 7 blocks CE in this assay (Sumanas and Ekker, 2001). In case syntenin supports noncanonical Wnt signaling by interacting with Fz 7, we assumed Xsyntenin-a overexpression should be able to rescue partial XFz 7 loss-of-function. Five nanograms of XFz 7 MO was the lowest dose required for an efficient block of elongation (Figure 6, A and B). This block was rescued by coinjection of a low dose of Xsyntenin-a RNA (200 pg, a dose having no effect on CE as shown in Figure 5E) but not by coinjection of 200 pg of control GFP RNA (Figure 6, C and D). This low dose of Xsyntenin-a RNA was also sufficient to rescue the effect of XFz 7 down-regulation in whole embryos (data not shown), indicating syntenin is a downstream mediator of XFz 7 signaling.

To situate syntenin in the Fz 7 noncanonical Wnt signaling, we investigated a relationship with the CDC42 signaling

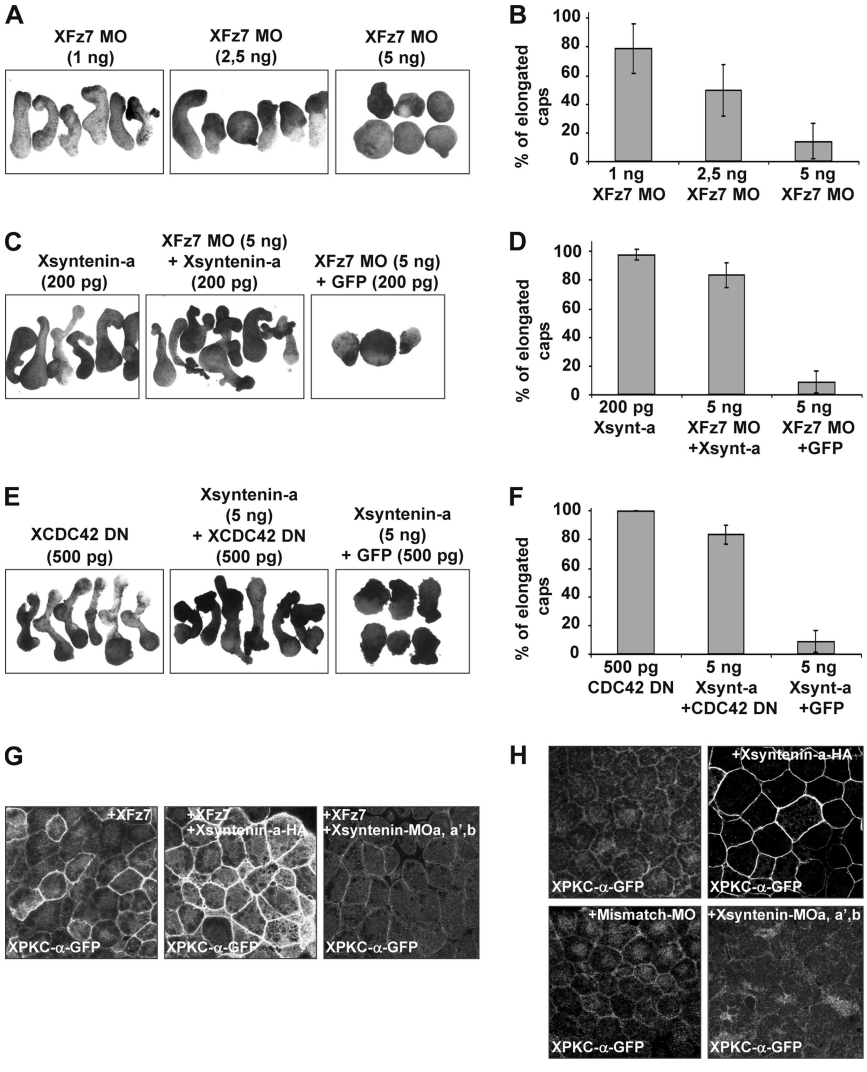

Figure 6. Xsyntenin supports the XFz 7/XPKCα/XCDC42 branch of the noncanonical Wnt signaling pathway. (A, C, and E) Pictures of stage 19 animal cap explants originating from embryos injected at two-cell stage with different components as indicated on top, dissected at stage 9, and cultured in the presence of 50 ng/ml activin. Note that the block of elongation observed with MOs for XFz 7 (A, right image) is rescued by the coinjection of a low dose of Xsyntenin-a RNA (C, middle picture). Note that the block of elongation induced by a high dose of Xsyntenin-a RNA is rescued by the coinjection of RNA encoding a DN form of the small GTPase XCDC42 (compare E, middle, with Figure 5F). (B, D, and F) Results are expressed as the mean percentage of elongated caps per condition; bars represent standard deviations. (G and H) Confocal micrographs of *Xenopus* animal caps at stage 9 showing the subcellular distribution of XPKCα-GFP as indicated at the bottom. (G) Comparison of the XPKCα-GFP