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Profilin is an effector for Daam1 in non-canonical Wnt signaling and is required for vertebrate gastrulation

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Non-canonical Wnt signaling plays important roles during vertebrate embryogenesis and is required for cell motility during gastrulation. However, the molecular mechanisms of how Wnt signaling regulates modification of the actin cytoskeleton remain incompletely understood. We had previously identified the Formin homology protein Daam1 as an important link between Dishevelled and the Rho GTPase for cytoskeletal modulation. Here, we report that Profilin1 is an effector downstream of Daam1 required for cytoskeletal changes. Profilin1 interacted with the FH1 domain of Daam1 and was localized with Daam1 to actin stress fibers in response to Wnt signaling in mammalian cells. In addition, depletion of Profilin1 inhibited stress fiber formation induced by non-canonical Wnt signaling. Inhibition or depletion of Profilin1 in vivo specifically inhibited blastopore closure in *Xenopus* but did not affect convergent extension movements, tissue separation or neural fold closure. Our studies define a molecular pathway downstream of Daam1 that controls Wnt-mediated cytoskeletal reorganization for a specific morphogenetic process during vertebrate gastrulation.

KEY WORDS: Profilin, *Xenopus*, Daam1

INTRODUCTION

Establishment of the vertebrate body plan depends on cell movements during gastrulation. This process generates the extended axis of the embryo through directed cell migration, termed 'convergent extension movements' during which cells polarize, elongate, align and intercalate, resulting in mediolateral narrowing (convergence) and anteroposterior lengthening (extension) of the axis (Keller, 2002). The directionality of these cell movements results from stabilization of protrusions (lamellopodia) on the mediolateral surfaces (Wallingford et al., 2000). Blastopore closure occurs as a result of the sequential circumferential shortening that is mediated by a 'purse string' mechanism that constricts the circumference of the blastopore (Keller et al., 2003). In subsequent primary neurulation the lateral edges of the neural plate elevate until they appose at the midline and fuse to form the neural tube; convergent extension movements provide the force for this process (Wallingford and Harland, 2002). The molecular mechanisms that control convergent extension movements, blastopore closure and neural tube folding and closure are incompletely understood, but modification of the cytoskeleton via the non-canonical Wnt signaling pathway is required for these processes (Keller, 2002; Wallingford et al., 2002).

The non-canonical Wnt signaling pathway, also termed the β-catenin independent pathway or the planar cell polarity (PCP) pathway, regulates gastrulation cell movements among other processes, and is mediated by the PDZ and DEP domains of Dishevelled (Dvl) (Wallingford and Habas, 2005). This pathway regulates cell movements through modification of the actin cytoskeleton and appears to be independent of transcription (Veeman et al., 2003; Wallingford and Habas, 2005). The specificity of Wnt ligands for distinct branches of the pathway remains poorly deciphered but Wnt5a was first identified as a regulator of gastrulation (Moon et al., 1997). However, Wnt5a can also activate canonical signaling (He et al., 1997; Mikels and Nusse, 2006). Likewise, Wnt3a can activate both canonical and non-canonical signaling (Cadigan and Liu, 2006). Additional known components of the non-canonical pathway include Wnt11, Fz, Dvl, Daam1, Rho, Rac, Jun kinase (JNK), Strabismus and Prickle (Habas and Dawid, 2005; Wallingford and Habas, 2005). In this pathway the Wnt signal is mediated through Fz independently from LRP5/6 (He et al., 2004) and the pathway bifurcates downstream of Dvl into two parallel branches that lead to the activation of the small GTPases Rho and Rac (Habas et al., 2003; Habas et al., 2001; Tahinci and Symes, 2003). Signaling to Rho involves Daam1, which binds to the PDZ domain of Dvl (Habas et al., 2001), leads to the activation of the Rho associated kinase Rock, and mediates cytoskeletal reorganization (Marlow et al., 2002; Veeman et al., 2003; Wallingford et al., 2002). The second branch requires the DEP domain of Dvl but not Daam1 and activates the Rho family GTPase, Rac, which in turn stimulates JNK activity (Habas et al., 2003; Li et al., 1999; Yamanaka et al., 2002).

Daam1 is a member of the Formin family of proteins that are central players in cytoskeletal reorganization (Alberts, 2002; Wallar and Alberts, 2003). Formin proteins contain three major domains termed the GTPase binding domain (GBD), Formin Homology 1 (FH1) and Formin Homology 2 (FH2) domains (Alberts, 2002). It is proposed that Formin proteins exist in the cytoplasm in an auto-inhibited state, which is mediated by binding of the C-terminal Diaphanous auto-inhibitory domain (DAD) to the amino terminus (Alberts, 2002; Higgs, 2005). It has been proposed that activated Rho-GTP binds to the GBD domain, releasing the protein from auto-inhibition, followed by binding of the FH1 and FH2 domains to effectors to elicit cytoskeletal changes. The FH2 domain can polymerize actin filaments and serves an actin nucleation function (Higgs, 2005; Kovar et al., 2006; Kovar and Pollard, 2004). One

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molecule known to bind the FH1 domain of the Formin proteins is Profilin, which binds the Formin mDia1 (Evangelista et al., 2002; Frazier and Field, 1997; Severson et al., 2002).

Profilin is an evolutionarily conserved actin binding protein that is involved in actin polymerization (Watanabe and Higashida, 2004; Witke, 2004). Genetic studies in *Drosophila* have uncovered roles for Profilin1 in oogenesis, spermatogenesis, bristle and eye formation (Cooley et al., 1992; Verheyen and Cooley, 1994) and in neuronal cells for axonal guidance and dendritic spine morphology (Witke, 2004). Among the three mammalian profilins, Profilin1 is essential for cytokinesis and mouse knockout mutants die by the two- to eight-cell stage (Witke et al., 2001; Witke, 2004). Profilin1 interacts with the FH1 domain of Formin proteins and serves an actin monomer delivery and capping function (Higgs, 2005; Kovar and Pollard, 2004; Zigmond, 2004). Whether this Profilin-Formin interaction is required for a morphogenetic process in vivo remains unknown. To date, no signaling pathway is known to require Profilin1, and the function of Profilin1 during embryogenesis remains poorly defined.

Here we report the identification of Profilin1 as an interacting partner of Daam1 and a functional component of the non-canonical Wnt signaling pathway. Profilin1 binds to the FH1 domain of Daam1 and colocalizes with Daam1 to actin stress fibers in response to Wnt stimulation. Depletion of Profilin1 inhibits Wnt- and Daam1-mediated stress fiber formation. *Xenopus* Profilin1 is expressed in the embryo at a time and place consistent with a role in gastrulation. Overexpression or depletion of Profilin1 results in inhibition of blastopore closure but convergent extension, tissue separation and neural fold closure are unaffected. Furthermore, Profilin1 has no role in canonical Wnt signaling and mesoderm specification. Together, our studies reveal a branch point in the non-canonical Wnt pathway that controls a specific aspect of vertebrate gastrulation.

MATERIALS AND METHODS

Materials

Monoclonal antibodies (mAbs) against HA (F-7), RhoA (26C4), Dvl2 (10B5), Myc (9E10), and polyclonal Abs (pAbs) against RhoA (CAT119) and Myc (N-262) were from Santa Cruz Biotechnology. mAbs against Rac and Cdc42 were from Transduction Laboratories, and against Flag (M2) was from Sigma. Alexa Fluor anti-mouse and anti-rabbit Abs, Texas Red X-Phalloidin and Oregon Green-Phalloidin were from Molecular Probes (Eugene, OR). Anti-β-catenin antibody was from Transduction Laboratories (San Diego, CA).

Plasmids and oligonucleotides

The human Daam1 and fragments of Daam1 were generated by restriction digestion or a PCR approach, and subcloned in pCS2+MT (for the Myc tag at the N terminus) or pcDNA-HA (for the HA tag at the amino terminus), or pCS2+GFP vector (kindly provided by Dr Jeffrey Miller, University of Minnesota). Rat Profilin1 (isolated from our screen) and *Xenopus* Profilin1 (isolated by a PCR approach from a *Xenopus* Stage 10.5 cDNA library) were cloned into pCS2+MT or pCS2+GFP. Details of plasmids are available upon request.

The dsRNAi oligonucleotides for Profilin1 or control GFP oligonucleotides were synthesized using the Dicer Kit (Ambion) and purified following the manufacturer's instructions.

The XProfilin1 Morpholino oligonucleotide (MO) complementary to the translational initiation site, 5′-TGTAGCCGTTCCAAGACATTGTTGT-3′, was synthesized by Gene Tools. A MO with a random sequence was used as the negative control.

Yeast two-hybrid screen

A rat brain cDNA library (Clonetech) was screened using the c-Daam1 fragment of Daam1 (Fig. 1A) as the bait. 3.9 million independent clones were screened, and 12 overlapping Profilin1 fragments, in addition to other positives, were obtained.

Transfections

All were carried out with HEK293T cells or NIH3T3 cells. Cells in a six-well plate were transfected using the calcium-phosphate method, Polyfect reagent (Qiagen), or Dicer transfection reagent (for RNAi experiments) with 1-2 μg of each indicated plasmid or 500 ng-1 μg annealed RNAi oligo plus 1 μg plasmid. Transfected DNA amounts were equalized via vectors without inserts.

Wnt conditioned media

Wnt3a-transfected, Wnt5a-transfected or control L cells were obtained from ATCC and cultured according to the suppliers instructions. Serum-free Wnt3a, Wnt5a and L cell condition medium were prepared according to the manufacturers instructions. Purified Wnt3a protein was purchased from R&D systems and used at a concentration of 250 ng/ml.

Antibody generation

Anti-Daam1 antibodies were generated in rabbits against a GST fusion protein containing amino acids 967-1078 of human Daam1. The Daam1 specific antibody was affinity purified using the GST-Daam1 fusion protein by standard methods.

Immunocytochemistry

This was carried out as described previously (Capelluto et al., 2002; Habas et al., 2001). Images were obtained using an Olympus IX70 fluorescent microscope with 100X objective lens (Melville, NY) or a Zeiss Axiovert 100 microscope (Oberkochen, Germany). For quantification of localization of Daam1 or Profilin1 to stress fibers, a base line of 10 stress fibers per cell was used and the merged image of Daam1 or Profilin1 onto these fibers was counted as a positive. For quantification of the effects of depletion of Profilin1 and Wnt- and Daam1 mediated stress fiber induction, a base line of 10 stress fibers per cell was used to score, thus any cell containing more than or less than10 fibers was scored as an increase or decrease respectively. These experiments were repeated at least three times and scoring was done in a blind manner so that the scorer had no knowledge of the sample being scored.

Embryo manipulations, RT-PCR, in situ hybridization and explant assays

These were performed as described (Habas et al., 2003; Habas et al., 2001; Kato et al., 2002). Embryo injections were done with in vitro transcribed RNAs. Convergent extension assays in explants were performed as described (Habas et al., 2003) using 5 ng/ml activin. Keller explant assays were performed as described (Shih and Keller, 1992). Tissue separation assays were performed as described (Hukriede et al., 2003).

