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Wnt/Frizzled Activation of Rho Regulates Vertebrate Gastrulation and Requires a Novel Formin Homology Protein Daam1

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Summary

Wnt signaling via the Frizzled (Fz) receptor controls cell polarity and movement during development, but the molecular nature of Wnt/Fz polarity signal transduction remains poorly defined. Here we report that in human cells and during Xenopus embryogenesis, Wnt/Fz signaling activates the small GTPase Rho, a key regulator of cytoskeleton architecture. Wnt/Fz activation of Rho requires the cytoplasmic protein Dishevelled (Dvl) and a novel Formin homology protein Daam1. Daam1 binds to both Dvl and Rho, and mediates Wnt-induced Dvl-Rho complex formation. Inhibition or depletion of Daam1 prevents Wnt/Fz activation of Rho and of Xenopus gastrulation, but not of β-catenin signaling. Our study illustrates a molecular pathway from Wnt/Fz signaling to Rho activation in cell polarity signal transduction.

Introduction

The establishment of cell polarity is a central issue in biology. Cell polarity controls cell division, morphology, movement, and function. While the epithelial apical-basal polarity is well characterized, less is known about the polarity within the epithelia plane, referred to as *p*lanar *c*ell *p*olarity (PCP) (Adler, 1992; Gubb, 1993). An example of PCP is illustrated by the sensory hair cell, which detects sound/motion via graded stereocilia elaborated like a staircase on its apical surface. Disruption of this PCP results in human deafness (Eaton, 1997). Genetic studies of PCP in Drosophila wing and eye development have revealed a group of "core" PCP genes (Shulman et al., 1998; Mlodzik, 1999), most notably *frizzled* (*Dfz1*) and *dishevelled* (*dsh*). *Dfz1* encodes a serpentine receptor that is hypothesized to be a receptor for an unknown extracellular PCP signal, whereas *dsh* encodes a cytoplasmic scaffolding protein (Boutros and Mlodzik, 1999). In vertebrates, Fz/Dsh PCP signaling is essential for cell polarity and movement during gastrulation (Sokol, 2000).

Fz and Dsh families of proteins are components of the conserved Wnt/β-catenin signaling pathway that controls cell fate and proliferation (Wodarz and Nusse, 1998; Miller et al., 1999). Secreted Wnt ligands, upon binding to Fz receptors and the coreceptor LRP5/6 (Wehrli et al., 2000; Tamai et al., 2000; Pinson et al., 2000), signal via Dsh to stabilize β-catenin, leading to the activation of β-catenin-dependent transcription. However, Fz/Dsh PCP function in Drosophila and vertebrates requires neither β-catenin nor other components involved in β-catenin signaling (Strutt et al., 1997; Axelrod et al., 1998; Boutros et al., 1998; Tada and Smith, 2000; Heisenberg et al., 2000; Wallingford et al., 2000), suggesting a distinct transduction pathway. More than a dozen core PCP genes in Drosophila have been identified, many of which encode proteins of unknown functions, including several multi-pass transmembrane proteins (Shulman et al., 1998), implying that PCP regulation is complex. Interestingly, core PCP gene products also include Rho, Rac (Strutt et al., 1997; Boutros et al., 1998; Eaton et al., 1996; Fanto et al., 2000), and the Rho-associated kinase (Drok) (Winter et al., 2001). The Rho family of GTPases, including Rho, Rac, and Cdc42, are molecular switches that regulate cytoskeleton and transcription via cycling between inactive GDP-bound and active GTP-bound forms (Hall, 1998; Kaibuchi et al., 1999). Although *RhoA* genetically interacts with *Dfz1* and *dsh* (Strutt et al., 1997), whether/how Wnt signaling is coupled to Rho function is unknown, and the molecular nature of PCP signal transduction remains obscure.

Here we report that Wnt/Fz signaling directly activates RhoA. Wnt/Fz activation of RhoA requires Dsh and Daam1 (*D*ishevelled-*a*ssociated *a*ctivator of *m*orphogenesis), a novel Formin-homology (FH) protein. Daam1 binds to both Dsh and RhoA, and mediates Wnt-induced Dsh-RhoA complex formation. Daam1 function is essential for Wnt/Fz PCP signaling during Xenopus gastrulation.

Results

Wnt/Fz Signaling Activates Rho

RhoA genetically interacts with *Dfz1*/*dsh* in Drosophila (Strutt et al., 1997), but whether DFz1/Dsh signaling regulates RhoA activity is not known. Previous studies have demonstrated that Rho activation in vivo can be detected via a GST-RBD [*R*ho *b*inding *d*omain, fused with the glutathione S-transferase (GST)] fusion protein that recognizes the GTP-bound Rho (Ren et al., 1999). Using this assay, we found that Fz proteins, upon expression in human 293T cells, exhibit distinct RhoA activation capabilities (Figure 1A). Fz7 is implicated in both the PCP and β-catenin pathways (Sumanas et al., 2000; Medina et al., 2000; Djiane et al., 2000), and indeed activates RhoA (Figure 1A). Fz1 similarly activates RhoA (Figure 1A) and β-catenin signaling (Yang-Snyder et al., 1996) By contrast, Fz5 activates β-catenin signaling (He et al., 1997) but not RhoA, whereas Fz2 activates neither β-catenin nor RhoA (Figure 1A), but may activate a Ca^{2+}-dependent pathway (Sheldahl et al., 1999). Interestingly, Fz1 and Fz7, but not Fz2, can result in Dsh plasma membrane translocation (Rothbacher et al., 2000), which

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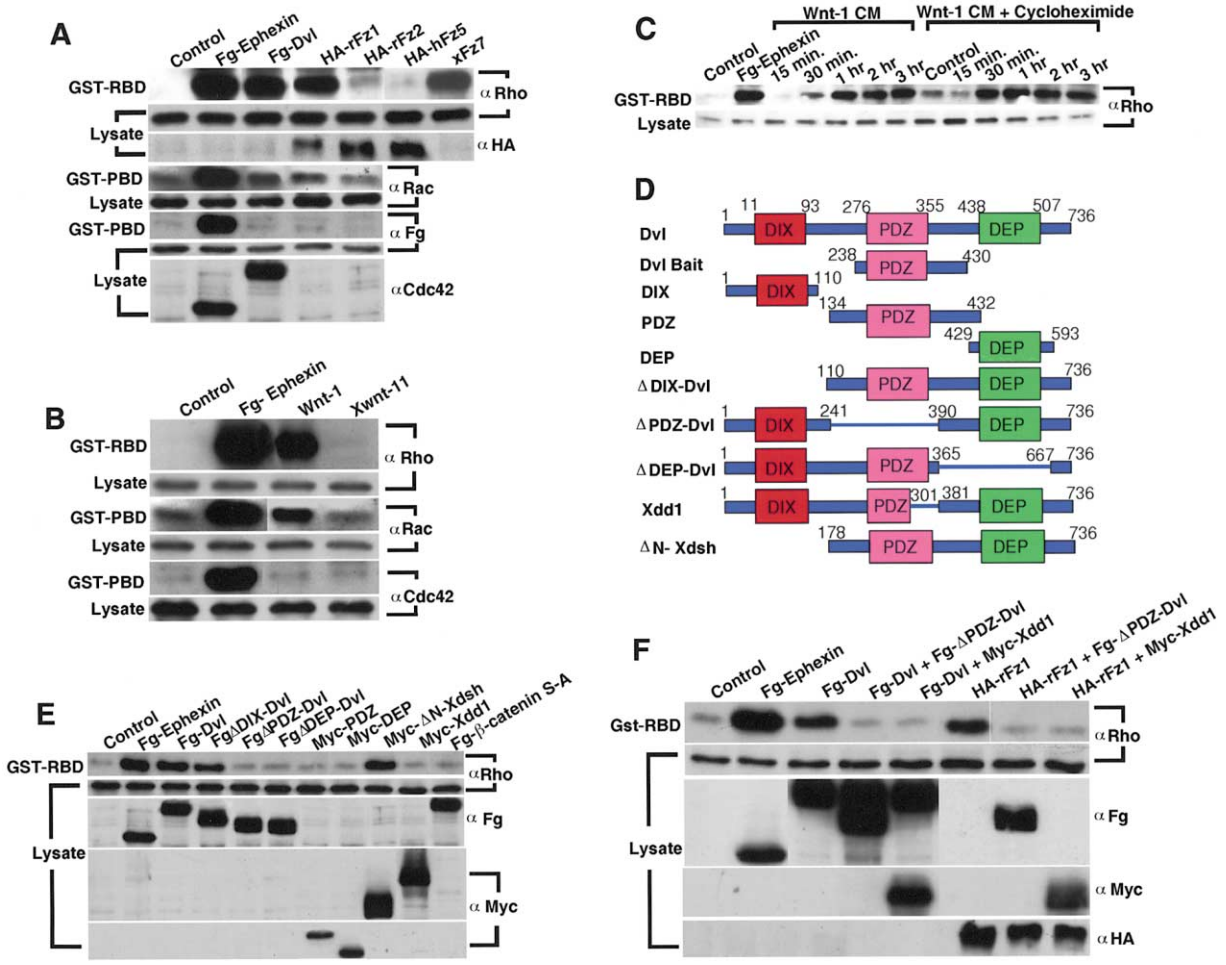

