$G\alpha_o$ mediates WNT-JNK signaling through Dishevelled 1 and 3, RhoA family members, and MEKK 1 and 4 in mammalian cells

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Summary

In *Drosophila*, activation of Jun N-terminal Kinase (JNK) mediated by Frizzled and Dishevelled leads to signaling linked to planar cell polarity. A biochemical delineation of WNT-JNK planar cell polarity was sought in mammalian cells, making use of totipotent mouse F9 teratocarcinoma cells that respond to WNT3a via Frizzled-1. The canonical WNT–β-catenin signaling pathway requires both $G\alpha_o$ and $G\alpha_q$ heterotrimeric G-proteins, whereas we show that WNT-JNK signaling requires only $G\alpha_o$ protein. $G\alpha_o$ propagates the signal downstream through all three Dishevelled isoforms, as determined by epistasis experiments using the Dishevelled antagonist Dapper1 (DACT1). Suppression of either Dishevelled-1 or Dishevelled-3, but not Dishevelled-2, abolishes WNT3a activation of JNK. Activation of the small GTPases RhoA, Rac1 and Cdc42 operates downstream of Dishevelled, linking to the MEKK 1/MEKK 4-dependent cascade, and on to JNK activation. Chemical inhibitors of JNK (SP600125), but not p38 (SB203580), block WNT3a activation of JNK, whereas both the inhibitors attenuate the WNT3a–β-catenin pathway. These data reveal both common and unique signaling elements in WNT3a-sensitive pathways, highlighting crosstalk from WNT3a-JNK to WNT3a–β-catenin signaling.

Supplementary material available online at
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Key words: WNT, β-catenin, JNK, Heterotrimeric G-proteins, Frizzled, $G\alpha_o$, Dishevelled, Planar cell polarity

Introduction

WNT proteins are secreted, palmitoylated and glycosylated ligands that act to regulate signaling pathways in development, including the canonical (WNT–β-catenin) and non-canonical [planar cell polarity (PCP) and WNT–cyclic-GMP–Ca^{2+}] pathways operating through the G-protein-coupled receptors Frizzled (FZ) (Bhanot et al., 1996; Liu et al., 1999). For the canonical pathway, in the absence of WNT, cellular β-catenin is degraded by a protein complex that includes, among other proteins, Axin, the product of the *adenomatous polyposis coli* gene, and the Ser/Thr protein kinase glycogen synthase kinase 3β (GSK3β), whose phosphorylation of β-catenin targets the phosphoprotein for proteasome-mediated degradation. WNT3a binding to Frizzled-1 (FZ1) leads to activation of the G-proteins $G\alpha_o$ and $G\alpha_q$ and of the phosphoprotein Dishevelled (DVL), resulting in reduced GSK3β activity and ultimately to increased accumulation of β-catenin. Nuclear accumulation of β-catenin follows stimulating activation of lymphoid-enhancer factor/T-cell factor (LEF/TCF)-sensitive transcription of developmentally related genes (Behrens et al., 1996; Molenaar et al., 1996). For the non-canonical pathways, WNT proteins regulate cyclic GMP, phosphodiesterase and Ca^{2+} (Wang and Malbon, 2003) as well as Jun N-terminal kinase (JNK) and PCP (Barrow, 2006). Recently, an obligate role for the heterotrimeric G-protein G_o has been revealed in WNT-JNK signaling to PCP in the fly (Katanaev et al., 2005). The phosphoprotein Dishevelled (fly DSH/vertebrate DVL) mediates not only the canonical WNT–β-catenin pathway but also the PCP pathway in both *Drosophila* and vertebrates (Axelrod et al., 1998; Boutros et al., 1998; Heisenberg et al., 2000; Sokol, 1996). Genetic epistasis experiments of the WNT canonical pathway made possible in the fly reveal DSH to be downstream of G_o (Katanaev et al., 2005). For mammals, biochemical delineation of the WNT-JNK pathway – for example the role, if any, for G-proteins or the individual roles of the three mammalian DVL proteins (only a single gene encoding DSH is found in the fly) – has not been reported.

Here, we attempt a biochemical description of the major features of mammalian WNT-JNK signaling by application of several complementary strategies. We detail an obligate role for $G\alpha_o$, DVL1 and DVL3, small-molecular-weight GTPases of the Rho family and mitogen-activated protein kinase kinase kinase 1 (MEKK 1)- and mitogen-activated protein kinase kinase kinase 4 (MEKK 4)-dependent signaling to JNK. In addition, we reveal divergence between the WNT3a signaling pathway controlling β-catenin accumulation and LEF/TCF-sensitive transcription and that regulating the activation of JNK. We also reveal some crosstalk from JNK to the WNT–β-catenin signaling pathway.