RESULTS

Identification of Profilin1 as a binding partner for Daam1

We had shown that a fragment of Daam1 termed C-Daam1 can induce Rho activation and cytoskeletal changes (Habas et al., 2001) (Fig. 1A). In order to identify downstream effectors for Daam1 for these processes, we performed a yeast two hybrid screen using C-Daam1 as bait. From this screen, we isolated 12 overlapping clones of Profilin1 as a C-Daam1-interacting protein.

To delineate interactions between Profilin1 and Daam1 outside of yeast, we examined Profilin1 interaction with Daam1 by co-immunoprecipitation using epitope-tagged wild type and mutant proteins expressed in mammalian HEK293T cells (Fig. 1A). We found that Profilin1 binds to full length Daam1 and C-Daam1, which contains the FH1 and FH2 domains, but not to N-Daam1, which contains the amino-terminal domain (Fig. 1B). Using smaller fragments of C-Daam1 harboring the FH1 or FH2 domains separately (Fig. 1A), we localized the Profilin1 interacting domain to the FH1-containing fragment of Daam1 but not to the FH2-containing fragment (Fig. 1B).

We next examined whether endogenous Profilin1 interacts with epitope-tagged Daam1. For these experiments, we utilized a commercial Profilin1 antibody (Cytoskeletal Labs) that is functional

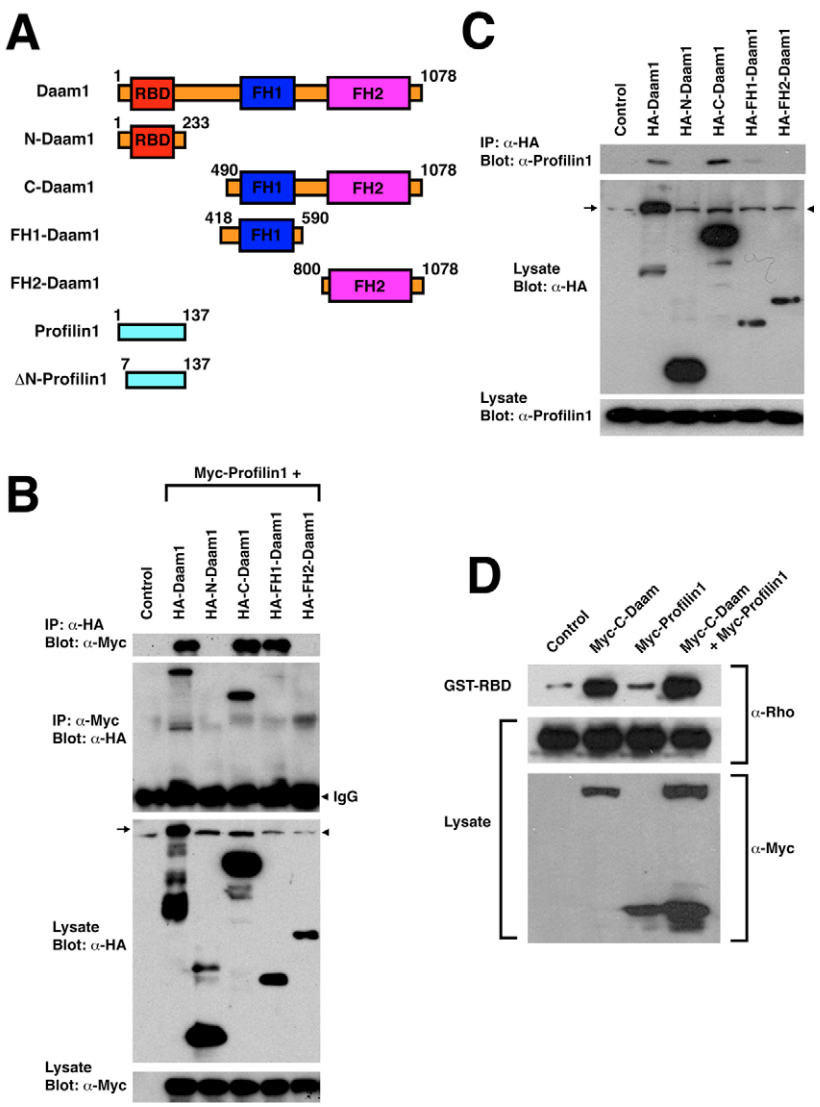

Fig. 1. Profilin binds to the FH1 domain of Daam1. (**A**) Schematic diagram of the Daam1 and Profilin1 constructs showing domains, with numbers indicating amino acid positions. (**B**,**C**) Co-immunoprecipitation experiments. Plasmids encoding tagged-Daam1 or Daam1 fragments and Profilin1 were cotransfected into HEK293T cells, and lysates were immunoprecipitated (IP) with indicated Abs. Precipitates were immunoblotted with indicated Abs. (B) Profilin1 interacts with Daam1, C-Daam1 and the FH1 domain of Daam1 but not N-Daam1 or the FH2 domain of Daam1. Note the tagged FH1 domain migrates overlapping the IgG band and is not resolved and a nonspecific band comigrates at the same position as HA-Daam1 (arrowhead). (C) Endogenous Profilin1 is precipitated with epitope tagged-Daam1, C-Daam1 or the FH1 domain of Daam1 but not N-Daam1 or the FH2 domain of Daam1. (**D**) Profilin1 does not induce Rho activation or interfere with Rho activation induced by C-Daam1. Immunoprecipitation and Rho activation assays were carried out as described (Habas et al., 2003; Habas et al., 2001).

for western blotting but not immunoprecipitation or immunocytochemistry. In agreement with the above results, full-length Daam1 and C-Daam1 but not N-Daam1 interacted with endogenous Profilin1 (Fig. 1C). Additionally the FH1- but not the FH2-containing construct of Daam1 was found to interact with endogenous Profilin1 (Fig. 1C).

Profilin1 mediates cytoskeletal changes but not Rho activation downstream of Daam1

As C-Daam1 can mediate Rho activation and cytoskeletal changes (Habas et al., 2001), we tested whether Profilin1 functions in C-Daam1-mediated RhoA activation using the Rhotekin assay (Habas and He, 2006; Ren et al., 1999) and extracts of mammalian cells transfected with C-Daam1, Profilin1 or both. As in previous studies, C-Daam1 expression induced Rho activation but expression of Profilin1 did not (Fig. 1D). In addition, co-expression of Profilin1 did not interfere with C-Daam1-mediated Rho activation (Fig. 1D). This suggests that Profilin1 does not function in Daam1-mediated Rho activation.

We next examined the effects of Profilin1 on the actin cytoskeleton in NIH3T3 cells, using Wnt3a and Wnt5a conditioned media (CM) or Wnt3a protein. Previous studies have revealed a dramatic cytoskeletal reorganization of COS or B cells in response to Wnt3a stimulation (Endo et al., 2005; Qiang et al., 2003), but such effects on NIH3T3 cells were not reported. We found that treatment of NIH3T3 cells with Wnt3a CM but not control CM for 3 hours resulted in nuclear accumulation of β-catenin and robust induction of stress fibers (Fig. 2A). An identical effect on stress fiber induction and nuclear accumulation of β-catenin was observed with purified Wnt3a protein (Fig. 2A). We next examined the effects of Wnt5a CM. These studies revealed a robust induction of stress fiber formation but no nuclear accumulation of β-catenin (Fig. 2A). Lastly, a mutant construct of Dishevelled, ΔDIX-Dishevelled that is solely involved in non-canonical Wnt signaling (Habas et al., 2001; Tada and Smith, 2000; Wallingford et al., 2000), could induce the formation of stress fibers (Fig. 2B) without induction of nuclear β-catenin (not shown). These results demonstrate that NIH3T3 cells respond to non-canonical Wnt signaling with the induction of actin stress fibers.

Previous studies have revealed variable results on the effects and localization of Profilin1 to the actin cytoskeleton (Cao et al., 1992; Roy and Jacobson, 2004; Witke, 2004). We examined the localization of GFP-Profilin1 fusion protein in response to Wnt3a stimulation in NIH3T3 cells. Although GFP-Profilin1 was diffusely localized in the cytoplasm of NIH3T3 cells treated with control CM (Fig. 2C), treatment with Wnt3a CM resulted in colocalization of

GFP-Profilin1 with actin stress fibers (Fig. 2C,E). These studies suggest that Profilin1 may be a component of non-canonical Wnt signaling that modulates the actin cytoskeleton.

Profilin1 is required for Wnt/Daam1 mediated cytoskeletal reorganization

To investigate the role of Profilin1 in mediating non-canonical Wnt signaling, we explored the localization of Profilin1 and Daam1 in mammalian cells. Staining with affinity purified α-Daam1 polyclonal sera showed that Daam1 is localized mainly in the cytoplasm of NIH3T3 cells (Fig. 2D). In response to Wnt3a CM stimulation, Daam1 relocalized predominantly to actin stress fibers and to a lesser extent to the plasma membrane (Fig. 2D,E). As this response was similar to that of Profilin1 (Fig. 2C), we examined whether Daam1 and Profilin1 are colocalized in response to Wnt stimulation and found this to be the case for both transfected and endogenous Daam1 (Fig. 3A,B). This colocalization in response to Wnt stimulation suggest that Daam1 and Profilin1 may function in a common molecular pathway to mediate effects on the actin cytoskeleton

We next determined whether Profilin1 is required for Wnt- and Daam1-mediated cytoskeletal changes. We employed dsRNA-mediated interference (RNAi) to deplete endogenous Profilin1 by more than 60% without affecting the levels of β-catenin or Daam1 (Fig. 3C). Transfection of Profilin1 or control siRNA did not inhibit Top-flash reporter activation in NIH3T3 cells mediated by Wnt3a CM or Dishevelled (Fig. 3D). Importantly in cells depleted of Profilin1 stress fiber induction in response to Wnt3a CM, purified Wnt3a or ΔDIX-Dishevelled, but not in response to serum stimulation, was markedly decreased whereas the nuclear