Figure 1. RhoA Is a Direct Downstream Component of Wnt/Fz/Dvl Signaling

(A) Specific Fz proteins and Dvl2 (labeled as Dvl) activate RhoA and possibly Rac, but not Cdc42. Ephexin activates all three GTPases. GTP-bound Rho in cell lysates was precipitated using GST-RBD and detected by RhoA-specific antibodies (Abs). GTP-bound Rac and Cdc42 were precipitated using GST-PBD and detected by Rac- or Cdc42-specific Abs, respectively. Rho, Rac, and Cdc42 in lysates were detected by the above Abs. In all transfection experiments, products from transfected cDNAs were monitored by immunoblotting the lysate with Abs against Flag (Fg), hemagglutinin (HA), or Myc epitope. Fz1 and Fz2 (rat), Fz5 (human), and Fz7 (Xenopus) were used.

(B) Wnt-1 transfection activates RhoA and Rac but not Cdc42. Xenopus Wnt-11 activates none.

(C) Wnt-1 CM, but not control CM, activates RhoA in the absence of new protein synthesis. Cycloheximide does not affect Rho activation, but inhibits cyclinD1 induction (not shown).

(D) Dvl2 and Xdsh constructs. Numbers indicate amino acid positions and the thin line represents internal deletions.

(E) The Dvl2 PDZ and DEP domains, but not the DIX domain, are required for Rho activation. A stabilized β-catenin (S-A) has no effect on Rho activity but causes an 18-fold activation of a TCF-reporter gene (not shown).

(F) Either ΔPDZ-Dvl or Xdd1 blocks Rho activation by Fz or Dvl2.

correlates with Fz/PCP but not Wnt/β-catenin signaling (Axelrod et al., 1998, 2001; but see Boutros et al., 2000). Fz1 also activates Rac weakly but not Cdc42 (Figure 1A), as detected via an analogous assay using GST-PBD (*p*21 *b*inding *d*omain) that recognizes GTP-bound Rac or Cdc42 (Benard et al., 1999; Akasaki et al., 1999). Ephexin, which is a guanine nucleotide exchange factor (GEF) for Rho, Rac, and Cdc42 (Shamah et al., 2001), activates all three GTPases. Thus, specific Fz signaling activates RhoA and possibly Rac.

Wnt-11 regulates PCP in vertebrates (Tada and Smith, 2000; Heisenberg et al., 2000). We thus examined whether Wnt signaling activates RhoA. Surprisingly, Wnt-1, which has been studied primarily in β-catenin signaling, activates RhoA and Rac, but not Cdc42 (Figure 1B). Importantly, Wnt-1 conditioned medium (CM) induces RhoA activation, which is not blocked by cycloheximide, a protein synthesis inhibitor (Figure 1C). Thus, RhoA lies directly downstream of Wnt signaling. Xenopus Wnt-11 activates none of the GTPases in 293T cells (Figure 1B), possibly due to a lack of appropriate receptors since Wnt-11 activates RhoA in embryos (see below).

Fz/Dsh Activation of Rho Reflects PCP Signaling

Dsh is downstream of Fz signaling, and thus may be involved in RhoA activation. Indeed, mouse Dishevelled-2 (Dvl2) activates RhoA and Rac but not Cdc42 (Figure 1A). Other Dsh proteins, including Dvl1 and Dvl3,

and Xenopus and Drosophila Dsh also activate RhoA (not shown). If RhoA activation by Wnt/Fz and Dvl underlies PCP signaling, Fz activation of RhoA should require Dvl but not β-catenin, and Dvl activation of RhoA should correlate with PCP function. Dsh/Dvl proteins have three conserved domains: the amino DIX domain, the central PDZ domain, and the carboxyl DEP domain (Boutros and Mlodzik, 1999; Figure 1D). The DIX domain is essential for β-catenin signaling but dispensable for PCP, whereas both the PDZ and DEP domains are required for PCP function (Axelrod et al., 1998; Boutros et al., 1998; Tada and Smith, 2000; Heisenberg et al., 2000; Wallingford et al., 2000). We tested a panel of Dvl2 mutants (Figure 1D) for RhoA activation. ΔDIX-Dvl (lacking the DIX domain) is fully capable of activating RhoA, but neither ΔPDZ-Dvl (lacking the PDZ domain) nor ΔDEP-Dvl (lacking the DEP domain) is able to do so (Figure 1E). We also tested two Xenopus dsh mutants. ΔN-Xdsh (lacking the DIX domain) is functional in PCP signaling, and indeed activates RhoA, whereas Xdd1 (lacking a part of the PDZ domain) activates neither PCP signaling (Sokol, 1996; Tada and Smith, 2000; Heisenberg et al., 2000; Wallingford et al., 2000) nor RhoA (Figure 1E). Further, Xdd1 is a dominant negative inhibitor for the PCP pathway (Sokol, 1996), and indeed inhibits RhoA activation by Dvl2 (Figure 1F). Thus, we observed a full correlation between Dvl activation of RhoA and its cell polarity function.

We also examined whether Dsh mediates Fz activation of RhoA. We found that Xdd1 or ΔPDZ-Dvl blocks Fz activation of RhoA (Figure 1F), whereas neither β-catenin nor GSK-3 inhibition, which stabilizes β-catenin, activates RhoA or interferes with RhoA activation by Fz or Dvl (Figure 1E and not shown). Therefore, Fz activation of RhoA requires Dvl but not β-catenin, and is not a secondary effect due to β-catenin signaling.

These results provide direct evidence that RhoA is downstream of Wnt/Fz/Dsh signaling. We note that although the DEP domain of Dvl is required and sufficient for JNK (Jun N-terminal kinase) activation (Boutros et al., 1998; Li et al., 1999), it is not able to activate RhoA (Figure 1E), suggesting that JNK activation by Dsh is independent of RhoA.

A Formin-Homology (FH) Protein Daam1 that Binds to Dvl

Because Dvl2 PDZ domain is required for Fz/Dvl signaling to Rho, we searched, via a yeast two-hybrid screen, for proteins associated with the PDZ domain. We isolated two partial cDNAs that encode overlapping carboxyl terminal fragments (Figure 2E) of a novel protein, referred to as Daam1. The widely expressed human Daam1 protein contains 1078 amino acids (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/107/7/843/DC1), and belongs to the family of Formin homology (FH) proteins that have been implicated in cell polarity from yeast to human (Wasserman, 1998). Formin is the product of the *limb deformity* locus and required for limb morphogenesis in mice (Woychik et al., 1990). Daam1 shares 22% to 30% identity with, and thus is distantly related to, several known mammalian FH proteins. Like other FH proteins, Daam1 contains a central proline-rich FH1 domain and a more carboxyl FH2 domain, and represents a novel subfamily that includes a closely related Daam2, Xenopus and zebrafish Daam, and a Drosophila ortholog dDaam. The Daam subfamily exhibits extensive similarity both within and outside the FH1 and FH2 domains, including the amino and carboxyl terminal regions (Supplemental Figure S1). Since several FH proteins bind to Rho, Rac, or Cdc42 (Wasserman, 1998), Daam1 may also bind Rho GTPases.