Results

WNT3a activates JNK in mouse F9 cells

Totipotent mouse embryonal F9 cells transfected to express rat Frizzled-1 (rFZ1) provide an optimal system for the biochemical analysis of WNT-FZ1 signaling (Malbon, 2005). WNT3a stimulates the formation of primitive endoderm, characteristic of early mouse development (Liu et al., 1999). We tested whether WNT3a stimulation of F9 cells expressing rFZ1 is capable of

activating JNK by immunoblotting SDS-PAGE gel blots with antibodies specific for phospho-Jun, the primary product of JNK activation, as well as by measuring the AP-1–luciferase gene reporter activity (Fig. 1). Addition of purified WNT3a to the rFZ1 cultures stimulates activation of JNK and was adopted as the primary read-out for JNK activity. The activation of JNK in response to WNT3a is rapid, reaching a peak within 15 minutes of treatment with WNT3a. In either wild-type F9 cells or F9 cells expressing the empty vector (pcDNA) alone, WNT3a fails to stimulate activation of JNK (Fig. 1A). Similarly, WNT3a showed robust stimulation of AP-1–luciferase reporter activity in rFZ1-expressing cells (Fig. 1B). By contrast, treating the cells with WNT5a, which activates Frizzled-2, failed to activate JNK (supplementary material Fig. S1). The ability of WNT3a to stimulate JNK activity also was measured in human embryonic kidney 293 cells (HEK 293 cells). Similar to rFZ1-expressing cells, wild-type HEK 293 cells showed a sharp stimulation of JNK activity and LEF/TCF-sensitive transcription upon WNT3a treatment (supplementary material Fig. S2A,B). The expression of Frizzled-1 along with other Frizzled homologs in HEK 293 cells was confirmed by RT-PCR (supplementary material Fig. S2C). Similar activation of JNK by WNT3a has also been reported in WEHI-231, BAJI and Raji B-cells (Shi et al., 2006) as well as in HEK cells (Habas et al., 2003). Thus, F9 cells expressing rFZ1 were adopted as a model for studies of the WNT3a-JNK signaling pathway in mammalian cells.

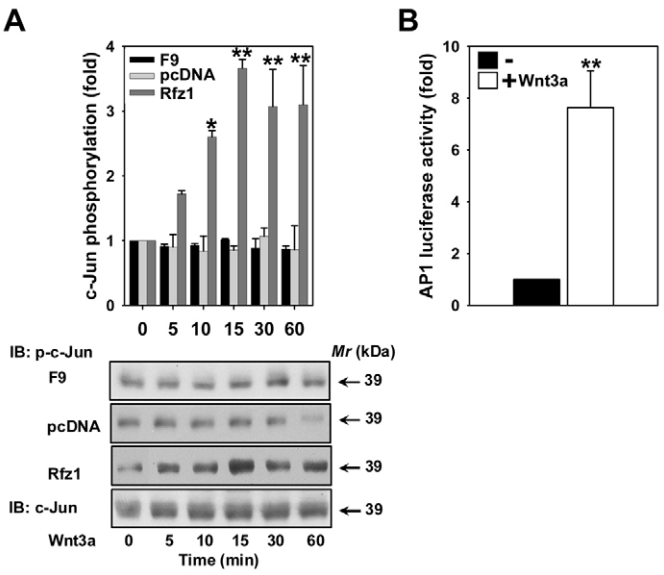

Fig. 1. WNT3a stimulates activation of JNK and the AP-1–luciferase reporter in F9 cells. (A) Confluent wild-type F9 clones (F9) or F9 cells transfected with pCDNA3.1 alone (pcDNA) or F9 cells stably expressing rat Frizzled-1 (rFZ1) were treated with purified WNT3a for 0 to 60 minutes and JNK activation was measured by immunoblotting total lysates with phospho-specific antibodies against Jun. (B) rFZ1-expressing cells were transfected with 30 ng of AP-1–luciferase reporter plasmid in a 12-well plate for 24 hours, followed by 24 hours of serum starvation. The cells then were treated with or without WNT3a for 7 hours and luciferase gene reporter assays were performed, as described in the Materials and Methods. The upper panel displays the mean values±s.e.m. obtained from three independent experiments; the lower panel displays corresponding representative blots. For the AP-1-luciferase assay, the data represent the mean values±s.e.m. obtained from three independent replicate experiments. *P<0.05; **P<0.01 versus the time 'zero' control.