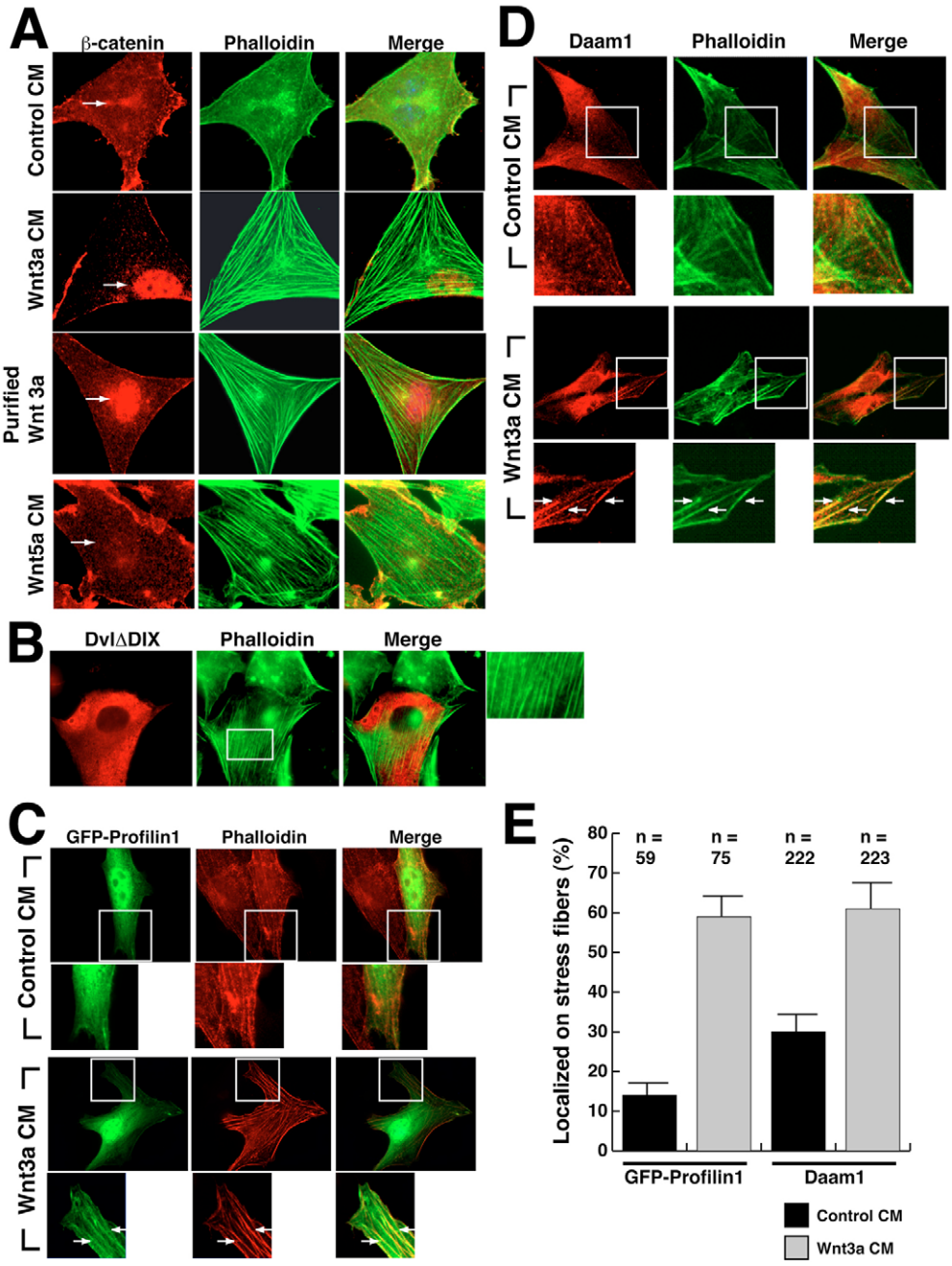

Fig. 2. Subcellular localization of Profilin1 and Daam1 in response to Wnt stimulation. (**A**) NIH3T3 cells respond to Wnt3a and Wnt5a but not control conditioned media (CM). After 3 hours of exposure of cells to Wnt3a CM or Wnt3a protein, endogenous β-catenin (red) is localized in the nucleus and stress fiber induction is indicated by Phalloidin staining (green). After 3 hours of exposure to Wnt5a CM, no nuclear localization of β-catenin is seen but stress fiber induction is observed. Position of the nucleus is indicated by arrow. (**B**) Expression of Flag-ΔDIX-Dishevelled (red) induces stress fiber formation (green) in NIH3T3 cells. (**C**) Subcellular localization of GFP-Profilin1 in NIH3T3 cells. GFP-Profilin1 is localized diffusely in the cytoplasm of NIH3T3 cells in the presence of control CM but associates with actin stress fibers (arrow) (red) after 3 hours Wnt-3a CM stimulation. (**D**) Endogenous Daam1 (red) is localized diffusely in the cytoplasm of NIH3T3 cells but is localized to actin stress fibers (arrow) (green) after 3 hours Wnt3a CM stimulation. (**E**) Quantification of the studies of C and D. Number of cells evaluated is shown at the top of each bar. Note in B,C,D magnifications of boxed areas (white) are shown below each respective panel.

accumulation of β-catenin was unaffected (Fig. 4A-C). Additionally in cells transfected with C-Daam1, which induces stress fiber formation (Habas et al., 2001), a significant reduction of stress fiber formation was observed in cells depleted of Profilin1 (Fig. 5A,B). These studies demonstrate that Profilin1 is required for Wnt- and Daam1-mediated cytoskeletal changes.

Expression pattern of Profilin1 during *Xenopus* development

To help elucidate the in vivo role of Profilin1, we examined the expression pattern of Profilin1 during *Xenopus* embryogenesis. XProfilin1 shares 48.7% identity with the rat and human proteins (see Fig. S1A in the supplementary material). RT-PCR analysis showed that Profilin1 is expressed maternally and throughout development (see Fig. S1B in the supplementary material). The spatial pattern of Profilin1 gene expression visualized by in situ hybridization revealed a dynamic expression profile in the developing embryo especially in regions associated with morphogenetic movements. Profilin1 was expressed in the animal pole of the fertilized egg (see Fig. S1C in the supplementary material). At the blastula stage, Profilin1 was observed circumferentially around the blastopore lip and in the involuting mesodermal cells (see Fig. S1C in the supplementary material). During the neurula stage Profilin1 was expressed in the neural folds and anterior neural plate and during later development was expressed at higher levels in the brain, eyes and spinal cord (see Fig. S1C in the supplementary material). We note that the expression pattern of Profilin1 overlaps with that of Daam1 (Nakaya et al., 2004).

Profilin1 overexpression interferes with *Xenopus* gastrulation

To elucidate the function of Profilin1 in vivo, we examined the effects of misexpression of Profilin1 during *Xenopus* development. Injection of Profilin1 RNA into the two ventral marginal blastomeres of the four-cell embryo had no effect on *Xenopus* development in a concentration range of 100 pg to 1 ng (Fig. 6A,E). In contrast injection of Profilin1 RNA into the dorsal marginal zone of the four-cell embryo resulted in severe gastrulation defects in a dosage dependent manner whereas injection of LacZ RNA had no significant effect (Fig. 6A,E). In Profilin1-injected embryos anterior structures including the head and eyes were reduced and the neural

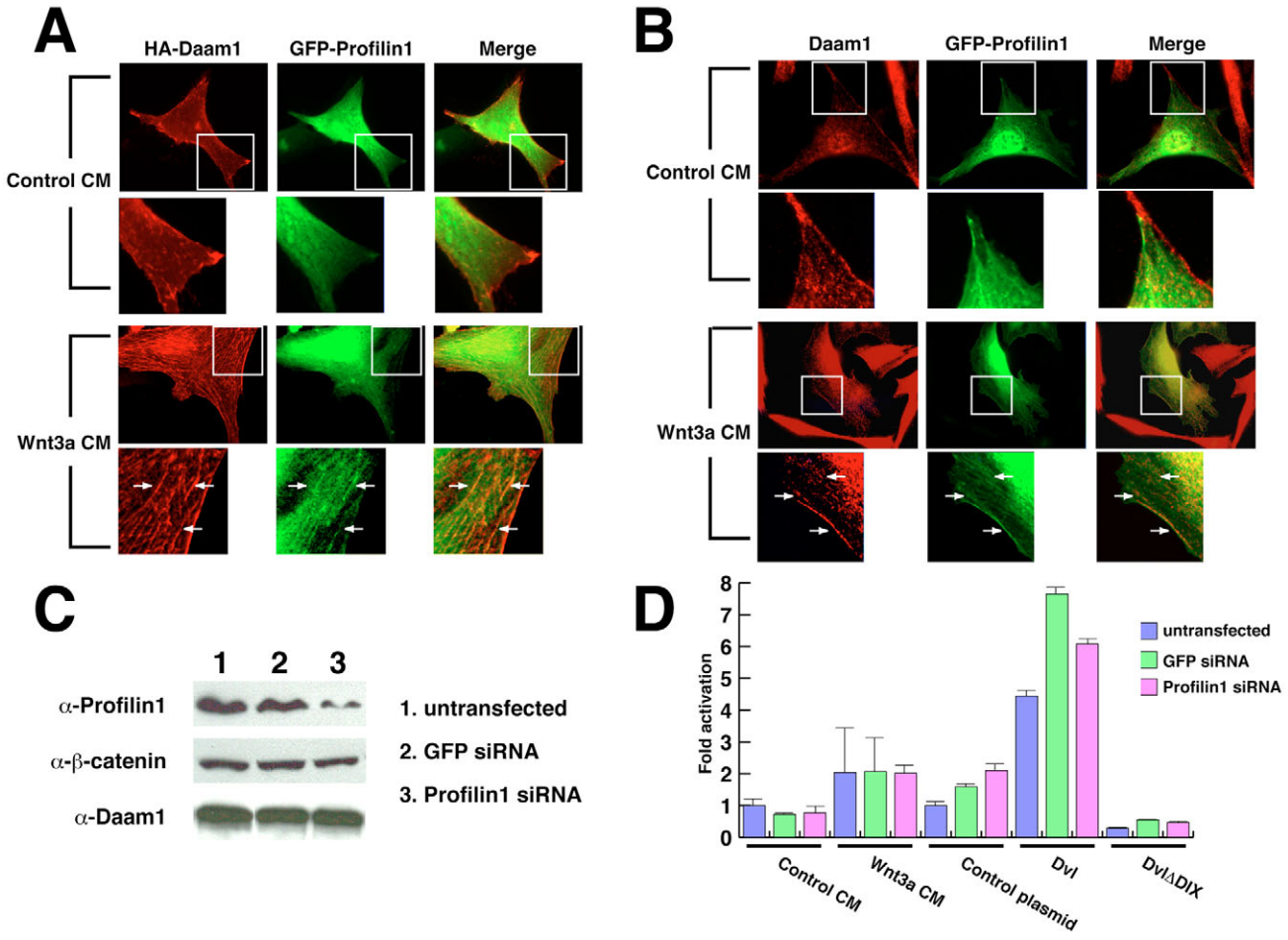

Fig. 3. Wnt stimulation induces a colocalization of Daam1 and Profilin1. (**A**) Epitope-tagged Daam1 (HA) or (**B**) endogenous Daam1 (red) colocalizes with GFP-Profilin1 to structures resembling actin stress fibers (arrow) in response to 3 hours Wnt3a but not control CM stimulation. Note in A and B, magnifications of the boxed areas (white) are shown below each respective panel. (**C**) Transfection of Profilin1 siRNA but not a control GFP siRNA reduces the level of endogenous Profilin1 in NIH3T3 cells. β-catenin and Daam1 are used as loading controls. (**D**) Top-Flash assay reveals Wnt3a CM and Dishevelled but not control CM or ΔDIX-Dishevelled induces reporter activation in NIH3T3 cells. Transfection of control or Profilin1 siRNA does not interfere with reporter activation by either Wnt stimulation or Dishevelled expression.