Daam1 Mediates Wnt-Induced Dvl-Rho Complex Formation

We first examined whether Daam1 interacts with Dvl using epitope-tagged Dvl2, Daam1, or their mutant forms (Figures 1D and 2E). Daam1 binds to both PDZ and DEP domains, which are required for PCP signaling, but not to the DIX domain (Figure 2A). Consequently, Daam1 binds to Dvl2, ΔDIX-Dvl, ΔPDZ-Dvl, or ΔDEP-Dvl (Figure 2C and not shown). The yeast two-hybrid screen suggests that Daam1 carboxyl terminal region is responsible for Dvl binding. Indeed, C-Daam1, which contains the tail region plus the FH1 and FH2 domains (Figure 2E), interacts with Dvl2 (Figures 2A and 2D), whereas N-Daam1, which is the amino terminal quarter (Figure 2E), does not (Figure 2D). Daam1 binding to Dvl is specific, because another FH protein, mDia2, or its mutant ΔGBD-mDia2, which lacks the amino terminus and thus is analogous to C-Daam1 (Tominaga et al., 2000), does not interact with Dvl2 (Figure 2B), reflecting distinct carboxyl terminal regions of Daam1 and mDia2.

We next examined whether Daam1, like some FH proteins, binds to Rho GTPases. Daam1 or N-Daam1, but not C-Daam1, binds to both GDP and GTP-bound RhoA in vitro, with stronger binding toward GTP-bound RhoA (Figure 2F). Daam1 exhibits little, if any, binding to Rac or Cdc42, whereas PAK (p21 activated kinase) binds to either GTP-loaded Rac or Cdc42, but not Rho (Figure 2F). These results suggest that Daam1 contains a Rho binding domain at its amino terminal region.

Since Daam1 binds to Dvl and RhoA, we tested whether Daam1 forms a ternary complex with Dvl and RhoA. We immunoprecipitated the endogenous RhoA from 293T cells treated with or without Wnt-1 CM. Strikingly, we found that the endogenous Dvl protein is associated with RhoA only upon Wnt stimulation (Figure 2G). The Wnt induced Dvl-RhoA complex requires the endogenous Daam1 protein, because this complex is abolished (Figure 2G) by the expression of either N-Daam1, which binds RhoA but not Dvl, or T-Daam1, which is a fragment from the two-hybrid screen (Figure 2E) and thus binds Dvl but not RhoA. These results suggest that Wnt signaling induces a Dvl-Rho complex formation that is assembled via Daam1.

Daam1 Is Required for Wnt/Fz/Dvl Activation of Rho

We next examined whether Daam1 function is required for Rho activation by Wnt/Fz/Dvl signaling. Since either N-Daam1 or T-Daam1 disrupts the Dvl-Rho complex formation, they may represent dominant negative Daam1 mutants. Indeed, either N-Daam1 or T-Daam1, which do not induce RhoA activation, blocks RhoA activation by Wnt-1 CM, Fz, or Dvl, but not by Ephexin (Figures 3A and 3B and not shown), suggesting that Daam1 function

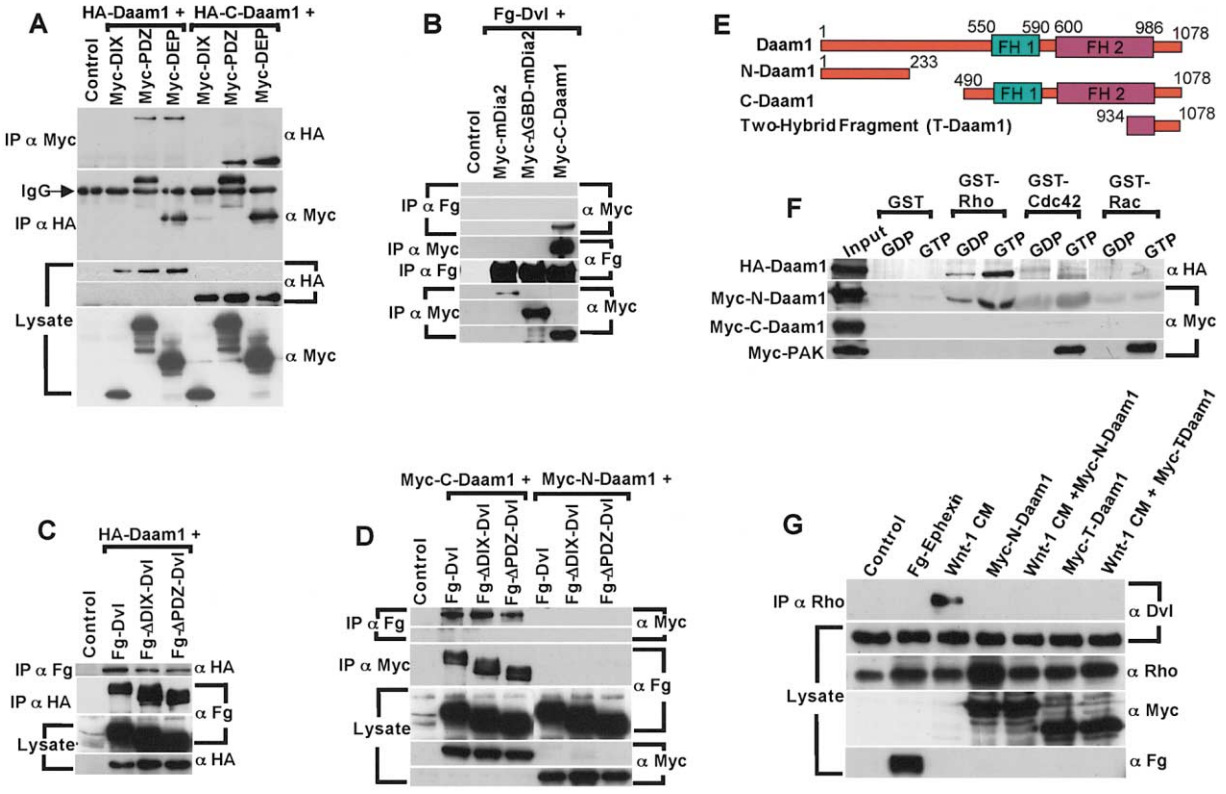

Figure 2. Wnt Induction of a Dvl-Rho Complex Assembled via Daam1

(A–D) Coimmunoprecipitation. Plasmids for tagged Dvl and Daam1 or their mutants were cotransfected, and cell lysates were immunoprecipitated (IP) with indicated Abs. Precipitates were then immunoblotted with indicated Abs (shown on the right side). (A) Daam1 and C-Daam1 bind to the Dvl2 PDZ and DEP domains, but not to the DIX domain. (B) C-Daam1, but neither mDia2 nor ΔGBD-mDia2, binds to Dvl2. (C and D) Daam1 and C-Daam1, but not N-Daam1, bind to Dvl2, ΔDIX-Dvl, or ΔPDZ-Dvl.

(E) Daam1 and mutants. Numbers indicate amino acid positions. T-Daam1 corresponds to one of the two fragments from the yeast two-hybrid screen (the other starts from amino acid position 941).

(F) Daam1 and N-Daam1 interact with GST-Rho-GDP and GST-Rho-GTP in vitro. Lysates from cells transfected with cDNAs for Daam1, its mutants, and PAK were precipitated with GST, GST-Rho, -Rac, or -Cdc42 preloaded with GDP or GTP, and bound Daam1, mutants, or PAK were detected via immunoblotting. (G) Wnt-1 CM induces the endogenous Dvl-RhoA complex formation, which is abolished by either N-Daam1 or T-Daam1 expression. Ephexin, which activates RhoA, does not induce Dvl-RhoA association. Rho was immunoprecipated, and coprecipitated Dvl was detected via immunoblotting via Dvl Abs. Cells were treated with Wnt-1 CM for 3 hours.

is specifically required downstream of Dvl in Wnt/Fz activation of Rho. We also employed double-strand (ds) RNA mediated interference (RNAi) (Elbashir et al., 2001) to deplete endogenous Daam1 protein. The ds RNAi oligo for Daam1 reduces the protein level of Daam1, but not of RhoA, β-catenin (Figure 3C) or Dvl2 (not shown). Importantly, the RNAi oligo inhibits RhoA activation by Wnt-1, Fz, or Dvl, but not by Ephexin (Figure 3C). A control ds RNAi oligo has no effect on Daam1 or RhoA protein level or RhoA activation (Figure 3C). Thus, Daam1 is essential for Wnt/Fz/Dvl activation of RhoA.

An Activated Daam1 Induces Rho Activation in a Rho-GEF-Dependent Manner

Previous studies suggest that deletion of the amino terminal domain results in constitutive activation of FH proteins by releasing an intramolecular inhibition (Watanabe et al., 1999; Tominaga et al., 2000; Westendorf, 2001). C-Daam1 thus may represent an activated form of Daam1. Indeed, while the full length Daam1 is inactive, C-Daam1 overexpression causes activation of RhoA, but not of Rac or Cdc42 (Figure 3D). C-Daam1 activation of RhoA is not affected by ΔPDZ-Dvl (Figure 3A), which inhibits RhoA activation by Fz or Dvl (Figure 1F), further demonstrating that Daam1 functions downstream of Dvl in Rho activation.