Heterotrimeric G-proteins mediate activation of JNK in response to WNT3a

Activation of heterotrimeric G-proteins has been shown to be obligate for the WNT–β-catenin pathway in mammalian cells, *Xenopus*, zebrafish embryos, as well as in *Drosophila* (Malbon, 2005). To date, however, there is no biochemical evidence for the involvement of heterotrimeric G-proteins in WNT-JNK signaling. To ascertain whether WNT-JNK signaling was operating through heterotrimeric G-proteins, we made use of treatment with pertussis toxin, which inactivates members of the G_o/G_i G-protein family (including G_{i1}, G_{i2}, G_{i3}, G_o and G_t) by catalyzing ADP-ribosylation of the alpha-subunit of these G-proteins. Pretreating the cells with pertussis toxin abolishes the ability of WNT3a to activate JNK (Fig. 2A). As $G\alpha_o$ has been shown previously to be a major target of pertussis toxin action in the WNT3a–β-catenin pathway in F9 cells (Bhanot et al., 1996; Liu et al., 1999), we tested its potential role in WNT3a-JNK signaling directly. We suppressed expression (through knockdown) of individual G-protein alpha subunits. Cells were treated for 72 hours with short-interfering RNAs (siRNAs) designed specifically to suppress the alpha-subunit of $G\alpha_o$, $G\alpha_q$ or $G\alpha_{11}$. Under these conditions of siRNA treatment, suppression of G-protein alpha subunits by ~70% or more was achieved (Fig. 2). Treatment with siRNAs targeting $G\alpha_o$ abolishes the ability of WNT3a to stimulate JNK activation in the treated cells (Fig. 2B). The obligate requirement for $G\alpha_q$ and $G\alpha_o$ for WNT3a-stimulation of β-catenin accumulation provoked us to evaluate the role of $G\alpha_q$ in the WNT3a-JNK pathway (Fig. 2C). Treating cells with siRNAs targeting $G\alpha_q$, unlike those targeting $G\alpha_o$, had no effect on the WNT3a-stimulated JNK activity (Fig. 2B,C). These data demonstrate a crucial divergence, at the level of heterotrimeric G-proteins, of the WNT–β-catenin and WNT-JNK pathways in mammalian cells. Treating the cells with siRNAs targeting an additional, but unrelated, G-protein alpha subunit, $G\alpha_{11}$, also showed no effect on the ability of WNT3a to activate JNK (Fig. 2D). If the hypothesis that $G\alpha_o$ mediates activation of JNK by WNT3a is correct, expression of a constitutively active (CA), GTPase-deficient mutant (Q205L substitution) of $G\alpha_o$ would be expected to activate JNK, even in the absence of WNT3a. Transient expression of the Q205L mutant version of $G\alpha_o$ indeed stimulates JNK activity, mimicking the effect of WNT3a stimulation (Fig. 2E). Taken together, these observations demonstrate that the WNT-JNK signaling pathway is mediated by the G-protein $G\alpha_o$ in mammalian cells.

Role of DVL-1, -2 and -3 in WNT3a-stimulated activation of JNK

Dishevelled is a cytoplasmic phosphoprotein that acts downstream of the WNT-FZ-$G\alpha_o$ signaling axis (Katanaev et al., 2005). Unlike the genome of the fly that encodes a single DSH protein, the mouse genome encodes three Dishevelled isoforms designated DVL1, DVL2 and DVL3. All three DVL proteins were found to be expressed in mouse F9 cells (Fig. 3). We were keen to investigate whether the individual DVL proteins play unique or common roles in WNT3a-JNK signaling (Fig. 3). siRNAs specifically targeting each isoform were designed, tested for their specificity and then employed to selectively suppress individual DVL isoforms in rFZ1-expressing cells. The siRNA reagents specifically suppressed individual DVL proteins, achieving a >70% reduction in the expression of each protein (Fig. 3D). Scrambled siRNAs designed by the commercial supplier were tested as controls in some subsets; they showed no capacity to suppress either DVL expression or