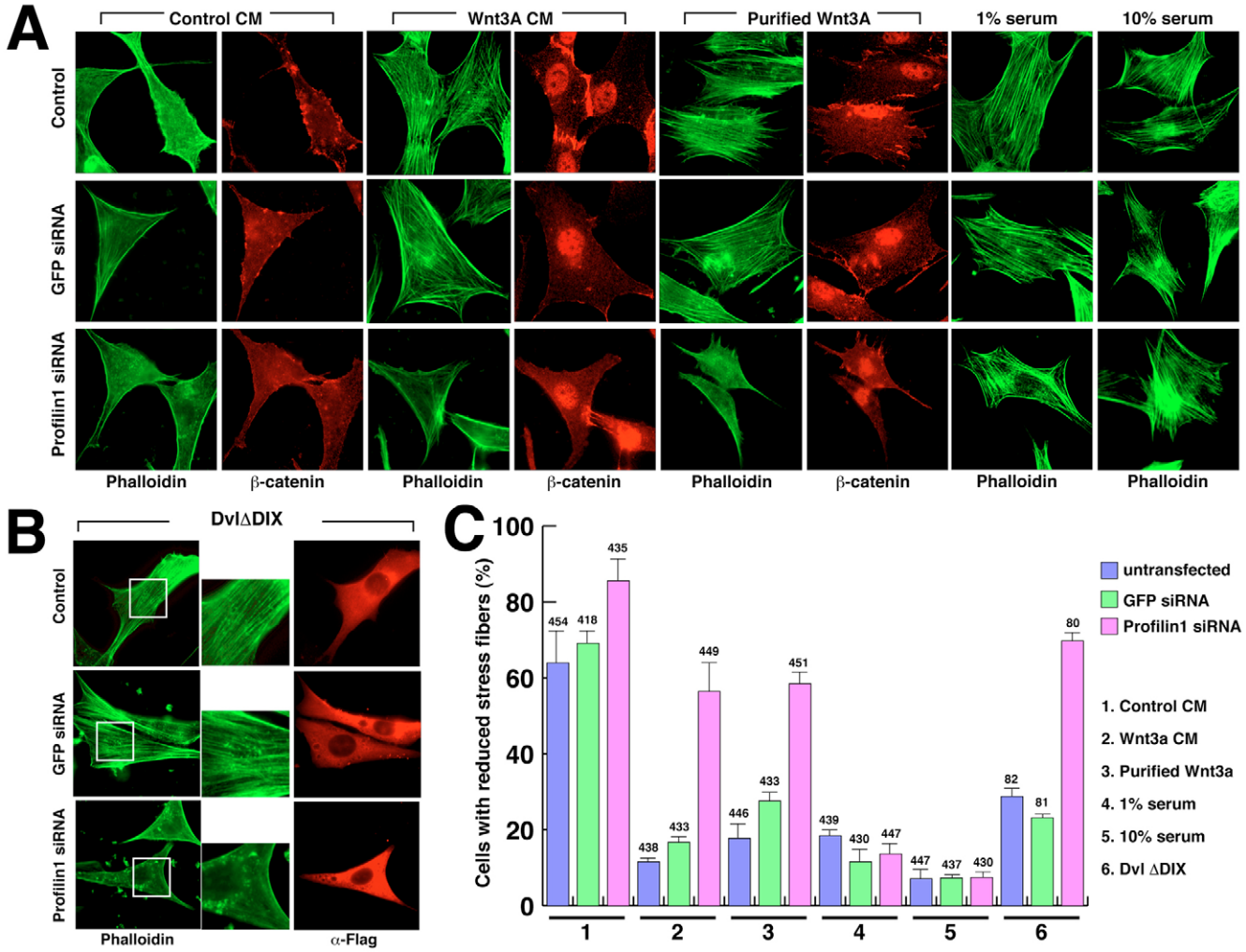

Fig. 4. Profilin1 is required for Wnt3a and Dishevelled-mediated stress fiber induction. (**A**) Transfection of Profilin1 siRNA but not GFP siRNA into NIH3T3 cells inhibits stress fiber induction (green) by 3 hours Wnt3a CM or Wnt3a protein stimulation. Note the Profilin1 siRNA does not inhibit stress fiber induction induced by serum stimulation. Nuclear translocation of endogenous β-catenin (red) induced by Wnt3a CM or purified Wnt3a protein stimulation is not affected by Profilin1 siRNA. (**B**) Transfection of Profilin1 siRNA but not GFP siRNA into NIH3T3 cells inhibits stress fiber induction (green) induced by ΔDIX-Dishevelled expression (red), magnifications of the boxed areas (white) are shown at the side of each respective panel. (**C**) Quantification of the results of A and B. Number of cells evaluated is shown at the top of each bar.

folds failed to close. This phenotype is suggestive of a role of Profilin1 in gastrulation cell movements and blastopore closure (Wallingford et al., 2002).

To study the role of Profilin1 in early *Xenopus* development by a loss-of-function approach, we designed an antisense MO to deplete the endogenous Profilin1 protein (Fig. 6B). As the commercial Profilin1 antisera did not recognize endogenous *Xenopus* Profilin1 by western blot analysis (not shown), we tested the efficiency of the Profilin1 MO to inhibit translation of a Myc-Profilin1 construct in the *Xenopus* embryo. We found a dose dependent suppression of translation of Myc-Profilin1, but not of Rho and Rac by injection of 25 and 50 ng of the Profilin1 MO and no effect was observed using a control MO (Fig. 6C,E). Injection of the Profilin1 MO or a control MO at a level of 50 ng into the marginal zone of the ventral two cells of the four-cell embryo had little effect on *Xenopus* development (Fig. 6D,E). Similarly injection of control MO into the dorsal cells had little effect but injection of 10-50 ng of the Profilin1 MO resulted in embryos with open neural folds in a dose dependent manner (Fig. 6D,E). The phenotype induced by injection of the maximal dose of 50 ng Profilin1 MO could be rescued by co-injection of 100 pg of a ΔN-Profilin1 RNA, a construct in which the MO binding site was deleted, but not by 100 pg of LacZ RNA (Fig. 6D,E). These experiments demonstrate the specificity of the Profilin1 MO and also demonstrate that Profilin1 is required for gastrulation.

Profilin1 and canonical Wnt signaling

To investigate whether Profilin1 can regulate canonical Wnt signaling, we performed secondary axis induction assays (McMahon and Moon, 1989; Sokol et al., 1991). Expression of Xwnt8 or Dsh ventrally in *Xenopus* embryos induced the formation of a secondary axis, and co-expression of Profilin1 had no effect on this induction (see Fig. S2A,B in the supplementary material). Furthermore, Profilin1 or Profilin1 MO had no effect on Xwnt-8 or Dsh induction of *Xnr3* and *Siamois* (Harland and Gerhart, 1997) (see Fig. S2C in the supplementary material). These results support the view that Profilin1, like Daam1, functions specifically in non-canonical Wnt signaling.

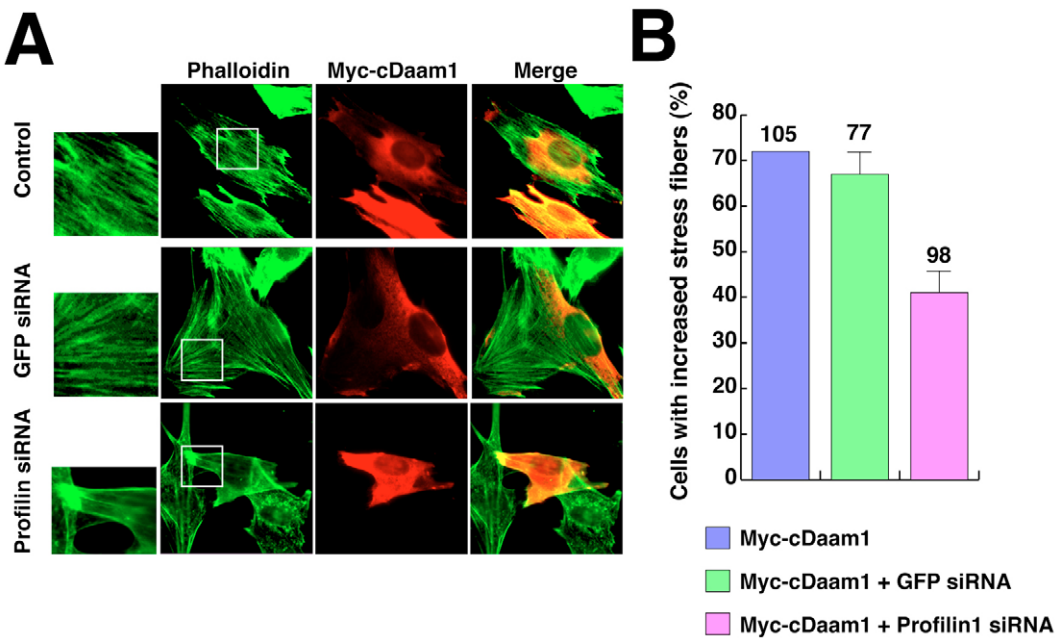

Fig. 5. Profilin1 is required for Daam1-mediated stress fiber induction. (**A**) Expression of Myc-tagged C-Daam1 (red) in NIH3T3 cells induces stress fibers (green), and this effect is abrogated by co-expression of Profilin1 siRNA but not GFP siRNA, magnifications of the boxed areas (white) are shown at the side of each respective panel. (**B**) Quantification of the effects on Profilin1 and GFP siRNA on stress fiber formation induced by C-Daam1 expression in NIH3T3 cells. Number of cells evaluated is shown at the top of each bar.

Profilin1 and mesodermal cell fate specification

One mechanism by which Profilin1 overexpression or depletion may affect gastrulation might involve the disruption of mesodermal cell fate specification. We tested this possibility by examining marker gene expression in animal cap explants treated with activin. Neither injection of Profilin1 RNA nor Profilin1 MO inhibited the expression of the panmesodermal marker *brachyury* (*Xbra*), dorsal mesodermal marker *chordin*, and ventrolateral mesodermal marker *Xwnt8* (see Fig. S2D in the supplementary material).