Rho, Rac, and Cdc42 elicit distinct morphological changes in cultured mammalian cells (Hall, 1998). Specifically, Rho activation induces stress fiber formation. We found that N-Daam1 disrupts, whereas C-Daam1 enhances, the formation of actin stress fibers (Figure 4A). Thus, the cell biological assay is consistent with the biochemical evidence that Daam1 regulates RhoA activity.

We next examined the potential mechanism of RhoA activation by C-Daam1. GEFs such as Ephexin are a major class of Rho activators (Hall, 1998; Kaibuchi et al., 1999). It is known that dominant negative GTPase mutants, such as RhoA-N19, Rac-N17, and Cdc42-N17, bind tightly to and thereby titrate specific GEFs, and thus can serve as indicators for GEF-dependent processes (Hart et al., 1994). We found that RhoA-N19, but neither Rac-N17 nor Cdc42-N17, blocks RhoA activation by

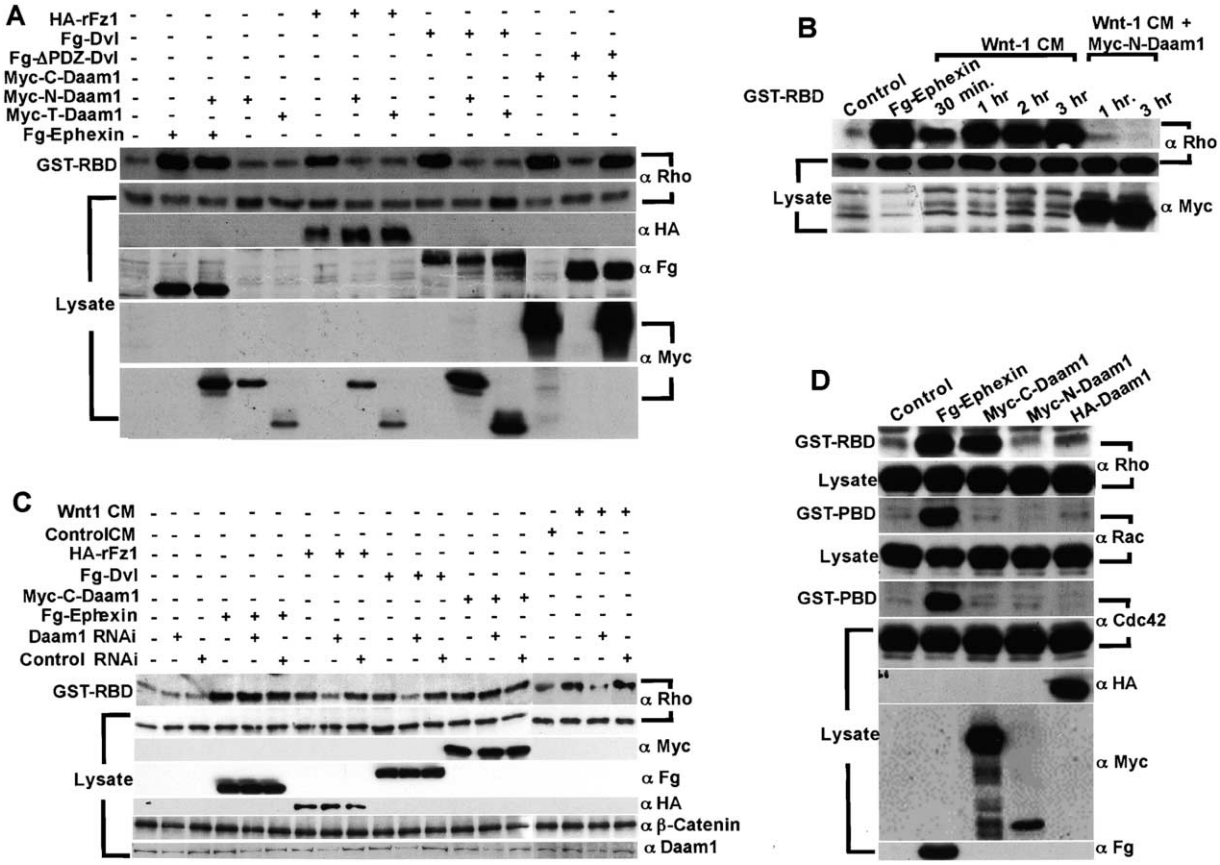

Figure 3. Daam1 Is Required for Rho Activation by Wnt/Fz/Dvl but Not by Ephexin

(A–C) Daam1 functions specifically downstream of Dvl in Rho activation. (A) Either N-Daam1 or T-Daam1 blocks RhoA activation by Fz or Dvl2 but not by Ephexin. ΔPDZ-Dvl2 does not inhibit RhoA activation by C-Daam1. (B) N-Daam1 blocks RhoA activation by Wnt-1 CM. (C) The Daam1 RNAi oligo, but not a control RNAi oligo, inhibits RhoA activation by Wnt-1 CM, Fz, or Dvl, but not by Ephexin or C-Daam1. Daam1 RNAi, but not the control RNAi, reduces the endogenous Daam1 protein to 36 ± 8% of the control level (24 samples of 4 independent experiments), but has no effect on the level of the endogenous RhoA, β-catenin, or Dvl2 protein (not shown), or of the cotransfected cDNA gene products. Note that the C-Daam1 mRNA does not contain the sequence to which the Daam1 RNAi oligo matches, and C-Daam1 protein synthesis is thus resistant to RNAi.

(D) C-Daam1 activates RhoA, but not Rac or Cdc42. The wild type Daam1 activates none.

Wnt/Fz/Dvl, and, importantly, C-Daam1 (Figure 4B). For comparison, RhoA-N19, Rac-N17, and Cdc42-N17 all inhibit Ephexin activation of RhoA (Figure 4B), and of Rac and Cdc42 (not shown), consistent with Ephexin being a GEF for Rho, Rac, and Cdc42 (Shamah et al., 2001). Thus, C-Daam1 activation of RhoA depends on a GEF activity that appears Rho-specific.

As C-Daam1 does not harbor an identifiable GEF motif, we asked whether C-Daam1 might recruit a Rho-GEF. Intriguingly, C-Daam1 in cell extracts is precipitated with GST-RhoA-N19, but barely with GST-Rac-N17 or -Cdc42-N17 (Figure 4C). Because C-Daam1 does not associate detectably with Rho-GTP or Rho-GDP (Figure 2F), these results are indicative of a C-Daam1-associated Rho-GEF, which favors nucleotide-free Rho mimicked by Rho-N19. For comparison, Ephexin is precipitated effectively by GST-Rho-N19, -Rac-N17, -Cdc42-N17, and by GST-Rho, -Rac, and -Cdc42 to a lesser extent (Figure 4C), consistent with Ephexin being a GEF for Rho, Rac, and Cdc42. Thus, C-Daam1 appears to recruit a Rho-GEF.

We also found that an activated form of mDia2, ΔGBD-mDia2 (Tominaga et al., 2000), induces RhoA activation as well (Figure 4D). Thus, FH proteins may activate Rho in other signaling pathways.

Daam1 Mediates Rho Activation by Wnt-11 during Xenopus Gastrulation

To elucidate Daam1 function in vivo, we examined whether Xenopus Daam1, which is expressed throughout embryogenesis (Figure 5A), mediates Wnt-11 PCP signaling in regulating gastrulation movements. Wnt-11 expression and morphogenetic movements occur predominantly in dorsal tissues. Consistently, we found that RhoA is activated in the *d*orsal, but not the *v*entral, *m*arginal *z*one (DMZ versus VMZ) in gastrula embryos (Figure 5B). Wnt-11/Fz PCP signaling is essential for dorsal RhoA activation, because various dominant negative mutants that inhibit Wnt-11 signaling, such as Dn-Xwnt-11 (Tada and Smith, 2000), extra-xFz7 (Djiane et al., 2000), Xdd1 (Sokol, 1996), and importantly, N-Daam1, each prevent RhoA activation in DMZ (Figure 5B). Conversely, activation of Wnt-11 PCP signaling by the ex-