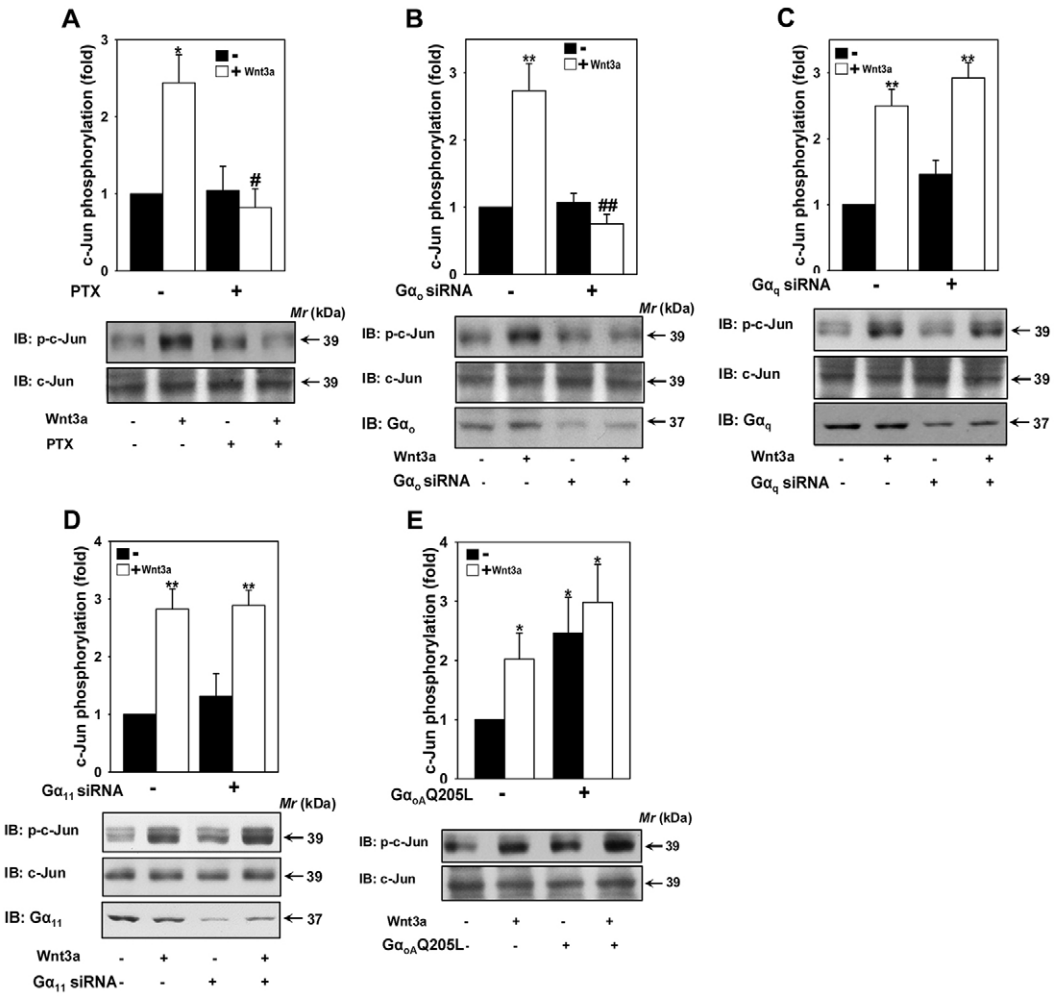

Fig. 2. Suppression of Gα$_o$, but not Gα$_q$ or Gα$_{11}$, abolishes, whereas expression of constitutively active Gα$_o$ mimics, the activation of JNK by WNT3a. (A) Confluent F9 cells expressing rFZ1 were treated with pertussis toxin (50 ng/ml) for 1 hour followed by WNT3a treatment for 15 minutes. Cell lysates were collected and JNK activity was assayed by probing Jun phosphorylation. (B-D) F9 cells expressing rFZ1 were treated with siRNAs specific for Gα$_o$ (B), Gα$_q$ (C) or Gα$_{11}$ (D) for 72 hours before treatment with WNT3a for 15 minutes. JNK activity was then determined by probing Jun phosphorylation. (E) F9 cells expressing rFZ1 were transfected with an expression vector harboring the Q205L Gα$_o$ mutant (0.25 μg/well in a 12-well plate). 48 hours after transfection, cells were treated with WNT3a for 15 minutes, cell lysates collected and probed with phospho-Jun-specific antibodies. The upper panel displays mean values±s.e.m. obtained from three independent experiments; the lower panel displays corresponding representative blots. *P<0.05; **P<0.01 versus the –WNT3a control; $^{\#}$, P<0.05; $^{\#\#}$, P<0.01 versus the +WNT3a control. The extent of knockdown of the expression of the G-protein alpha-subunit routinely was 70% or more.

WNT3a-induced JNK activation (data not shown). We then measured the ability of WNT3a to stimulate JNK activity in cells in which an individual DVL was suppressed. Suppression of DVL1 and DVL3 isoforms provoked a loss of the ability of WNT3a to stimulate JNK activity (Fig. 3A,C), whereas suppression of the DVL2 isoform interestingly had no effect on WNT3a-stimulated JNK activity (Fig. 3B). To test further the selectivity of the siRNAs for their targeted DVL proteins as well as the possible existence of 'off-target' effects of the siRNAs on WNT3a-induced activation of JNK, we performed 'rescue' experiment in the knocked-down cells, employing individual expression constructs harboring the entire coding region of the human DVL1 and human DVL3, each fused to a C-terminal sequence encoding EGFP. The expression of exogenous DVL rescued the ability of WNT3a to activate JNK in those cells in which the response had been blocked by knockdown of the targeted DVL (Fig. 3E,F). Thus, the ability of knock-down of either DVL1 or of DVL3 to block WNT3a-stimulated activation of JNK appears to reflect loss of the targeted DVL and is not an 'off-target' effect. The basal activity of JNK increased in the cells expressing the exogenous DVL (Fig. 3E,F), probably reflecting a functional consequence of 'over'-expression of the DVL protein. It should be noted that the effects of knockdown of DVL1, by contrast, could not be rescued by expression of hDVL3 and vice versa (data not shown).

To explore further the roles of individual DVL proteins in mediating the activation of JNK, we examined the effect of expression of exogenous DVL isoforms on JNK activity alone (in the absence of WNT3a). HA-tagged versions of DVL1, DVL2 and DVL3 were engineered into a mammalian expression vector and employed in transient transfections of rFZ1-expressing cells (supplementary material Fig. S3). Expression of DVL2 or DVL3 increases JNK activity (P<0.05 for DVL2; P<0.01 for DVL3). As the DVL proteins were each tagged with the hemagglutinin (HA) antigen, we could ascertain by immunoblotting their relative levels of expression in the F9 cells. The relative levels of HA-tagged DVL proteins expressed individually in these cells are as follows:

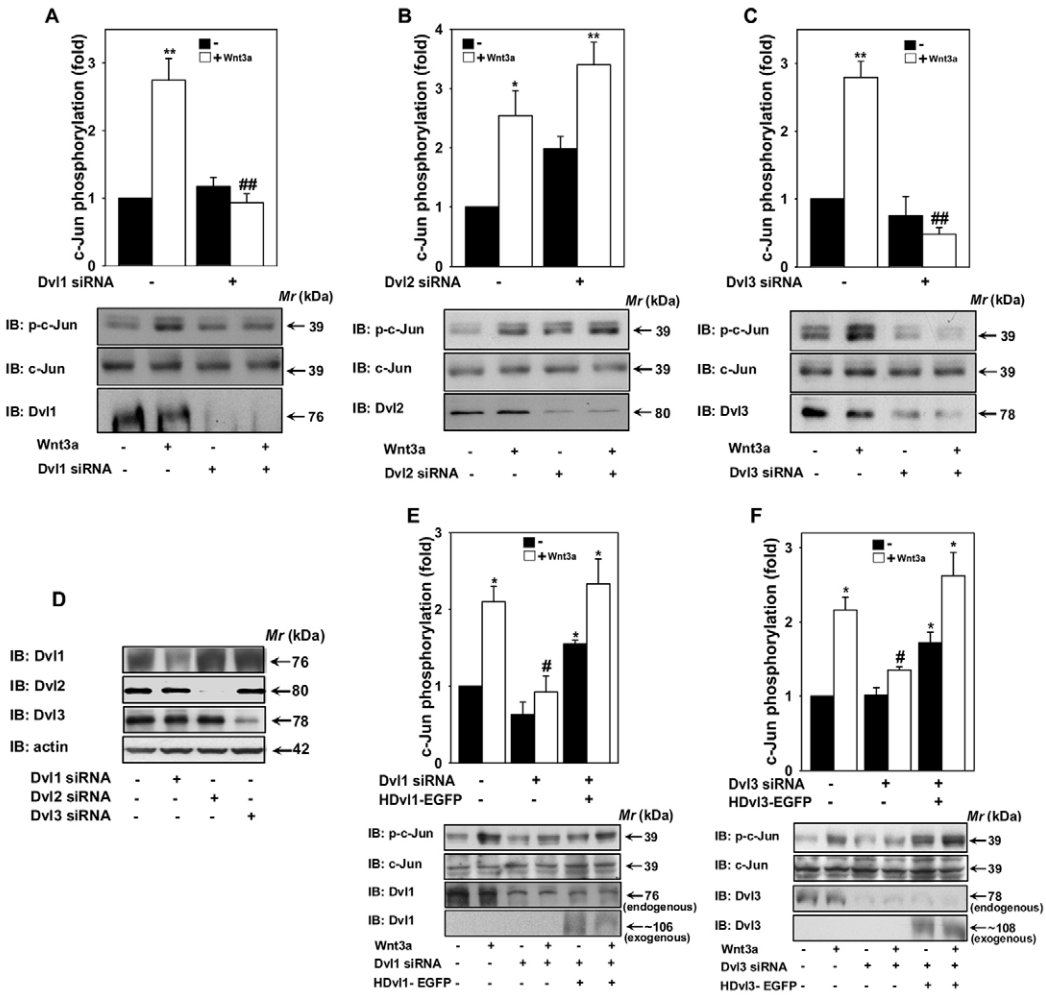

Fig. 3. Suppression of DVL1 and DVL3, but not of DVL2, abolishes the ability of WNT3a to stimulate JNK activation, whereas expression of exogenous human DVL1 and DVL3 rescues the effect. F9 cells expressing rFZ1 were treated with siRNAs designed to suppress the expression of DVL1 (A), DVL2 (B) and DVL3 (C) for 48 hours, and JNK activity was measured by probing Jun phosphorylation, as described in the Materials and Methods. Specific suppression of individual DVL isoforms was demonstrated by immunoblotting with isoform-specific antibodies. The extent of suppression of DVL isoforms is as follows; DVL1 (71%), DVL2 (81%) and DVL3 (85%). The siRNAs were specific to the particular isoform as no cross-reaction was detected (D). Rescue experiments were performed by transfection of human (h) DVL1 (E) or DVL3 (F) into rFZ1-expressing cells in which DVL1 and DVL3 were knocked-down, respectively, by siRNA treatment as described in the Materials and Methods. The upper panel displays mean values±s.e.m. obtained from three independent experiments; the lower panel displays the corresponding representative blots.*P<0.05; **P<0.01 versus –WNT3a control; $^{\#}$, P<0.05; $^{\#\#}$, P<0.01 versus +WNT3a control.

1:1.4:0.8 (DVL1:DVL2:DVL3). Although the overexpression level of DVL3 is the lowest, DVL3 overexpression resulted in the greatest level of JNK activation (supplementary material Fig. S3).

Gα$_o$ operates upstream of DVL

Epistasis experiments in the fly suggest that the G-protein Gα$_o$ is upstream of DSH in FZ-wingless signaling (Katanaev et al., 2005), whereas the precise positioning of G-proteins and Dishevelled for WNT signaling in mammals is unknown (Malbon, 2005). To resolve this issue, we made use of a Dishevelled-interacting protein, Dapper1 (DACT1), that acts as a negative modulator of WNT–β-catenin signaling by antagonizing DVL function (Cheyette et al., 2002). Dapper1 was expressed in F9 cells to test its effect on WNT3a-stimulated JNK activation. Cells were transiently transfected with an expression vector harboring a Myc-tagged version of mouse Dapper1 and the cells were tested for JNK activation in response to WNT3a. The expression of mouse Dapper1 was verified by immunoblotting with antibodies against Myc (Fig. 4A). Expression of mouse Dapper1 abolished the ability of WNT3a to activate JNK (Fig. 4A). We next tested whether DVL functions downstream of Gα$_o$ by comparing the ability of the constitutively active Q205L Gα$_o$ to activate JNK activity in the absence versus the presence of mouse Dapper1 expression (Fig. 4B). Expression of mouse Dapper1 effectively abolishes the ability of Q205L Gα$_o$ to stimulate JNK activation. Taken together, these observations suggest that, in the WNT3a-JNK signaling pathway, Gα$_o$ is upstream of the DVL proteins (Fig. 4B). We further probed whether there was a possible interaction of mouse Dapper1 with individual DVL proteins (Fig. 4C). Co-immunoprecipitations (co-IPs) were performed from lysates of F9 cells expressing an individual HA-tagged DVL isoform together with, or without, the Myc-tagged Dapper1. The cell lysates were mixed with antibodies against HA coupled to sepharose beads and the adsorbed HA-tagged DVL proteins were subjected to pull-