We next examined the expression and localization of mesendodermal and neural markers by in situ hybridization. Embryos were injected dorsally at the 4-cell stage with Profilin1 RNA, Profilin1 MO, control MO, ΔN-Profilin1 RNA or Profilin1 MO plus ΔN-Profilin1 RNA. The Profilin1 and Profilin1 MO injected embryos were abnormal, and the latter could be rescued by co-injecting ΔN-Profilin1 RNA as described above (Fig. 6D,E). At stage 10.5 Profilin1 and Profilin1 MO-injected embryos showed normal mesoderm and dorsal axis formation as assayed by the mesodermal marker *Xbra*, and dorsal *Gsc* and *Otx2* expression (Fig. 7). At stage 12 such embryos expressed *Xbra* surrounding a large blastopore that failed to close (Fig. 7). At stage 13 *Gsc* expression in control embryos was observed in the prechordal plate in the deep anterior mesendoderm whereas in Profilin1 and Profilin1 MO-injected embryos, *Gsc* expression remained near the open blastopore (Fig. 7). *Otx-2* was expressed anteriorly in both mesodermal and overlying neural tissues in control embryos at stage 13. However, in Profilin1 and Profilin1 MO-injected embryos, two separate *Otx-2* expression domains were seen (Fig. 7), one next to the open blastopore, which probably reflects expression in the anterior mesoderm that fails to involute, and the other in neural ectoderm that may be induced via planar neural induction. *Sox-2* is a pan-neural marker that marks the neural plate at stage 14. In Profilin1 and Profilin1 MO-injected embryos, *Sox-2* expression was seen in a broad dorsal region that surrounds the open blastopore but lacks neural plate morphology (Fig. 7).

These experiments demonstrate that overexpression or depletion of Profilin1 does not interfere with mesoderm specification.

Profilin1 and convergent extension movement

The phenotypes observed with overexpression or depletion of Profilin1 suggest a role in gastrulation. To delineate whether Profilin1 functions in convergent extension movements, we first examined the effects of overexpression or depletion of Profilin1 on activin-treated animal explants which exhibit morphogenetic elongation characteristic of gastrulation (Symes and Smith, 1987). We observed that expression or depletion of Profilin1 at doses that resulted in severe gastrulation phenotypes (Fig. 5A,D,E) had no effect on elongation of the explants (Fig. 8A,B).

This result was surprising as all known components of the non-canonical Wnt pathway including Wnt11 (Tada and Smith, 2000), Fz7 (Djiane et al., 2000), Dsh (Sokol, 1996), Daam1 (Habas et al., 2001), Rho (Habas et al., 2003; Tahinci and Symes, 2003) and Rac (Habas et al., 2003; Tahinci and Symes, 2003) potently inhibit convergent extension movements. We therefore tested the role of Profilin1 in convergent extension movements using dorsal marginal zone (Keller) explants, which more closely reflect the tissue undergoing convergent extension movements in vivo (Keller et al., 1985). Again we observed that expression or depletion of Profilin1 had no effect on elongation of the Keller explants (Fig. 8C,D), whereas dominant negative Dsh, Xdd1, potently inhibited elongation (Fig. 8C,D). These results demonstrate that Profilin1 is not required for convergent extension movements during gastrulation.

Profilin1 regulates blastopore closure

As our results above show, Profilin1 does not regulate convergent extension movements. The phenotypes we observed with expression or depletion of Profilin1 nevertheless suggest a role for this protein in gastrulation, which thus might function in tissue separation, blastopore closure, neural fold closure, or any combination thereof. To examine tissue separation, we injected Profilin1 RNA or MO and a tracer GFP RNA into the dorsal cells of the 4-cell embryo, explanted the axial mesodermal region of the injected embryos, and cultured the explants on wild type animal caps. No effect on tissue separation was observed (Fig. 8E,F). As a positive control we injected Xfz7, which was demonstrated to interfere with tissue

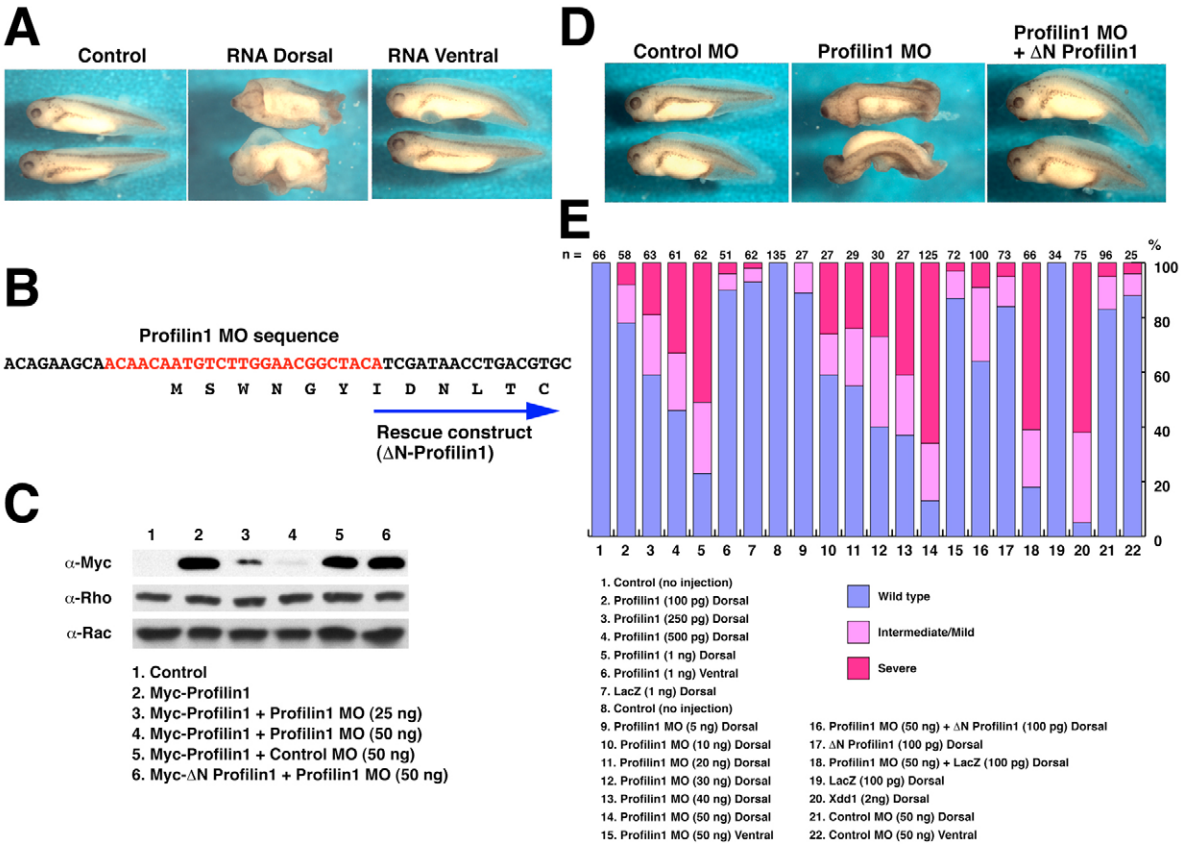

Fig. 6. XProfilin1 is required for gastrulation. (**A**) Injection of Profilin1 RNA dorsally but not ventrally inhibits gastrulation with the resulting embryos having open neural folds and reduced anterior structures. (**B**) XProfilin1 Morpholino (MO) sequence and diagram of the Profilin1 rescue construct (ΔN-Profilin1). (**C**) Injection of the XProfilin1 MO inhibits translation of Myc-tagged Profilin1 in a dose dependent way but does not affect the endogenous levels of Rho and Rac proteins. (**D**) Dorsal injection of XProfilin1 MO inhibits gastrulation and results in a similar gastrulation defect phenotype as overexpression of Profilin1 RNA (see A). This phenotype is reversed by injection of ΔN-Profilin1 with the XProfilin1 MO. (**E**) Quantitation of the phenotypic results from overexpression or depletion of XProfilin1. Injections were performed into the dorsal or ventral marginal zone of the 4-cell embryo, and phenotypes were scored at the tadpole stage.

separation (Winklbauer et al., 2001), and could confirm these observations (Fig. 8E,F). These studies indicate that Profilin does not regulate tissue separation during gastrulation.

We next examined the role of Profilin1 in neural fold closure. For this purpose we employed the strategy of Wallingford and Harland (Wallingford and Harland, 2002) by injecting Profilin1 RNA or MO into the dorsal medial or dorsal marginal cells of the 16-cell embryo. These injections selectively target the medial or lateral neural plate, respectively (Fig. 9A). These studies revealed that Profilin1 RNA and Profilin1 MO did affect neural fold closure when injected medially but not laterally (Fig. 9B). The effects of Profilin1 MO could be rescued by co-expression of the ΔN-Profilin1 RNA (Fig. 9B), and control MO injection dorsally or medially had no effect (not shown). However, the neural fold closure defects in these embryos were localized to the posterior of the embryos suggesting that the phenotype may reflect a defect in blastopore closure rather than a direct effect on neural fold closure. Indeed we observed that Profilin1 RNA or Profilin1 MO-injected embryos displayed a delay in blastopore closure, and in a majority of injected embryos blastopore closure failed (Fig. 6A,D). These data strongly suggests a role for Profilin1 specifically in blastopore closure rather than neural fold closure.

We next examined the process of blastopore closure directly using time lapse imaging after injection with Profilin1 RNA into the dorsal marginal zone of the four-cell embryo. These studies revealed a dramatic delay and in the majority of injected embryos a complete failure of blastopore closure (Fig. 9C). Depletion of endogenous Profilin1 using Profilin1 MO revealed a similar failure of blastopore closure and this phenotype could be rescued by co-injection of ΔN-Profilin1 (Fig. 9C).

Loss of Profilin1 and Daam1 synergistically inhibit blastopore closure

Lastly, we performed experiments to simultaneously deplete Profilin1 and Daam1 to test for interactive effects on gastrulation and blastopore closure, which would be expected if the two factors functions in the same pathway. Embryos were injected with sub-threshold levels of Daam1 MO or Profilin1 MO, which individually induce failure of blastopore closure and gastrulation in only a minority of the embryos (Fig. 9D). However, when both Daam1 and Profilin1 MO were co-injected, the number of affected embryos increased substantially (Fig. 9D). Taken together these studies suggest that Profilin1 functions with Daam1 in the non-canonical signaling pathway during gastrulation.