distribution upon the overexpression of XFz 7 alone (left), together with Xsyntenin-a-HA (middle), or together with X-syntenin MOs (right). (H) Comparison of the XPKCα-GFP distribution upon the overexpression of Xsyntenin-a-HA (top) or the down-regulation of X-syntenins (bottom). Note that Xsyntenin enhances the plasma membrane recruitment of XPKCα-GFP.

cascade. Djiane *et al*. (2000) showed that the block of CE induced by overexpression of XFz 7 in activin-treated animal caps can be rescued by coinjecting RNA encoding a dominant-negative (DN) XCDC42. Similarly, DN XCDC42 was able to rescue the block of CE induced by a high dose of Xsyntenin-a RNA (compare Figure 5F with Figure 6E, middle), whereas it had no effect on CE by itself (Figure 6E, left). A control experiment using GFP RNA (Figure 6E, right) underscored the specificity of the DN CDC42 effect. This indicates that syntenin overexpression might act through the CDC42 pathway. To further establish a role for syntenin in the CDC42 noncanonical Wnt signaling cascade, we investigated the relationship of syntenin with PKCα. Indeed, Choi and Han (2002) showed that CDC42 is downstream of PKCα activation in noncanonical Wnt signaling. Overexpression of XFz 7 in animal caps induces membrane recruitment of XPKCα (Medina *et al*., 2000). We found that Xsyntenin-a-HA overexpression increases the membrane recruitment of XPKCα-GFP by XFz 7, whereas Xsyntenin down-regulation decreases this recruitment (Figure 6G). Surprisingly, Xsyntenin-a-HA overexpression alone was sufficient to induce translocation of XPKCα-GFP to the plasma membrane (Figure 6H, top). Consistently, in complementary experiments, Xsyntenin MOs decreased the discrete membrane localization of XPKCα-GFP (Figure 6H, bottom).

Together, these data suggest that syntenin works as an activator of Fz 7 noncanonical Wnt signaling upstream of PKCα and CDC42.