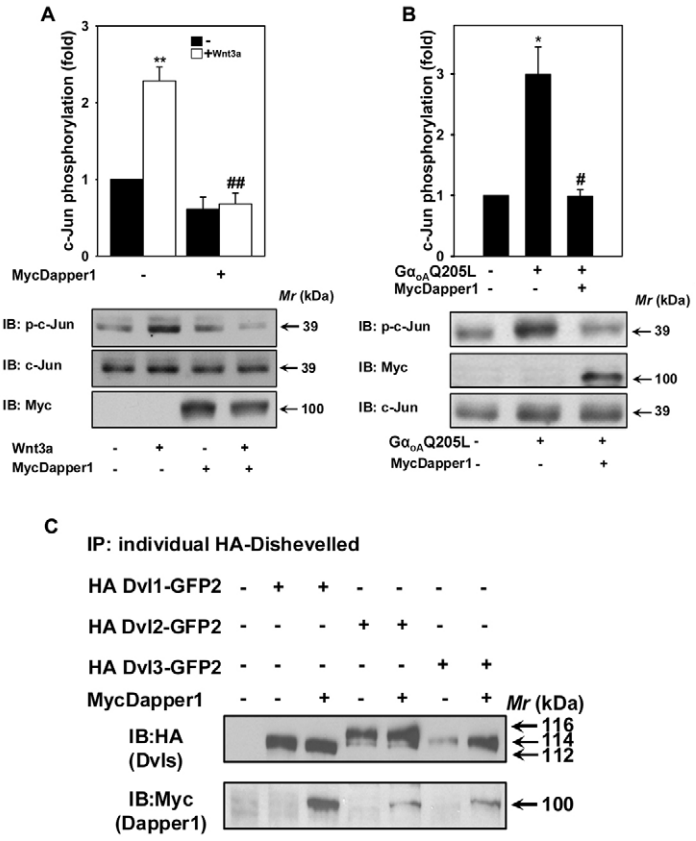

Fig. 4. Expression of Dapper1 abolishes the ability of WNT3a, as well as the ability of expression of Q205L $G\alpha_o$ to stimulate JNK activation. F9 cells expressing rFZ1 were either transfected (1 μg/well in a 12-well plate) with an expression vector harboring Myc-tagged mouse Dapper1 (A) or co-transfected with Myc-Dapper1 and Q205L $G\alpha_o$ (B) for 24 hours, and JNK activity was determined, as described in the Materials and Methods. The expression of Dapper1 was monitored by immunoblotting with antibodies against Myc. (C) F9 cells were either transfected (1 μg/well in a 6-well plate) with individual HA-DVL-GFP2 or co-transfected with HA-DVL-GFP2 and MycDapper1 (1 μg/well of each plasmid) for 24 hours followed by cell lysis and affinity pull-downs with anti-HA-sepharose beads. The interaction of Dapper1 with individual isoforms of DVL was made visible by probing the blots with the antibody against Myc. The expression of the exogenous DVL-GFP2 was established by stripping the Myc blot and probing with an antibody against HA. The upper panel displays mean values±s.e.m. obtained from three independent experiments; the lower panel displays the corresponding representative blots. The blot of the DVL-Dapper1 interaction is representative of two independent experiments that proved highly reproducible. *P<0.05; **P<0.01 versus –WNT3a control; $^{\#}$, P<0.05; $^{\#\#}$, P<0.01 versus +WNT3a control. The expression of DVL3 appears low in the absence of Dapper 1, but this was not a consistent observation.

downs. Dapper1 was identified in the pull-downs from DVL1, DVL2 and DVL3 (Fig. 4C). The levels of Dapper1 expression were found to be equivalent in all of the co-transfection experiments (data not shown). Similar interactions of Dapper1 with DVL1 (Cheyette et al., 2002) and DVL2 (Zhang et al., 2006) have been reported previously.