DISCUSSION

In this study we demonstrated that Profilin1 is an effector of Daam1 in non-canonical Wnt signaling. Profilin1 binds to the FH1 domain of Daam1 and colocalizes with Daam1 to actin stress fibers in

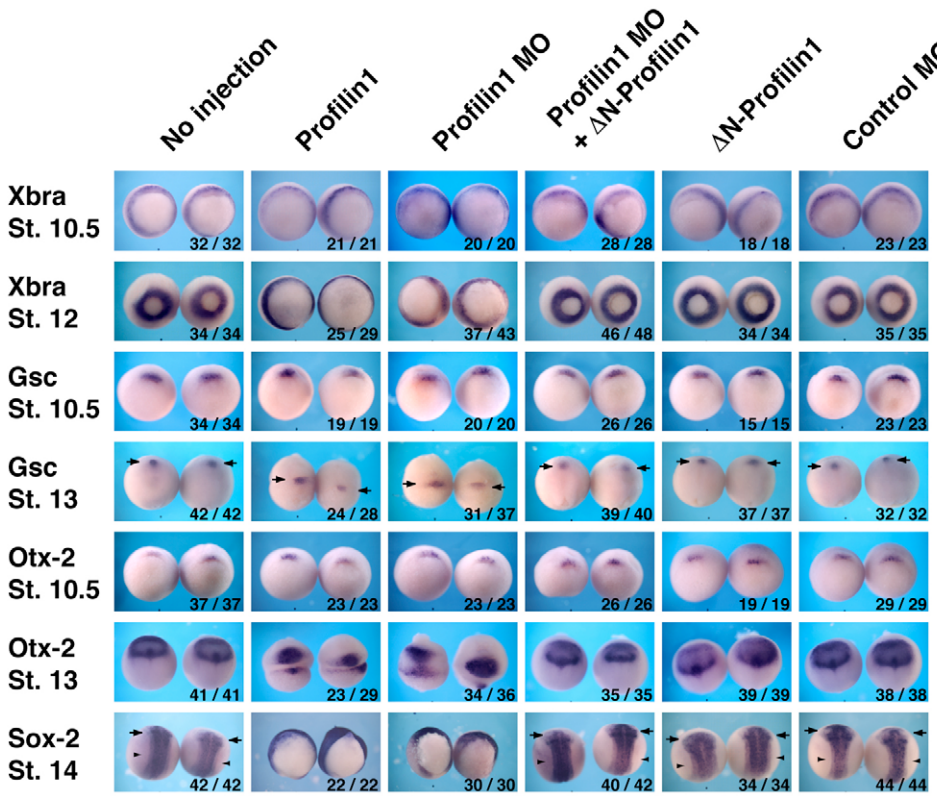

Fig. 7. Profilin1 does not interfere with mesoderm induction. Embryos injected dorsally with Profilin1 (2 ng) or XProfilin1 MO (50 ng) show normal mesoendoderm and neural induction, but abnormal tissue localization due to gastrulation defects. No effects were observed with injections of the control MO (50 ng) after gastrulation defects. The phenotype induced by the XProfilin1 MO can be rescued by co-expression of ΔN-Profilin1 (50 pg). Injected embryos show normal expression of mesodermal Xbra, and dorsal Gsc and Otx2 at st 10.5, but exhibit an Xbra expression surrounding a large blastopore that does not close at st 12. Gsc expression in control embryos at st 13 is observed in anterior mesoendoderm far from the closed blastopore (arrowheads), but in Profilin or XProfilin1 MO-injected embryo remains trapped near the open blastopore. Otx-2 is expressed anteriorly in both mesodermal and overlying neural tissues in control embryos at st 13 but in Profilin or XProfilin1 MO-injected embryos, two separate Otx-2 expression domains are obvious. Sox-2 is expressed in the neural plate at st 14 (arrowheads), but in Profilin or XProfilin1 MO-injected embryos, Sox-2 expression is seen surrounding the open blastopore.

response to Wnt stimulation. Depletion of Profilin1 specifically inhibits stress fiber formation induced by Wnt stimulation and Daam1 overexpression. We further showed that Profilin1 is required downstream of Daam1 for blastopore closure during *Xenopus* gastrulation. These findings illuminate a molecular pathway linking Wnt signaling to the regulation of the cellular cytoskeletal architecture.

Profilin1 is an effector for Daam1

Daam1 is a Formin protein that is required for non-canonical Wnt signaling (Habas et al., 2001). Daam1 functions as a link between Dishevelled and the Rho GTPase in mediating cytoskeletal changes required for gastrulation cell movements but how Daam1 accomplishes these effects is not known. It is likely that Daam1 acts as a scaffolding protein and utilizes independent domains (GBD, FH1, FH2, etc.) to recruit and regulate factors required for Rho activation and cytoskeletal changes.

Profilin1 was biochemically purified as one of the first actin binding proteins and Profilin1 can stimulate the polymerization of actin filaments in vitro but the signaling pathways that require Profilin1 for their function and morphogenetic process regulated by Profilin1 remained unknown (Witke, 2004). Here we report a functional role for Profilin1 in Wnt- and Daam1-mediated cytoskeletal changes and for blastopore closure during embryogenesis.

Profilin1 interacts with Daam1 as assayed by co-immunoprecipitation (Fig. 1B), and we demonstrate binding between endogenous Profilin1 and Daam1 indicating a physiological interaction (Fig. 1C). Profilin1 binds to the FH1 domain of Daam1 (Fig. 1B,C). An interaction between Profilin and the FH1 domain of Formins such as mDia1 has been reported previously and Profilin was implicated as an effector for Formin proteins in mediating actin polymerization (Wallar and Alberts, 2003; Watanabe et al., 1997). Recent studies have shown that the FH2 domain of Formins including mDia1 can stimulate the polymerization of actin filaments in vitro (Krebs et al., 2001) and it likely that this activity is coordinated with factors such as Profilin that bind to the FH1 domain for morphogenesis.

Profilin1 is a component of non-canonical Wnt signaling

The non-canonical Wnt pathway plays important roles in cell polarization and cytoskeletal reorganization. In mammalian cultured cells, stimulation through the non-canonical Wnt pathway induces shape changes and regulates motility (Endo et al., 2005; Qiang et al., 2003; Shibamoto et al., 1998; Torres and Nelson, 2000). Dishevelled is required in vivo in the formation and stabilization of lamellopodial protrusions that regulate cell movements during gastrulation, and the small GTPase Rho acts downstream of Dishevelled in this signaling

cascade (Endo et al., 2005; Wallingford et al., 2000). We showed that Profilin1 does not induce or inhibit Rho activation (Fig. 1D), suggesting that Profilin1 is required for cytoskeletal changes in addition to the Rho pathway downstream of Dishevelled and Daam1.

Characterizing the requirement for Profilin1 in cytoskeletal changes, we showed that Profilin1 and Daam1 are localized to actin stress fibers in response to Wnt stimulation in NIH3T3 cells (Fig. 2C,D). To our knowledge, this is the first time that a Formin protein or Profilin has been localized to actin stress fibers in mammalian cells. These observations suggest that the activity of Profilin1 may be regulated by a Wnt-dependent mechanism. Furthermore, depletion of Profilin1 abrogates stress fiber formation induced by Wnt, ΔDIX-Dishevelled or C-Daam1, demonstrating a requirement for Profilin1 in cytoskeletal changes mediated by non-canonical Wnt signaling (Fig. 4A-C and Fig. 5A,B). It is important to note that in these depletion studies dramatic effects on the actin cytoskeleton but no effects on the accumulation of nuclear β-catenin were observed (Fig. 4A), supporting the notion that morphological changes are independent of the canonical Wnt pathway.

Profilin1 is not required for canonical Wnt signaling

Wnt signaling branches into three main pathways downstream of Dishevelled and considerable effort has been expended to decipher how Dishevelled channels signaling into these pathways (Habas and Dawid, 2005; Wallingford and Habas, 2005). Previously we have demonstrated that Daam1 is not a component of canonical signaling (Habas et al., 2001) and we have shown here that Profilin1 did not induce a secondary axis, did not interfere with Wnt- or Dishevelled-induced secondary axis formation and did not inhibit target genes of the canonical pathway in animal explants (see Fig. S1A-C in the supplementary material). We therefore conclude that Profilin1 does not play a role in canonical Wnt signaling.

The gastrulation defects observed with overexpression or depletion of Profilin1 did not result from a failure of mesodermal specification, as expression levels of all mesodermal marker genes tested were unaffected by manipulating Profilin1. Likewise, expression levels of neural markers were not affected by manipulating Profilin1 levels (see Fig. S1D in the supplementary material and Fig. 7), although gastrulation defects led to spatial

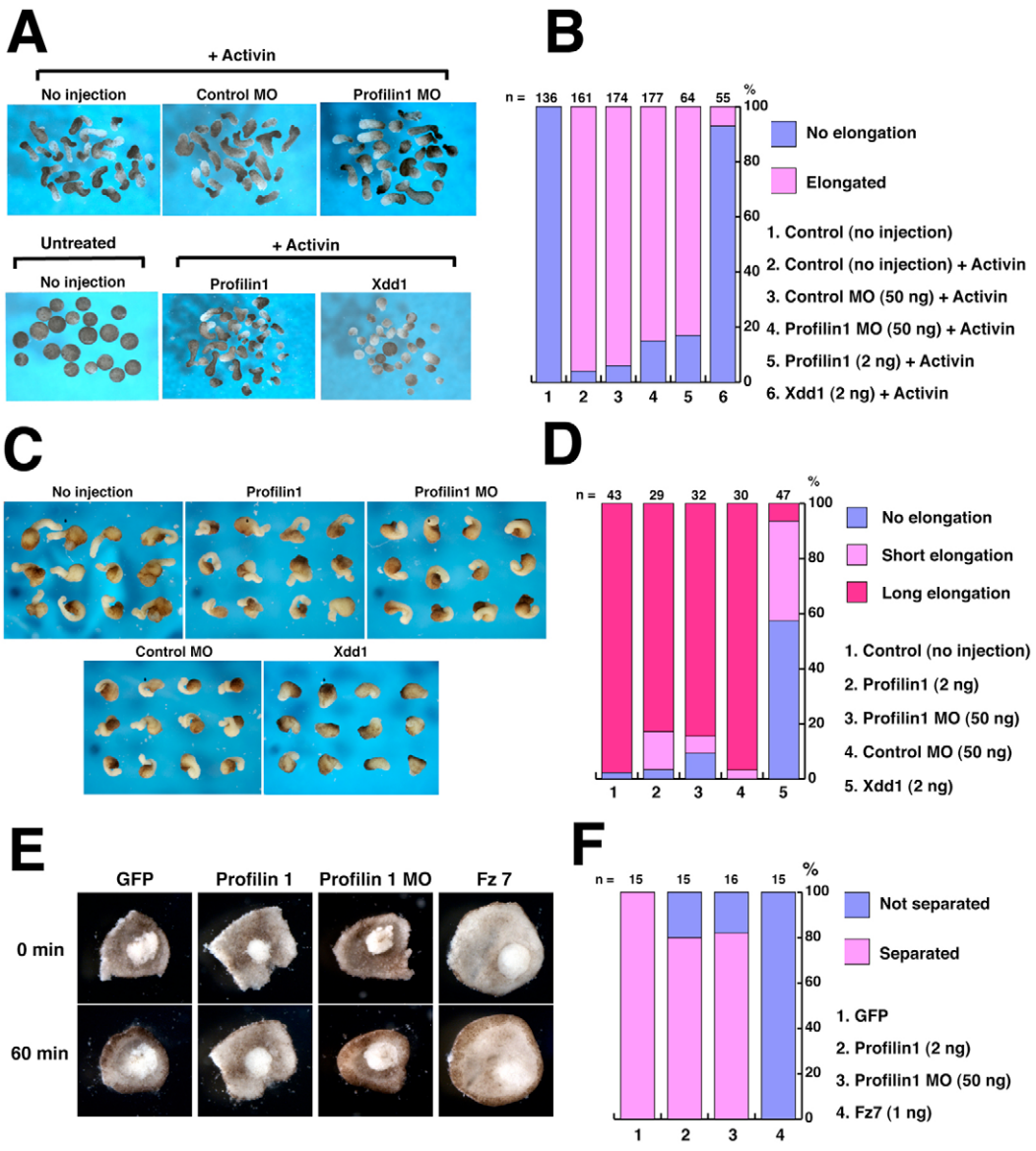

Fig. 8. Profilin1 is not required for tissue separation or convergent extension. (**A**) Overexpression (2 ng RNA) or depletion of Profilin1 (50 ng XProfilin1 MO) does not inhibit convergent extension in animal explants treated with activin or (**C**) in Keller explants whereas dominant negative Dishevelled (Xdd1, 2 ng) inhibits these movements. (**E**) Overexpression (2 ng RNA) or depletion of Profilin1 (50 ng XProfilin1 MO) does not inhibit tissue separation by 1 hr whereas injection of Xfz7 (1 ng) does. Quantitation of the convergent extension assays in (**B**) animal explants, (**D**) Keller explants and (**F**) separation assays. For animal explant assays, a length/width ratio of 2 or higher was scored as elongated. For Keller explants, a length/width ratio below 2 was scored as a short elongation and a length/width ratio of 2 or higher was scored as a long elongation.

mislocalization of both mesodermal and neural markers. These results are consistent with those of previous studies showing that mesodermal specification is unaltered by inhibition of non-canonical Wnt signaling (Djiane et al., 2000; Habas et al., 2001). We therefore conclude that Profilin1 does not affect mesodermal and neural specification in the embryo.