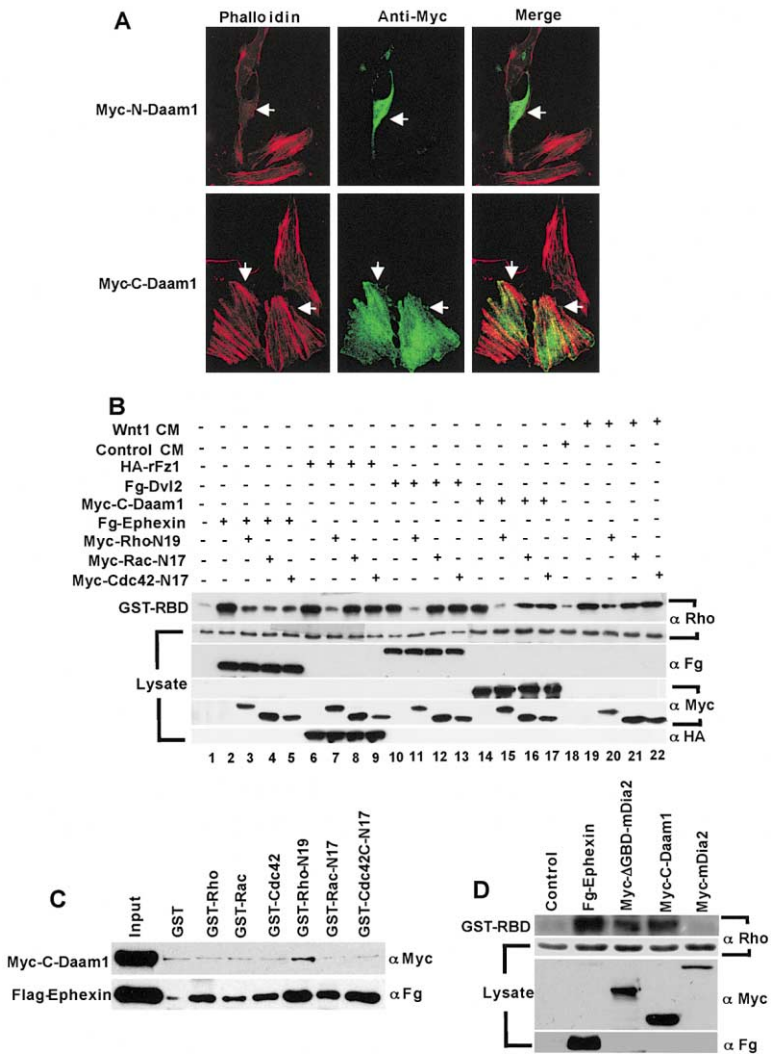

Figure 4. C-Daam1 Affects Stress Fiber Formation and Depends on, and May Associate with, a Specific Rho-GEF for Rho Activation

(A) N-Daam1 collapses, while C-Daam1 induces the formation of actin stress fibers (62 ± 6% and 60 ± 4% of transfected cells, respectively), as visualized with Phalloidin in HeLa cells. Arrows indicate transfected cells.

(B) Rho-N19, but neither Rac-N17 nor Cdc42-N17, blocks RhoA activation by Wnt, Fz, Dvl, or C-Daam1. Rho-N19, Rac-N17, and Cdc42-N17 all inhibit Ephexin activation of RhoA, and of Rac and Cdc42 (not shown).

(C) C-Daam1 in extracts of transfected cells associates with GST-Rho-N19, but not with GST-Rac-N17 or GST-Cdc42-N17, nor with GST-Rho, -Rac, or -Cdc42 (which are likely GDP-bound). Ephexin associates with all the above, but not with GST alone. "Input" represents 1% of C-Daam1 and 10% of Ephexin in extracts incubated with GST-fusion proteins. The low percentage of C-Daam1 precipitated with GST-Rho-N19 may reflect the limiting amount of the putative Rho-GEF associated with C-Daam1.

(D) ΔGDB-mDia2, but not mDia2, activates RhoA.

pression of Xwnt-11, Fz7, or Xdsh, and importantly, C-Daam1 is sufficient to activate RhoA in VMZ (Figure 5C). These results indicate that Wnt-11/Fz PCP signaling, mediated by Xdsh and XDaam1, controls RhoA activity during gastrulation.

Daam1 Regulates Xenopus Gastrulation

To study whether XDaam1 regulates gastrulation, we first examined activin-treated animal pole explants, which represent dorsal tissue and exhibit morphogenetic elongation characteristic of gastrulation (Figure 5D). This elongation is suppressed by N-Daam1, and by Dn-Xwnt-11, extra-xFz7, or Xdd1 as reported (Figure 5D and Supplemental Table S1 at http://www.cell.com/cgi/content/full/107/7/843/DC1). While C-Daam1 alone does not induce elongation (not shown) or affect elongation induced by activin, it fully rescues elongation inhibited by Dn-Xwnt-11, extra-xFz7, or Xdd1 (Figure 5D and Supplemental Table S1). We further employed a Morpholino oligo (MO) that is designed to specifically block endogenous XDaam1 protein synthesis. Inhibition of elongation is observed in explants from embryos injected with the XDaam1 MO, but not a control MO (Figure 5D and Supplemental Table S1). This inhibition is reversed by coinjection with either human Daam1 RNA or C-Daam1 DNA, which direct human Daam1 or C-Daam1 protein synthesis that is resistant to the MO interference (Figure 5D and Supplemental Table S1). Thus, both biochemical and embryological assays suggest that Daam1 functions downstream of Dvl in the Wnt-11/Fz PCP pathway.

Although N-Daam1 blocks elongation movements, N-Daam1 (or C-Daam1) does not inhibit activin induction of mesodermal genes, such as *Brachyury* (*Xbra*), *Goosecoid* (*Gsc*), *Chordin*, and *Xwnt-8* (Figure 5E), indicating that Daam1, like Wnt-11, XFz7, or Xdsh, regulates gastrulation movements without interfering with mesoderm formation (Sokol, 2000). In addition, Daam1, N-Daam1, or C-Daam1 neither induces nor affects Xwnt-8 induction of *Siamois* and *Xnr3* (Figure 5F and not shown), which are activated by Wnt/β-catenin signaling. Therefore, Daam1 function is specifically required for Wnt/Fz PCP but not Wnt/β-catenin signaling.

We further investigated Daam1 function during gastrulation in vivo. Embryos injected dorsally with N-Daam1 RNA develop normally prior to gastrulation (Figure 6A). But from the onset of gastrulation, these embryos exhibit