DISCUSSION

Here, we show that syntenin directly interacts with Fz 3, 7, and 8 (Figure 1B) and that it is coexpressed with cognate Fz during *Xenopus* early development (Figure 4, B–I). Syntenin seems to leave canonical Wnt signaling unaffected, because its overexpression had no effect on TOP/FOP flash reporter assays in cells, or on secondary axis formation in *Xenopus* (data not shown). Yet, syntenin overexpression or down-regulation both elicit a shortened trunk (Figure 5, M–O and Q). Such phenotype was also observed upon overexpression or down-regulation of components known to regulate noncanonical Wnt signaling, such as Fz 7 and strabismus (Djiane *et al*., 2000; Medina *et al*., 2000; Sumanas and Ekker, 2001; Darken *et al*., 2002). Moreover, syntenin overexpression increases c-jun phosphorylation in cultured cells (Figure 2E), and it blocks the activin-supported elongation of animal caps at high dose (Figure 5, F, G, and J), two additional pieces of evidence for a role in noncanonical Wnt signaling. At low dose, syntenin can rescue the Fz 7 down-regulation phenotype in activin-induced animal caps (Figure 6, C and D) and in whole embryos (data not shown), supporting a stimulating role for syntenin in noncanonical Wnt signaling. Consistently, syntenin recruits PKCα in animal caps (Figure 6H) as do Fz 7 and Wnt5a (Medina *et al*., 2000). Moreover, a DN CDC42 rescues the inhibitory effects of syntenin overexpression in animal caps (Figure 6, E and F), as it does for the inhibitory effects of the overexpression of noncanonical components such as Wnt11 and Fz 7 (Djiane *et al*., 2000). This indicates that syntenin functions downstream of Fz and upstream of the PKCα/CDC42 cascade (Choi and Han, 2002; Penzo-Mendez *et al*., 2003).

By what direct mechanisms could syntenin operate to stimulate Fz 7-dependent noncanonical Wnt signaling? Mutational analysis shows that the syntenin–Fz 7 interaction requires the C-terminal PDZBM of Fz 7 (Figure 1B) and that this motif must be intact to recruit syntenin to the membrane in cells (Figure 2, A–C) and in *Xenopus* animal caps (Figure 4, N–P). This leaves the membrane proximal PDZBM available for Dsh interaction. Consistently, syntenin does not affect the membrane recruitment of Dsh by Fz 7 (Figure 4, Q–R). Syntenin overexpression or down-regulation shows no significant effect on Dsh membrane localization in animal caps (data not shown) in contrast to what it does to PKCα (Figure 6H). This means that the stimulation of noncanonical Wnt signaling by syntenin might be Dsh-independent. This is reminiscent of what has been reported by Winklbauer *et al*. (2001), where Fz 7 signals through trimeric G proteins and PKCα independently from Dsh to control cell-sorting behavior in the mesoderm. Domain mapping shows that the syntenin–Fz 7 interaction requires the PDZ1 domain of syntenin (Figure 1, C and D). Because syndecans interact preferentially with the PDZ2 domain of syntenin (Grootjans *et al*., 2000), one could envision that syntenin serves as a scaffold bridging syndecan-signaling complexes to Fz (Figure 7). Syntenin can be recruited to the plasma membrane by syndecans (Zimmermann *et al*., 2002) and by Fz 7 (Figure 2B). In addition, syndecan, Fz 7 and syntenin largely colocalize in clusters at the membrane when coexpressed in cultured cells, suggesting they might form one functional unit (data not shown). Remarkably, the syntenin expression pattern in *Xenopus* early embryos overlaps with that reported for syndecan-4 (Munoz *et al*., 2006). Syndecan-4 can bind and activate PKCα (Oh *et al*., 1998; Lim *et al*., 2003). Syntenin might