Profilin1 is required for blastopore closure

Vertebrate gastrulation involves a dynamic series of cell polarization and migration events that mediate blastopore closure, axial extension and neural fold closure (Wallingford et al., 2002). This morphogenetic process is regulated by non-canonical Wnt signaling, and Dishevelled is an important component of this pathway (Tada and Smith, 2000; Wallingford et al., 2000). We have previously shown that a Wnt-11/Fz/Xdsh/Daam1/Rho signaling pathway regulates convergent extension movements during gastrulation (Habas et al., 2001), and many studies have implicated the non-canonical Wnt pathway in blastopore closure, convergent extension movements, tissue separation and neural fold closure (Veeman et al., 2003; Wallingford et al., 2002). Whether distinct effectors are specifically utilized in the different morphogenetic processes has remained unclear (Keller, 2002; Keller et al., 2003).

We have examined the role of Profilin1 in each of these morphogenetic processes. In animal cap explants treated with activin or in Keller explants, expression or depletion of Profilin1 has no effect on elongation (Fig. 8A-D). We tested for a role of Profilin1 in tissue separation and observed no effects (Fig. 8E,F). We further tested the role of Profilin1 in neural fold closure using targeted injections to direct overexpression or depletion of Profilin1 in different regions and observed effects on neural fold closure exclusively in the posterior region of the embryo (Fig. 9B),

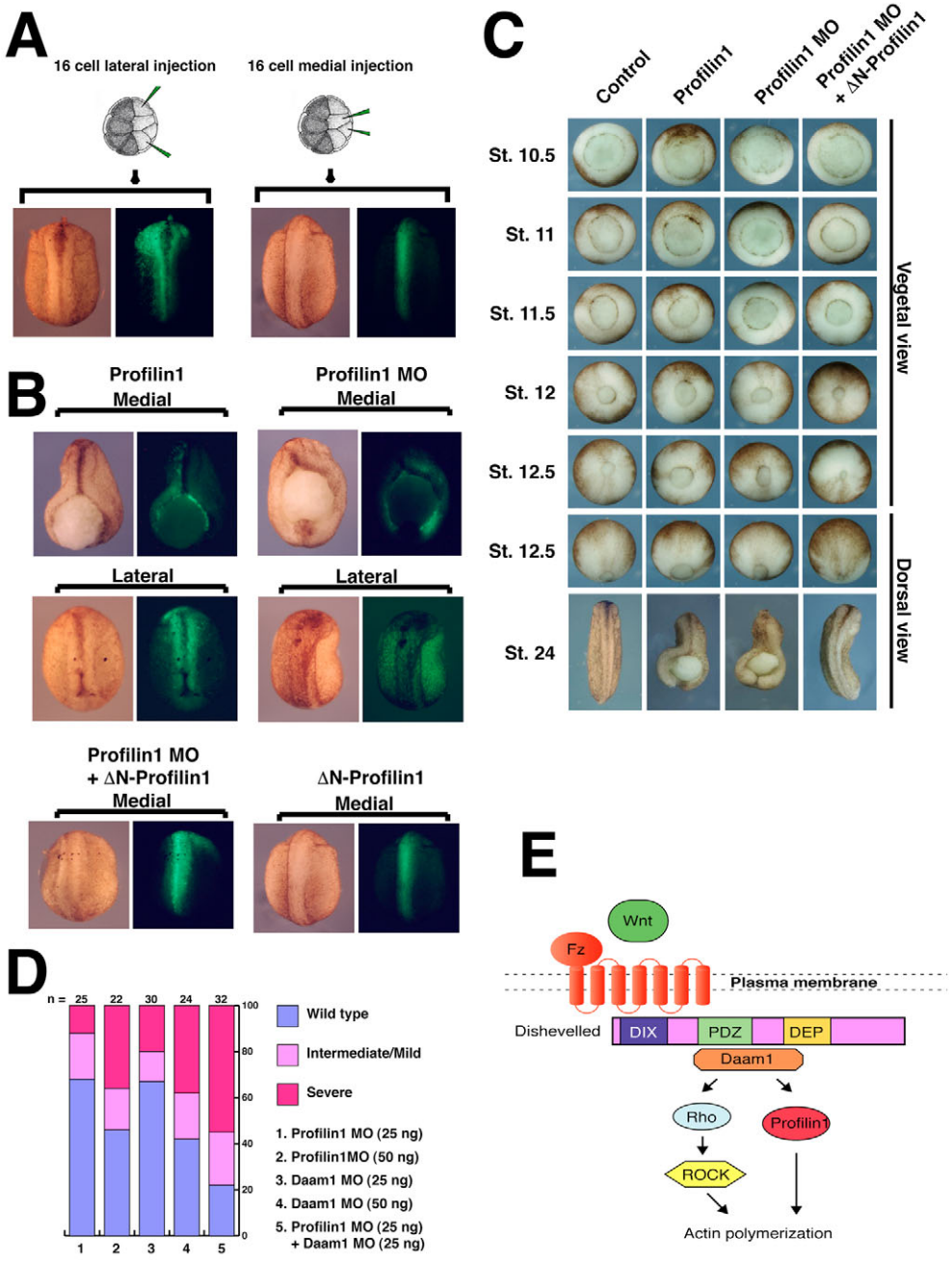

Fig. 9. Profilin1 is specifically required for blastopore closure. (**A**) Schema of the injection approach to overexpress or deplete Profilin1 in or lateral to the neural folds, adapted from Wallingford and Harland (Wallingford and Harland, 2002). RNA (50 pg) encoding membrane-localized GFP injected into the lateral blastomeres at the 16-cell stage targets expression lateral of the neural folds whereas injection into the medial blastomeres targets expression to the neural folds. (**B**) Targeted overexpression of Profilin1 RNA (2 ng) or depletion of Profilin1 (50 ng MO) in the neural folds does not inhibit anterior neural fold closure but leads to open neural folds in the posterior, reflecting a failure of blastopore closure. This phenotype can be rescued by co-expression of ΔN-Profilin1 (50 pg). Lateral overexpression or depletion of Profilin1 has no effect on neural fold closure. (**C**) Time lapse images of embryos injected with Profilin1 RNA (2 ng) or XProfilin1 MO (50 ng) showing a delay and eventual failure of blastopore closure. The XProfilin1 MO phenotype is rescued by co-injection of 50 pg ΔN-Profilin1 RNA. (**D**) Depletion of XProfilin1 and XDaam1 synergistically inhibit gastrulation. Quantitation of the phenotypes observed with separate or co-injections of XProfilin1 MO and/or XDaam1 MO, Embryos with an open blastopore and significantly reduced anterior posterior (AP) axis were scored as severe embryos. Embryos with a small open blastopore or delayed blastopore closure and a shortened AP axis or bent body axis were scored as intermediate to mild. (**E**) A model for how Profilin1 functions in the non-canonical Wnt signaling pathway (see Discussion).

indicative of an indirect effect through inhibition of blastopore closure. Embryos overexpressing or depleted in Profilin1 show delayed blastopore closure (Fig. 9C), which can explain the defects observed in such embryos. A failure of blastopore closure will result in a failure of the neural fold to close in the posterior although axial extension will be normal if convergent extension is unaffected.

These results are intriguing in the context that no single component of the non-canonical Wnt pathway has been shown previously to be required for blastopore closure alone. Force generation for blastopore closure probably involves the actin cytoskeleton (Keller et al., 1985), and we suggest that Profilin1 mediates a signal derived from the non-canonical Wnt pathway to the actin cytoskeleton. The identification of Profilin1 as a molecular component specifically required for blastopore closure provides a branch point in the non-canonical pathway for this specific morphogenetic event.

The molecular basis for cytoskeletal changes during gastrulation

It is clear that cell motility during gastrulation requires dynamic changes to the cytoskeleton and to cell polarity, involving polarization of the migrating cells for mediolateral intercalation and convergent extension movements (Keller, 2002; Keller et al., 2003; Wallingford et al., 2002). Additionally these movements are dependent on the stabilization of protrusions termed lamellipodia, which are controlled by Dishevelled (Wallingford et al., 2000). However, what factors control the active assembly and disassembly of the microtubule network and cellular actin cytoskeleton are poorly resolved. Recent studies have identified a dynamic requirement for changes to the microtubule cytoskeleton and a Rho-GEF was identified for this process (Kwan and Kirschner, 2005). Furthermore, the Formin protein Daam1 mediates signaling from Dishevelled to the actin cytoskeleton for convergent extension movements (Habas et al., 2001). We propose here that Daam1 utilizes the effector molecule Profilin1 to mediate actin polymerization for cell motility during gastrulation. As Wnt signaling induces a colocalization of Daam1 and Profilin1 to actin stress fibers and depletion of Profilin1 abrogates induction of actin stress fibers in response to Wnt stimulation, this Daam1/Profilin1 complex is probably required for reorganization of the actin cytoskeleton during blastopore closure. The 'purse string' mechanism of blastopore closure requires force generation executed by the actin cytoskeleton (Keller et al., 2003) and we propose this is mediated by the action of Daam1 and Profilin1. Indeed a recent study has revealed a role for Daam1 in *Drosophila* in actin polymerization during tracheal development (Matusek et al., 2006). The mechanism of this dynamic control over the actin cytoskeleton by Daam1 and Profilin1 will require a detailed investigation of the contribution of the individual domains within Daam1 to its function during gastrulation as well as the identity of other effector molecules such as the Rho-GEF, which triggers Rho activation in response to Wnt stimulation.