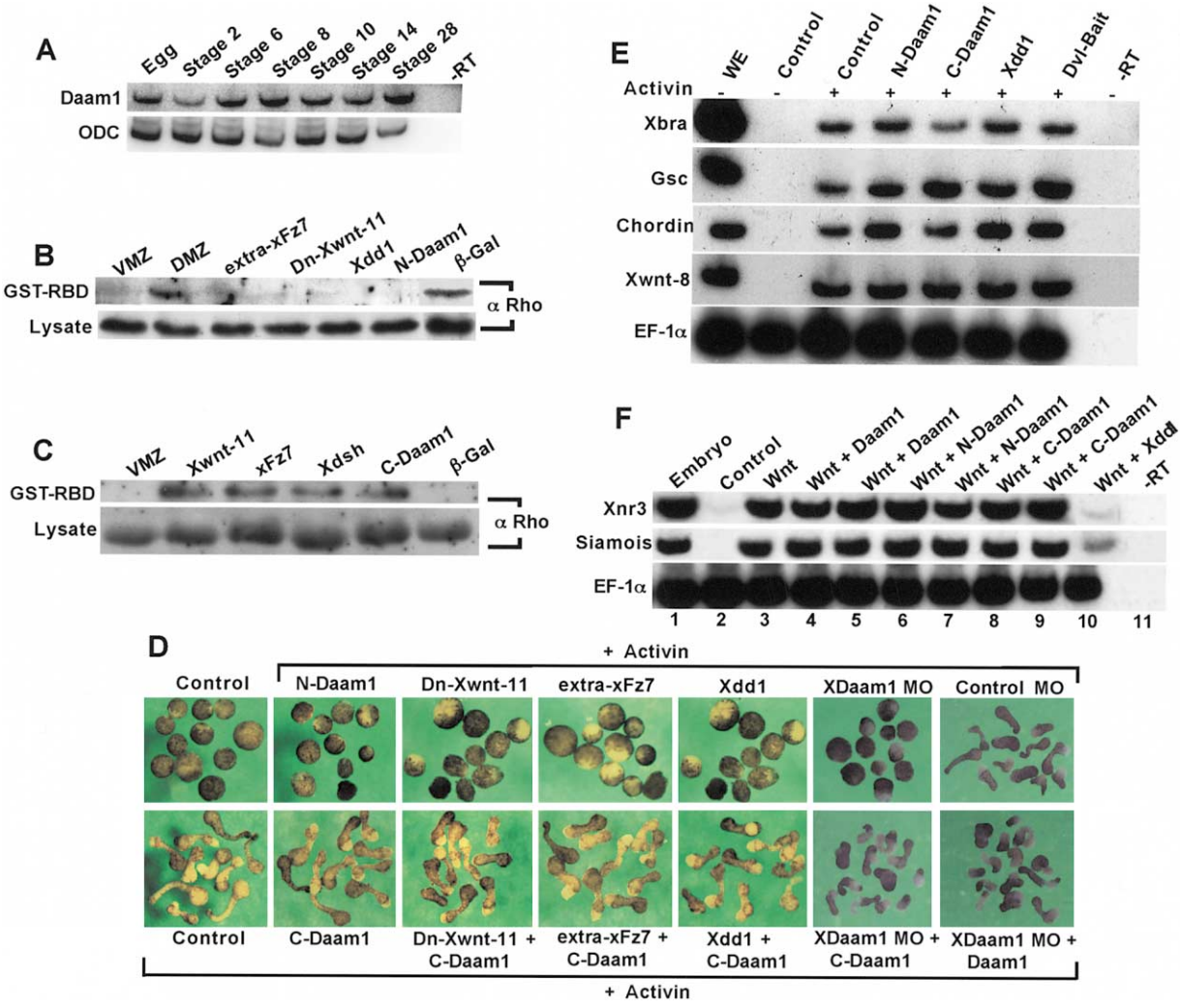

Figure 5. Daam1 Is Required for Dorsal RhoA Activation and Morphogenetic Movements of Animal Pole Explants

(A) Xenopus Daam1 is expressed maternally and throughout embryogenesis as monitored by RT-PCR. ODC is used as an internal control. −RT: without reverse transcriptase. In situ hybridization suggests Daam1 expression throughout embryogenesis (not shown).

(B) Endogenous RhoA activation is detected in DMZ but not in VMZ at stage 10.5, and is inhibited in DMZ by RNAs for Dn-Xwnt-11 (1 ng), extra-xFz7 (1 ng), Xdd1 (1 ng), or N-Daam1 (2 ng), but not by β-galactosidase (β-gal) RNA (2 ng).

(C) RhoA is activated in VMZ by RNAs for Xwnt-11 (200 pg), xFz7 (200 pg), or Xdsh (1 ng), or C-Daam1 DNA (200 pg), but not by β-gal RNA.

(D) In animal pole explants treated with activin, N-Daam1 RNA (2 ng) inhibits elongation, whereas C-Daam1 DNA (200 pg) rescues elongation inhibited by RNAs for Dn-Xwnt-11, extra-xFz7, and Xdd1, or by the XDaam1 MO (40 ng). Human Daam1 RNA (2 ng) also rescues the inhibition by XDaam1 MO.

(E) Neither N-Daam1 nor C-Daam1 affects mesodermal gene induction by activin in animal pole explants, as assayed by RT-PCR at stage 10.5. EF-1α was used as an internal control. WE: whole embryo.

(F) RNA for Daam1 or N-Daam1 (1 or 2 ng), or C-Daam1 DNA (100 or 200 pg), does not affect Xwnt-8 (20 pg) induction of *Xnr3* and *Siamois* in animal pole explants at stage 10.5. Xdd1 RNA (1 ng) inhibits *Siamois* and *Xnr3* induction by Xwnt-8, reflecting dual roles of Xdsh in β-catenin and PCP signaling.

severe morphogenetic defects manifested by a large and open blastopore, exposed endoderm cells, and a shortened body axis and microcephaly (Figure 6A and Supplemental Table S2 at http://www.cell.com/cgi/content/full/107/7/843/DC1). Characteristic of Fz PCP signaling (Tada and Smith, 2000; Wallingford et al., 2000; Djiane et al., 2000), embryos injected dorsally with C-Daam1 also exhibit severe gastrulation abnormalities (Supplemental Table S2). Importantly, embryos injected with the XDaam1 MO, but not the control MO, show similar gastrulation defects, which are rescued by coinjection with human Daam1 RNA (Figure 6A and Supplemental Table S2). Embryos injected ventrally with N-Daam1 RNA or the XDaam1 MO develop rather normally (not shown). Using mesoendodermal and neural markers, we found that embryos injected dorsally with N-Daam1 RNA, while expressing normal levels of specific marker genes, exhibit defects in positioning and morphology of mesoendodermal and neural tissues (Figure 6B). These phenotypes, reflecting normal cell fate specification but abnormal gastrulation, are similar to those resulted from Dn-Xwnt-11, extra-xFz7, or Xdd1,

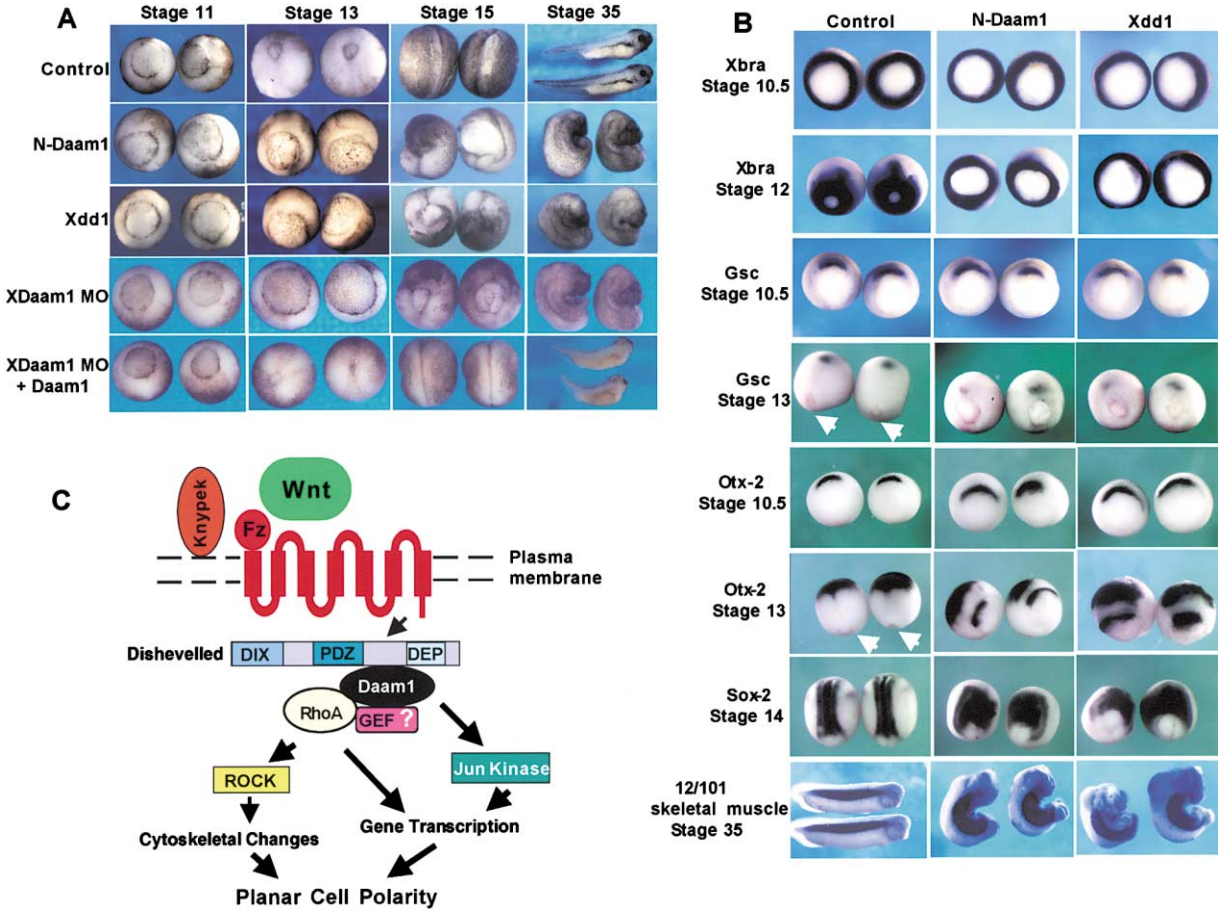

Figure 6. An Essential Role of Daam1 in Gastrulation and in the Wnt/Rho Signaling Pathway

(A) N-Daam1 or XDaam1 MO inhibits gastrulation. RNA (2 ng) for N-Daam1 or Xdd1 (for comparison) or Xdaam1 MO (40 ng) was injected dorsally at 4-cell stage (st). XDaam1 MO effect was rescued by coinjection with human Daam1 (2 ng), but not β-gal (2 ng) RNA.