Small-molecular-weight GTPases function in WNT3a activation of JNK

Signaling elements downstream from DVL for the WNT-JNK pathway in the fly, *Xenopus* and mammalian cells include the members of the small-molecular-weight GTPases (Fanto et al., 2000; Habas et al., 2003; Kim and Han, 2005; Kishida et al., 2004; Shi et al., 2006), including Rho, Rac1 and Cdc42. Similarly, $G\alpha_{13}$ regulates JNK activity through an interaction of two signaling cascades involving small-molecular-weight GTPases coupled downstream to a mitogen-activated protein kinase cascade (Lee et al., 2004). First, we examined the activation status of RhoA and two other GTPases, Rac1 and Cdc42, in rFZ1-expressing cells following stimulation with WNT3a. The activation of RhoA, Rac1 and Cdc42 was probed using assays designed to 'pull-down' specifically only the activated forms of each GTPase. Treatment of rFZ1-expressing cells with WNT3a for 10 minutes resulted in robust activation of all three of the small-molecular-weight GTPases (Fig. 5A). We then examined the role of these GTPases in the WNT3a-JNK pathway of F9 cells by expressing the well-known dominant-negative (DN) mutant forms of either RhoA (T19N), Rac1 (T17N) and Cdc42 (T17N). Each of the DN-GTPases was HA tagged and their expression in F9 cells was verified by immunoblotting with antibodies against HA (Fig. 5B). Expression of RhoA (T19N), Rac1 (T17N) or Cdc42 (T17N) dominant-negative mutants abolishes the ability of WNT3a to activate JNK (Fig. 5B). Furthermore, we also tested the roles of these GTPases on WNT3a-induced Jun phosphorylation by knockdown of the expression of each using siRNAs to rule out the effect of probable alterations to the cell physiology that might occur during overexpression of dominant-negative molecules and to understand their specific roles. Similar to the effects of expression of dominant-negative mutant forms of the GTPases, suppression of either RhoA, Rac1 or Cdc42 by siRNA also abolished WNT3a-induced Jun phosphorylation (Fig. 5C). Taken together, these experiments demonstrate the obligate roles of RhoA, Rac1 and Cdc42 in the WNT3a-JNK pathway. Conversely, expression of the constitutively active mutant form of either RhoA (Q63L), Rac1 (Q61L) or Cdc42 (Q61L) alone in rFZ1-expressing cells stimulates activation of JNK in the absence of WNT3a (supplementary material Fig. S4). Taken together, these observations suggest an obligate role for Rho-family GTPases in WNT3a-JNK signaling. DVL3-induced phosphorylation of Jun was attenuated by expression of dominant-negative RhoA (Fig. 5D), suggesting that DVL proteins operate upstream of small G-proteins in the WNT3a-FZ1-JNK pathway.

Role of MAPK proteins in WNT3a-FZ1-JNK signaling

Small-molecular-weight GTPases of the Rho family have been linked to JNK activation through the mitogen-activated protein (MAP) kinase cascade (Lee et al., 2004; Wang et al., 2002). Based upon these earlier studies, we employed two approaches: (1) expression of dominant-negative mutant forms of MEKK proteins or treatment with siRNAs targeting specific MEKK proteins in rFZ1-expressing cells, and (2) addition of specific chemical inhibitors of MAP kinases to interrogate the MAP kinase cascade linking WNT3a-stimulated small-molecular-weight GTPases to JNK activation. The effects of expression of dominant-negative forms of MEKK 1 (DN-MEKK 1) and MEKK 4 (DN-MEKK 4) in rFZ1-expressing cells on the ability of WNT3a to activate JNK was investigated (Fig. 6A). Expression of either DN-MEKK 1 or DN-MEKK 4 blocks the activation of JNK by WNT3a (Fig. 6A). Similar to the effects of dominant-negative mutants of MEKK proteins, specific depletion of MEKK 1 and MEKK 4 by siRNA treatment also abolished the WNT3a-