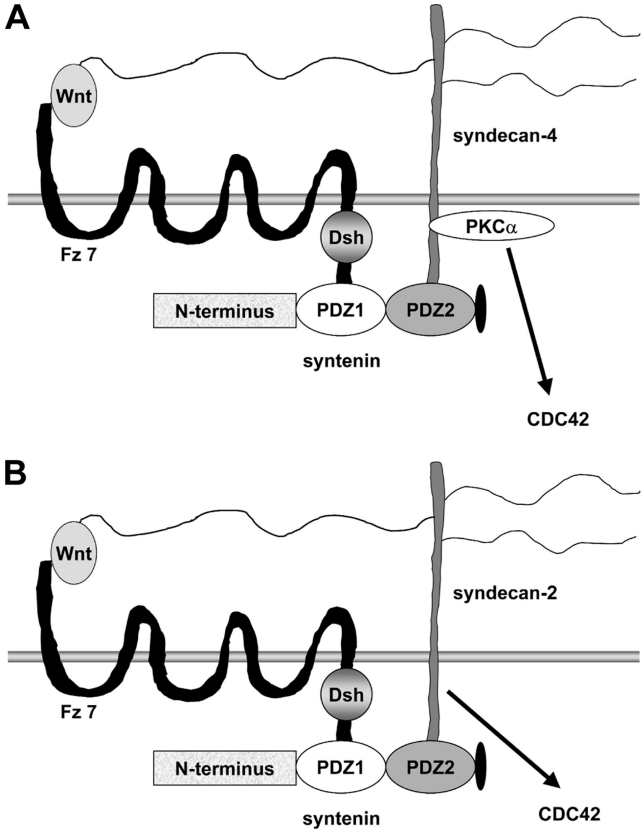

Figure 7. Model for the role of syntenin scaffolding in noncanonical Wnt signaling. Syntenin interacts with Fz 7 via its PDZ1 domain and with Syndecan-4 (A) or Syndecan-2 (B) via its PDZ2 domain. Syndecan and/or Fz 7 can recruit syntenin to the plasma membrane. (A) Syndecan-4 interacts directly with PKCα. Syndecan-4 triggers the activation of PKCα. PKCα functions upstream of CDC42 in noncanonical Wnt signaling. (B) Syndecan-2 might also activate CDC42. See *Discussion* for details and references.

serve as a scaffold for Fz 7-syndecan-4-PKCα complexes, and this might explain how it stimulates noncanonical Wnt signaling (Figure 7A). Interestingly, Munoz *et al*. (2006) recently showed that syndecan-4 is required for noncanonical Wnt signaling in *Xenopus* and that syndecan-4 interacts, functionally and physically, with Fz 7 and Dsh (Munoz *et al*., 2006). Although we do not have evidence for the importance of Dsh in Fz 7-syntenin signaling (see above), we cannot exclude it. Regardless, our in vitro findings show syntenin can provide a direct link between Fz 7 and syndecan. Additionally, syntenin effects might take place via syndecan-2, a syndecan that activates CDC42 (Granes *et al*., 1999) (Figure 7B). We could not identify a clear increase in CDC42-GTP content upon syntenin overexpression in MCF-7 cells (data not shown). Yet, syntenin might be important for the appropriate targeting of CDC42 to Fz signaling complexes rather than for increasing CDC42-GTP steady-state levels.

Syntenin seems to be indispensable in vertebrates, because its down-regulation interferes with *Xenopus* (Figure 5, K–Q) and zebrafish (Lambaerts, K., Mortier, E., Degeest, G., Luyten, A., and Zimmerman, P., unpublished data) early development. Yet, it is not essential for all aspects of noncanonical Wnt/Fz signaling, because *Drosophila melanogaster* and *Caenorhabditis elegans* lack a syntenin orthologue in their genome. Interestingly, it was shown that syntenin is overexpressed in several human cancer cell lines (Koo *et al*., 2002) and that it is a positive regulator of metastasis by promoting cell invasion and migration, in particular in melanoma cells (Koo *et al*., 2002; Boukerche *et al*., 2005). For this process, the PDZ domains of syntenin seem essential (Koo *et al*., 2002; Meerschaert *et al*., 2007). Although the canonical Wnt pathway plays a role in carcinogenesis (Polakis, 2000), more recently the noncanonical Wnt pathway has been implicated in metastasis (Weeraratna *et al*., 2002; Katoh, 2005; Dissanayake *et al*., 2007). Aberrant regulation of Wnt5a/PKCα activation has also been implicated in melanoma metastasis (Weeraratna *et al*., 2002; Dissanayake *et al*., 2007). Syntenin PDZ domains might thus represent an attractive drug target for cancer therapy as it has been proposed for Dsh (Shan *et al*., 2005; Fujii *et al*., 2007).

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