(B) Embryos injected dorsally with N-Daam1 or Xdd1 RNA. N-Daam1 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-sized blastopore and missing from the involuting notochord at st 12. Gsc expression in control embryos at st 13 is observed in anterior mesoendoderm far from the closed blastopore (arrowheads), but remains in a dorsal region close to the open blastopore in N-Daam1 injected embryo. Otx-2 is expressed anteriorly in both mesodermal and overlying neural tissues in control embyos at st 13. In N-Daam1 injected embryos, two separate Otx-2 expression domains are obvious. A bisection shows that the dorsal margin expression near the blastopore reflects the anterior mesoderm that fails to involute, and the other is in neuroectoderm. Sox-2 is expressed in the neural plate at st 14. In N-Daam1 injected embryos, Sox-2 expression is seen in a broad dorsal region that surrounds the open blastopore and lacks neural plate morphology. Finally, mAb 12/101, which is muscle-specific, detects normal amount of muscle formation along a severe shortened/bent body axis in N-Daam1 injected embryos. N-Daam1 phenocopies Xdd1 in all cases.

(C) A model for the Wnt/Rho signaling pathway (see Discussion).

demonstrating an essential role of Daam1 in Wnt-11 PCP signaling during gastrulation.

Discussion

We demonstrated that Wnt/Fz signaling via Dvl activates RhoA, a key regulator of cell polarity. We identified a novel FH protein, Daam1, which mediates Wnt/Fz activation of RhoA via the assembly of a Wnt-induced Dvl-RhoA complex. We further showed that Daam1 is essential for Wnt/Fz activation of RhoA and for Xenopus gastrulation. These findings elucidate a molecular pathway linking Wnt signaling to Rho in governing cytoskeletal architecture and polarity.

Wnt/Fz Signaling Activates RhoA

Genetic studies have implicated DFz1, Dsh, RhoA, Rac, Drok (ROCK), and several other cytoplasmic and transmembrane proteins in PCP establishment in Drosophila (Shulman et al., 1998; Mlodzik, 1999; Winter et al., 2001). While DFz1, Dsh, and a seven-pass transmembrane cadherin (Flamingo) are interdependent on one another for polarized localization (Strutt, 2001; Axelrod, 2001; Shimada et al., 2001), whether/how PCP gene products function in a common pathway or multiple parallel pathways is unknown. Our results that Fz/Dvl signaling activates RhoA thus provide a direct biochemical link from Fz signaling to Rho. Fz activation of RhoA requires Dsh/Dvl but not β-catenin (Figure 1E), corroborating with the genetic evidence that Fz/Dsh PCP and Wnt/β-catenin signaling are distinct pathways (Axelrod et al., 1998; Boutros et al., 1998). Although genetic analyses have not implicated Wnt signaling in Fz PCP or Rho GTPase regulation, we demonstrated that Wnt-1 and Wnt-11 activate RhoA in human and Xenopus, in a manner independent of new protein synthesis (Figures 1B, 1C, 5B,

and 5C). Thus, Rho activation is a direct Wnt response and likely represents a key pathway for Wnt regulation of cytoskeleton. Wnt/Fz/Dvl activation of RhoA in 293T cells recapitulates many aspects of PCP signaling in embryos, establishing a simple cell model for Wnt/Fz PCP signaling that has been previously refractory to biochemical analyses.

Wnt-11 is implicated in PCP signaling, and indeed induces and is required for RhoA activation during gastrulation. Wnt-1 activation of RhoA is unexpected, however, as Wnt-1 has thus far been associated only with β-catenin signaling. Wnt-1 and certain Fz receptors activate both Rho and β-catenin signaling (Figures 1A–1C), raising the possibility that Wnt-1 (and perhaps other Wnt) functions are mediated by Rho and β-catenin. Several observations are consistent with this notion. First, Wingless (Wnt-1) is required for cell shape changes during Drosophila dorsal closure in a manner that is distinct from β-catenin (McEwen et al., 2000). Second, mammary cells transformed by Wnt-1 show morphological changes, which often reflect activities of Rho GTPases. Third, a stabilized β-catenin does not fully mimic Wnt-1 effects in fibroblasts (Young et al., 1998). Finally, Wnt-1, but not β-catenin, induces the expression of *Wrch-1*, which encodes a Cdc42-related molecule (Tao et al., 2001). Whether Wnt-1 induction of *Wrch-1* is mediated via RhoA remains to be examined.

Daam1: An FH Protein Required for Wnt/Fz Activation of RhoA

We identified a novel Dvl-binding protein, Daam1, a member of FH proteins that have been implicated in cell polarity in eukaryotes (Wasserman, 1998). Daam1 binds to Dvl and RhoA, but not to Rac or Cdc42 (Figures 2A–2F and 4C). Importantly, Wnt signaling induces the formation of a Dvl-RhoA complex that is assembled by Daam1 (Figure 2G). Interference of Daam1 function via Daam1 mutants that disrupt Wnt-induced Dvl-RhoA complex formation, or depletion of Daam1 protein via RNAi, inhibits RhoA activation by Wnt/Fz/Dvl, but not by Ephexin (Figures 3A–3C). Thus Daam1 is specifically required downstream of Dvl in Wnt/Fz activation of RhoA.

Overexpression of C-Daam1, which lacks the amino terminal region and appears constitutively active, induces RhoA, but not Rac or Cdc42, activation (Figure 3D). C-Daam1 activation of RhoA depends on a Rho-GEF, as it is blocked by Rho-N19 (Figure 4B). C-Daam1 is found in a complex with Rho-N19, but not with Rho-GDP or Rho-GTP, nor with any forms of Rac or Cdc42 (Figures 2F and 4C). Thus, C-Daam1 may recruit a Rho-GEF. Another activated FH protein also causes RhoA activation (Figure 4D), suggesting that at least some FH proteins have a capability to promote Rho activation, likely mediated by the conserved FH1 and FH2 domains. FH1 is proline-rich and can interact with Src-homology 3 (SH3) and WW motifs (which mediate protein interactions), the actin-binding protein profilin (Wasserman, 1998), the Src kinase (via SH3) (Tominaga et al., 2000), and Rac (Westendorf, 2001), and possibly other proteins. No binding proteins for FH2 are yet known. It will be critical to identify proteins, including a putative Rho-GEF associated with C-Daam1.

The activated C-Daam1 reveals an autoinhibition mechanism similar to that found in other FH proteins, which are maintained in an inactive state by an intramolecular binding between the amino terminal region and the carboxyl autoregulatory domain (Watanabe et al., 1999; Nakano et al., 1999; Alberts, 2001; Westendorf, 2001). Dvl, which specifically binds to the carboxyl terminus of Daam1 but not of another FH protein (Figure 2B), likely releases such an intramolecular inhibition, thereby allowing Daam1 to function in RhoA activation. Consistent with this notion, expression of the Daam1 carboxyl terminus (T-Daam1) prevents Dvl activation of RhoA (Figure 3A).

The Daam1 amino terminus binds to Rho-GDP or Rho-GTP (Figure 2F), suggesting a role for Daam1 as a scaffolding protein to recruit Rho-GDP (via the amino terminus) and a Rho-GEF (via the C-Daam1 portion), thereby enhancing Rho-GTP formation. The Daam1 amino terminus binds Rho-GTP with apparently higher affinity, raising an intriguing possibility of positive feedback control, a theme common in cell polarization. Polarity establishment relies on signal amplifications that interpret a small difference in a polarity signal field into a polarized cellular response (Gulli and Peter, 2001). DFz1 exhibits a polarized localization that depends on Dsh function (Strutt, 2001; Axelrod, 2001), suggesting a positive feedback loop. Rho-GTP binding to the Daam1 amino terminus may stabilize Daam1 in its activated state, or recruit/activate additional Daam1, thereby promoting an amplification of Rho activation. Such a feedback loop would resemble one in pheromone-induced polarity in yeast. The mating pheromone, via its serpentine receptor and the trimeric G protein, recruits and activates a GEF specific for Cdc42. Activated Cdc42, in turn, is required for the GEF localization, thereby leading to further and polarized Cdc42 activation (Gulli and Peter, 2001). We note that we cannot rule out the possibility that Daam1 may function primarily in such a feedback control. In this scenario, Wnt/Fz signaling initiates Rho activation without Daam1, and the activated Rho together with Dvl recruits/activates Daam1 to amplify Rho activation. In any event, Daam1 function is essential for Rho activation triggered by Wnt/Fz signaling.