A model for Profilin1 function in non-canonical Wnt signaling

We propose a model of non-canonical Wnt signaling during gastrulation in which Dishevelled binds to Daam1 and Profilin1 is recruited to a Dishevelled/Daam1 complex (Fig. 9E). As Profilin1 is an actin polymerization factor, it can mediate cytoskeletal changes required for blastopore closure. In *Xenopus* gastrulation, Daam1 also leads to Rho and ROCK activation that independently results in modulation of the actin cytoskeleton (Fig. 9E). Wnt signaling through Dishevelled but not involving Daam1 also activates Rac, which is required independently for execution of the full array of gastrulation movements (Habas et al., 2003). Our results suggest that different aspects of gastrulation movements require different combinations of separate or overlapping signals that are generated as branches of the non-canonical Wnt pathway.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/21/4219/DC1

References

Alberts, A. S. (2002). Diaphanous-related Formin homology proteins. *Curr. Biol.* **12**, R796.

Cadigan, K. M. and Liu, Y. I. (2006). Wnt signaling: complexity at the surface. *J. Cell Sci.* **119**, 395-402.

Cao, L. G., Babcock, G. G., Rubenstein, P. A. and Wang, Y. L. (1992). Effects of profilin and profilactin on actin structure and function in living cells. *J. Cell Biol.* **117**, 1023-1029.

Capelluto, D. G., Kutateladze, T. G., Habas, R., Finkielstein, C. V., He, X. and Overduin, M. (2002). The DIX domain targets dishevelled to actin stress fibres and vesicular membranes. *Nature* **419**, 726-729.

Cooley, L., Verheyen, E. and Ayers, K. (1992). chickadee encodes a profilin required for intercellular cytoplasm transport during Drosophila oogenesis. *Cell* **69**, 173-184.

Djiane, A., Riou, J., Umbhauer, M., Boucaut, J. and Shi, D. (2000). Role of frizzled 7 in the regulation of convergent extension movements during gastrulation in Xenopus laevis. *Development* **127**, 3091-3100.

Endo, Y., Wolf, V., Muraiso, K., Kamijo, K., Soon, L., Uren, A., Barshishat-Kupper, M. and Rubin, J. S. (2005). Wnt-3a-dependent cell motility involves RhoA activation and is specifically regulated by dishevelled-2. *J. Biol. Chem.* **280**, 777-786.

Evangelista, M., Pruyne, D., Amberg, D. C., Boone, C. and Bretscher, A. (2002). Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. *Nat. Cell Biol.* **4**, 32-41.

Frazier, J. A. and Field, C. M. (1997). Actin cytoskeleton: are FH proteins local organizers? *Curr. Biol.* **7**, R414-R417.

Habas, R. and Dawid, I. B. (2005). Dishevelled and Wnt signaling: is the nucleus the final frontier? *J. Biol.* **4**, 2.

Habas, R. and He, X. (2006). Activation of Rho and Rac by Wnt/frizzled signaling. *Methods Enzymol.* **406**, 500-511.

Habas, R., Kato, Y. and He, X. (2001). Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1. *Cell* **107**, 843-854.

Habas, R., Dawid, I. B. and He, X. (2003). Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. *Genes Dev.* **17**, 295-309.

Harland, R. and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol.* **13**, 611-667.

He, X., Saint-Jeannet, J. P., Wang, Y., Nathans, J., Dawid, I. and Varmus, H. (1997). A member of the Frizzled protein family mediating axis induction by Wnt-5A. *Science* **275**, 1652-1654.

He, X., Semenov, M., Tamai, K. and Zeng, X. (2004). LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development* **131**, 1663-1677.

Higgs, H. N. (2005). Formin proteins: a domain-based approach. *Trends Biochem. Sci.* **30**, 342-353.

Hukriede, N. A., Tsang, T. E., Habas, R., Khoo, P. L., Steiner, K., Weeks, D. L., Tam, P. P. and Dawid, I. B. (2003). Conserved requirement of Lim1 function for cell movements during gastrulation. *Dev. Cell* **4**, 83-94.

Kato, Y., Habas, R., Katsuyama, Y., Naar, A. M. and He, X. (2002). A component of the ARC/Mediator complex required for TGF beta/Nodal signalling. *Nature* **418**, 641-646.

Keller, R. (2002). Shaping the vertebrate body plan by polarized embryonic cell movements. *Science* **298**, 1950-1954.

Keller, R., Davidson, L. A. and Shook, D. R. (2003). How we are shaped: the biomechanics of gastrulation. *Differentiation* **71**, 171-205.

Keller, R. E., Danilchik, M., Gimlich, R. and Shih, J. (1985). The function and mechanism of convergent extension during gastrulation of Xenopus laevis. *J. Embryol. Exp. Morphol. Suppl.* **89**, 185-209.
Kovar, D. R. and Pollard, T. D. (2004). Insertional assembly of actin filament barbed ends in association with formins produces piconewton forces. *Proc. Natl. Acad. Sci. USA* **101**, 14725-14730.
Kovar, D. R., Harris, E. S., Mahaffy, R., Higgs, H. N. and Pollard, T. D. (2006). Control of the assembly of ATP- and ADP-actin by formins and profilin. *Cell* **124**, 423-435.
Krebs, A., Rothkegel, M., Klar, M. and Jockusch, B. M. (2001). Characterization of functional domains of mDia1, a link between the small GTPase Rho and the actin cytoskeleton. *J. Cell Sci.* **114**, 3663-3672.
Kwan, K. M. and Kirschner, M. W. (2005). A microtubule-binding Rho-GEF controls cell morphology during convergent extension of Xenopus laevis. *Development* **132**, 4599-4610.
Li, L., Yuan, H., Xie, W., Mao, J., Caruso, A. M., McMahon, A., Sussman, D. J. and Wu, D. (1999). Dishevelled proteins lead to two signaling pathways. Regulation of LEF-1 and c-Jun N-terminal kinase in mammalian cells. *J. Biol. Chem.* **274**, 129-134.
Marlow, F., Topczewski, J., Sepich, D. and Solnica-Krezel, L. (2002). Zebrafish Rho kinase 2 acts downstream of Wnt11 to mediate cell polarity and effective convergence and extension movements. *Curr. Biol.* **12**, 876-884.
Matusek, T., Djiane, A., Jankovics, F., Brunner, D., Mlodzik, M. and Mihaly, J. (2006). The Drosophila formin DAAM regulates the tracheal cuticle pattern through organizing the actin cytoskeleton. *Development* **133**, 957-966.
McMahon, A. P. and Moon, R. T. (1989). Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis. *Cell* **58**, 1075-1084.
Mikels, A. J. and Nusse, R. (2006). Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biol.* **4**, e115.
Moon, R. T., Brown, J. D. and Torres, M. (1997). WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet.* **13**, 157-162.
Nakaya, M. A., Habas, R., Biris, K., Dunty, W. C., Jr, Kato, Y., He, X. and Yamaguchi, T. P. (2004). Identification and comparative expression analyses of Daam genes in mouse and Xenopus. *Gene Expr. Patterns* **5**, 97-105.
Qiang, Y. W., Endo, Y., Rubin, J. S. and Rudikoff, S. (2003). Wnt signaling in B-cell neoplasia. *Oncogene* **22**, 1536-1545.
Ren, X. D., Kiosses, W. B. and Schwartz, M. A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* **18**, 578-585.
Roy, P. and Jacobson, K. (2004). Overexpression of profilin reduces the migration of invasive breast cancer cells. *Cell Motil. Cytoskeleton* **57**, 84-95.
Severson, A. F., Baillie, D. L. and Bowerman, B. (2002). A Formin Homology protein and a profilin are required for cytokinesis and Arp2/3-independent assembly of cortical microfilaments in C. elegans. *Curr. Biol.* **12**, 2066-2075.
Shibamoto, S., Higano, K., Takada, R., Ito, F., Takeichi, M. and Takada, S. (1998). Cytoskeletal reorganization by soluble Wnt-3a protein signalling. *Genes Cells* **3**, 659-670.
Shih, J. and Keller, R. (1992). Patterns of cell motility in the organizer and dorsal mesoderm of Xenopus laevis. *Development* **116**, 915-930.
Sokol, S., Christian, J. L., Moon, R. T. and Melton, D. A. (1991). Injected Wnt RNA induces a complete body axis in Xenopus embryos. *Cell* **67**, 741-752.
Sokol, S. Y. (1996). Analysis of Dishevelled signalling pathways during Xenopus development. *Curr. Biol.* **6**, 1456-1467.
Symes, K. and Smith, J. C. (1987). Gastrulation movements provide an early marker of mesoderm induction in *Xenopus laevis*. *Development* **101**, 339-349.
Tada, M. and Smith, J. C. (2000). Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* **127**, 2227-2238.
Tahinci, E. and Symes, K. (2003). Distinct functions of Rho and Rac are required for convergent extension during Xenopus gastrulation. *Dev. Biol.* **259**, 318-335.
Torres, M. A. and Nelson, W. J. (2000). Colocalization and redistribution of dishevelled and actin during Wnt-induced mesenchymal morphogenesis. *J. Cell Biol.* **149**, 1433-1442.
Veeman, M. T., Axelrod, J. D. and Moon, R. T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev. Cell* **5**, 367-377.
Verheyen, E. M. and Cooley, L. (1994). Profilin mutations disrupt multiple actin-dependent processes during Drosophila development. *Development* **120**, 717-728.
Wallar, B. J. and Alberts, A. S. (2003). The formins: active scaffolds that remodel the cytoskeleton. *Trends Cell Biol.* **13**, 435-446.
Wallingford, J. B. and Harland, R. M. (2002). Neural tube closure requires Dishevelled-dependent convergent extension of the midline. *Development* **129**, 5815-5825.
Wallingford, J. B. and Habas, R. (2005). The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity. *Development* **132**, 4421-4436.
Wallingford, J. B., Rowning, B. A., Vogeli, K. M., Rothbacher, U., Fraser, S. E. and Harland, R. M. (2000). Dishevelled controls cell polarity during Xenopus gastrulation. *Nature* **405**, 81-85.
Wallingford, J. B., Fraser, S. E. and Harland, R. M. (2002). Convergent extension: the molecular control of polarized cell movement during embryonic development. *Dev. Cell* **2**, 695-706.
Watanabe, N. and Higashida, C. (2004). Formins: processive cappers of growing actin filaments. *Exp. Cell Res.* **301**, 16-22.
Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M. and Narumiya, S. (1997). p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J.* **16**, 3044-3056.
Winklbauer, R., Medina, A., Swain, R. K. and Steinbeisser, H. (2001). Frizzled-7 signalling controls tissue separation during Xenopus gastrulation. *Nature* **413**, 856-860.
Witke, W. (2004). The role of profilin complexes in cell motility and other cellular processes. *Trends Cell Biol.* **14**, 461-469.
Witke, W., Sutherland, J. D., Sharpe, A., Arai, M. and Kwiatkowski, D. J. (2001). Profilin I is essential for cell survival and cell division in early mouse development. *Proc. Natl. Acad. Sci. USA* **98**, 3832-3836.
Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S. and Nishida, E. (2002). JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep.* **3**, 69-75.
Zigmond, S. H. (2004). Formin-induced nucleation of actin filaments. *Curr. Opin. Cell Biol.* **16**, 99-105.