Daam1 is distantly related to several distinct mammalian FH proteins, such as FRL (Yayoshi-Yamamoto et al., 2000) (30% identity), FHOS (Westendorf et al., 1999) (27%), mDia1 (Watanabe et al., 1997) (28%), and mDia2 (Tominaga et al., 2000) (22%), whose functions in GTPase signaling remain to be fully understood. FRL and FHOS bind specifically to Rac in a nucleotide-independent manner, and an activated FHOS is antagonized by Rac and Rac mutants, leading to the suggestion that FRL and FHOS are scaffolding proteins linking Rac to other proteins (Yayoshi-Yamamoto et al., 2000; Westendorf, 2001). The mDia subfamily of FH proteins bind to Rho-GTP (and Rho-GDP in some cases), and are proposed to be Rho targets (Nakano et al., 1999; Watanabe et al., 1999; Tominaga et al., 2000). However, as actin fiber induction by the activated mDia can be blocked by inhibition of Rho in some instances (Nakano et al., 1999), and the activated mDia can cause RhoA activation (Figure 4D), the relationship between mDia and Rho, and between FH proteins and Rho GTPases in general, may be complex and needs further investigation.

Daam1 Is Required for Wnt/Fz PCP Signaling during Gastrulation

Vertebrate gastrulation involves polarization and intercalation of dorsal mesodermal cells along the mediolateral axis (convergence), resulting in the elongation of the anterioposterior axis (extension). This morphogenetic process is governed by Wnt-11 PCP signaling (Tada and Smith, 2000; Heisenberg et al., 2000; Wallingford et al., 2000). We found that in Xenopus gastrula, endogenous Rho activation is detected mainly in dorsal tissue, and is abolished when Wnt-11/Fz/Xdsh signaling or Daam1 function is inhibited. Conversely, ectopic Wnt-11/Fz/Xdsh signaling or C-Daam1 activates RhoA on the ventral side (Figures 5B and 5C). Thus, Wnt-11/Fz signaling, via Xdsh and Daam1, is necessary and sufficient for RhoA activation during gastrulation, consistent with the previous finding that interference of Rho function inhibits gastrulation (Wunnenberg-Stapleton et al., 1999). In an explant assay, inhibition or depletion of Daam1 perturbs morphogenetic movements, whereas C-Daam1 restores the movements even when Wnt-11/Fz or Xdsh is inhibited (Figure 5D). Daam1 thus functions downstream of Wnt-11/Fz/Xdsh in governing gastrulation. Finally, inhibition or depletion of Daam1 in the embryo blocks gastrulation and phenocopies the morphogenetic defects caused by inhibition of Wnt-11, Fz, or Xdsh signaling (Figures 6A–6B).

A Wnt/Rho Signaling Pathway

We suggest a molecular pathway for Wnt/Fz activation of Rho (Figure 6C), which we refer to as the Wnt/Rho pathway to distinguish it molecularly from Wnt/β-catenin and Wnt/Ca^{2+} pathways (Wodarz and Nusse, 1998; Miller et al., 1999). A Wnt signal activates a Fz receptor, which translocates Dsh to the plasma membrane and promotes Dsh-Daam1-RhoA complex formation and RhoA activation, likely via the recruitment of a RhoGEF by the Daam1 scaffolding protein. Activated RhoA generates polarized cytoskeleton remodeling via the ROCK kinase (Winter et al., 2001), and perhaps also induces changes in gene expression (Fanto et al., 2000). The zebrafish *knypek* gene product, a glypican, facilitates Wnt signal reception (Topczewski et al., 2001), whereas LRP5/6, which is the Fz coreceptor for Wnt/β-catenin signaling, participates in neither PCP signaling (Wehrli et al., 2000; Semenov et al., 2001) nor RhoA activation (not shown). Whether and how other PCP gene products function in the Wnt/Rho pathway or in parallel pathways remain to be elucidated.

Experimental Procedures

Antibodies

Monoclonal antibodies (mAbs) against HA (F-7), RhoA (26C4), Dvl2 (10B5), and 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 488 goat-anti-mouse Ab and Texas Red X-Phalloidin were from Molecular Probes.

Anti-Daam1 antibodies were generated in chickens against a GST fusion protein containing amino acids 967–1078 of the human Daam1. Egg IgY was purified using the Eggcellent Purification Kit (Pierce), and further affinity purified using the GST-Daam1. The final IgY specifically recognizes Daam1 (119 kD) (not shown).

Plasmids and Oligonucleotides

The mouse Dvl2 and mutants were generated by restriction digestions or site-directed mutagenesis, and subcloned in pCS2+ and/or pCS2+MT (for the Myc tag at the amino terminus). The Flag tag was introduced by PCR at the amino terminus. The human Daam1 cDNA was used with the exception of T-Daam1 (rat). N-Daam1, C-Daam1, and T-Daam1 were amplified by PCR, and like Daam1, were cloned into CS2+, CS2+MT, and/or pcDNA-HA (for the HA tag at the amino terminus). Details are available upon request.

The ds RNAi oligo for human Daam1 (and Daam2) were synthesized by Integrated DNA Technologies: 5′-GCAGGCCAUGCUGCA CUACTT-3′ (sense) and 5′-GUAGUGCAGCAUGGCCUGCTT-3′. A ds RNAi oligo for zebrafish squint gene of identical length was used as the negative control.

The XDaam1 MO complementary to the translational initiation site, 5′-GCCGCAGGTCTGTCAGTTGCTTCTA-3′, was synthesized by Gene Tools Inc. A MO with a random sequence was used as the negative control.

Yeast Two-Hybrid Screen

A rat brain cDNA library (Clontech) was screened using mDvl2 PDZ domain (Figure 1D) as the bait. 3.8 million independent clones were screened, 54 positives were obtained, of which two were Daam1 fragments (Figure 3E).

Transfections

All were done with HEK 293T cells except for Figure 4A (HeLa). Cells in a 6-well plate were transfected via the calcium phosphate method with 2 μg of each indicated plasmid with the exception of Rho-N19, Rac-N17, and Cdc42-N17 (3 μg each), or via Lipofectamine (Life Technologies) with 3 μg annealed RNAi oligo plus 2 μg plasmid. Transfected DNA amounts were equalized via vectors without inserts.

Rho, Rac, and Cdc42 Activation and Binding Assays

Cells were lysed 36 hours (or 72 hours for RNAi) posttransfection in the lysis buffer for Rho (Ren et al., 1999) or for Rac/Cdc42 (Benard et al., 1999). Xenopus DMZ or VMZ was dissected at stage 10.5 and lysed in the Rho lysis buffer. GST-RBD and GST-PBD binding assays were performed as described (Ren et al., 1999; Benard et al., 1999), and samples were resolved by 12% SDS-PAGE and immunoblotted with the anti-Rho, -Rac, or -Cdc42 mAb for cell lysates and anti-Rho pAbs for Xenopus explants. Cycloheximide was added at a final concentration of 10 μg/ml, starting one hour prior to Wnt-1 CM treatment.

Extracts of transfected cells were lysed and precipitated by GDP or GTP-loaded GST-Rho, -Rac or -Cdc42 (Figure 2F), or GST-Rho (or N19), -Rac (or N17) -Cdc42 (or N17) (Figure 4C) as described (Lu and Settleman, 1999). Samples were resolved by 12% SDS-PAGE and immunobloted with anti-Myc, -HA, -Flag mAbs.

Immunoprecipitation, Immunoblotting, and Immunocytochemistry

Cells were lysed 36 hours posttransfection in 0.5% NP40 lysis buffer for Dvl/Daam1 interactions or the Rho lysis buffer for Dvl/Rho complex detection. Lysates were precipitated with anti-Myc (pAbs), or anti-Flag, -HA, -Rho mAb, resolved by 12% SDS-PAGE and blotted with anti-Myc, -Flag, -HA, -Rho, or -Dvl2 mAb. Immunocytochemistry (Figure 4A) was performed as described (Tominaga et al., 2000).

Embryo Manipulations, RT-PCR, In Situ Hybridization, and Explant Assays

These were performed as described (Kato et al., 1999). Embryo injections were done with in vitro transcribed RNAs, except for C-Daam1, which was injected as DNA plasmid. Convergent extension assays in explants were performed as described (Sokol, 1996) using 20 ng/ml activin (final).

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