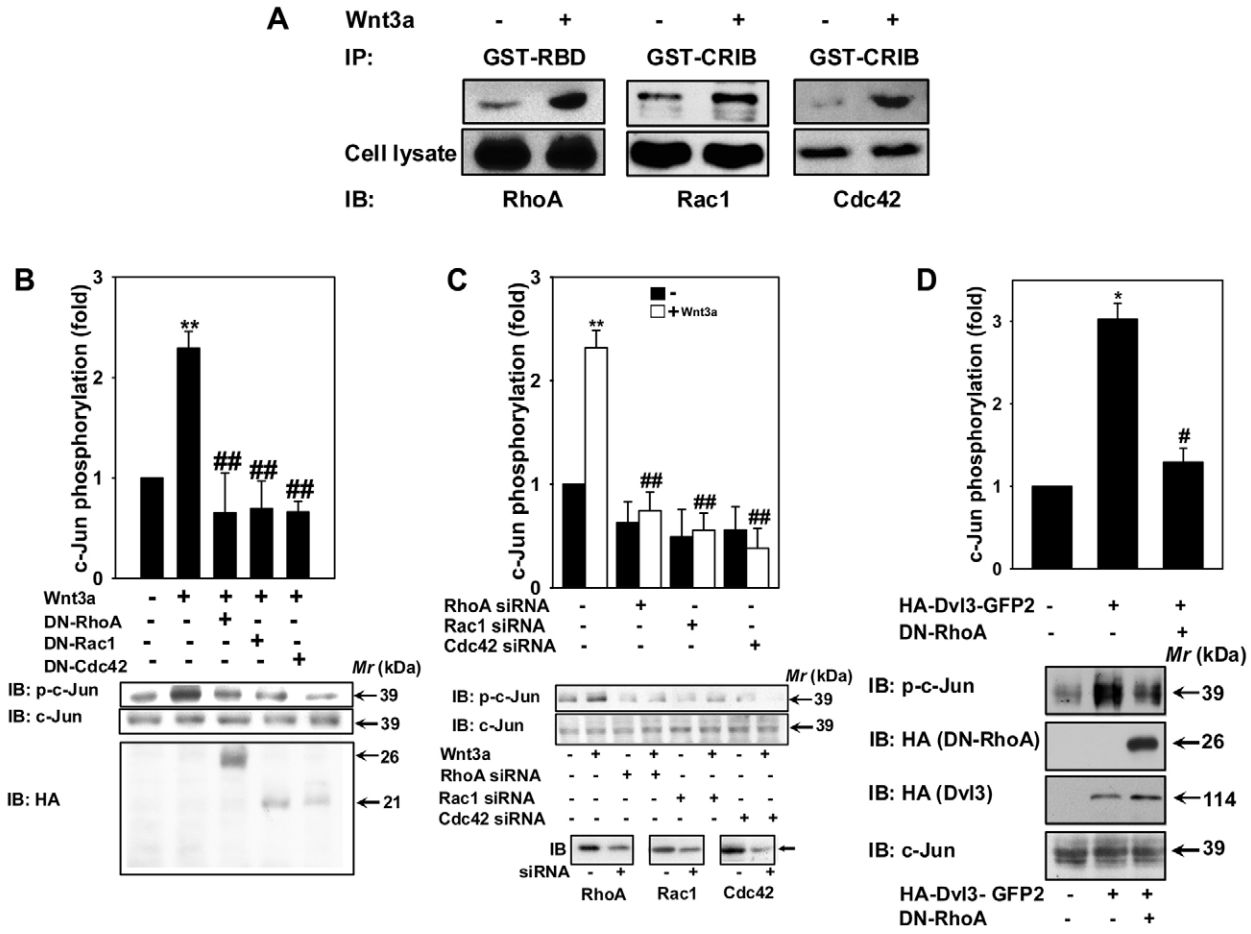

Fig. 5. WNT3a stimulates activation of small-molecular-weight GTPases, while expression of dominant-negative (DN) versions of small-molecular-weight GTPases or treatment of rFZ1-expressing cells with specific siRNAs targeting the same small GTPases abolish WNT3a stimulation of JNK activity. (A) F9 cells expressing rFZ1 were treated with WNT3a (100 ng/ml) for 10 minutes and activation of each of the small-molecular-weight GTPases was monitored as described in the Materials and Methods. (B,C) F9 cells expressing rFZ1 were transfected (1 μg/well in a 12-well plate) with dominant-negative versions of small-molecular-weight GTPases (B) or treated with siRNAs specific for RhoA, Rac1 and Cdc42 (C) for 24 hours and JNK activity was determined. (D) F9 cells stably expressing rFZ1 were transfected with DVL3-GFP2 alone or together with DN-RhoA for 24 hours, and JNK activity was determined. The upper panel displays mean values±s.e.m. obtained from three independent experiments; the lower panel displays the corresponding representative blots. The DVL3-RhoA epistasis experiment is representative of two independent experiments whose results were in strong agreement. The results displayed for the activation of small-molecular-weight GTPases are from a single experiment performed with triplicate sampling. These results are a representative of two separate experiments whose data were in high agreement. $^{**}P<0.01$ versus –WNT3a control; $^{\#\#}$, $P<0.01$ versus +WNT3a control.

induced Jun phosphorylation (Fig. 6B). As MEKK proteins regulate the activation of p38 and JNK through activation of the MAP kinase kinases MKK3 or MKK6 and MKK4 or MKK7, we probed the effects of specific inhibitors of p38 kinase (SB203580) and JNK (SP600125) on the ability of WNT3a to activate JNK (Fig. 6C). Treating rFZ1-expressing cells with the JNK inhibitor SP600125 (0.4 μM for 1 hour) effectively abolishes WNT3a-stimulated JNK activation. Treating rFZ1-expressing cells with the p38 inhibitor SB203580 (6 μM for 1 hour), in sharp contrast, actually stimulates basal levels of JNK activity (Fig. 6C). WNT3a, in combination with SB203580, yields JNK activation equivalent to either agent alone (Fig. 6C). Expression of dominant-negative MEKK 1 attenuates the Jun phosphorylation induced either by overexpression of DVL3 (Fig. 6D) or by overexpression of a constitutively active mutant of Cdc42 (Q61L) (Fig. 6E), suggesting that DVL proteins and small-molecular-weight GTPases operate upstream of MEKK proteins in the WNT3a-FZ1-JNK signaling cascade.

Divergence of WNT3a–β-catenin and WNT3a-JNK signaling

In mouse F9 cells, because WNT3a stimulates the $G\alpha_o$–DVL–β-catenin–LEF/TCF pathway (Liu et al., 2001; Liu et al., 2002) as well as the $G\alpha_o$–DVL–small-G-protein–JNK pathway (this study), so we investigated the impact of probes that regulate the WNT3a-JNK pathway on the function of WNT3a–β-catenin signaling. We focused on the possible role of small-molecular-weight G-proteins and MAP kinases. Expression of DN-RhoA, DN-Rac1 or DN-Cdc42 in each case blocked signaling of WNT3a to JNK activation (Fig. 5B), but expression of these mutants had either little (DN-Rac-1) or no (DN-RhoA, DN-Cdc42) significant effect on signaling by the WNT3a–β-catenin pathway (Fig. 7A).

Expression of either DN-MEKK 1 or DN-MEKK 4 effectively blocks the ability of WNT3a to signal to JNK (Fig. 6A) but not to effect LEF/TCF-sensitive transcription (Fig. 7B). The JNK inhibitor SP600125 not only blocks, as expected, the activation of JNK (Fig. 6C), but also attenuates WNT3a-stimulated LEF/TCF-

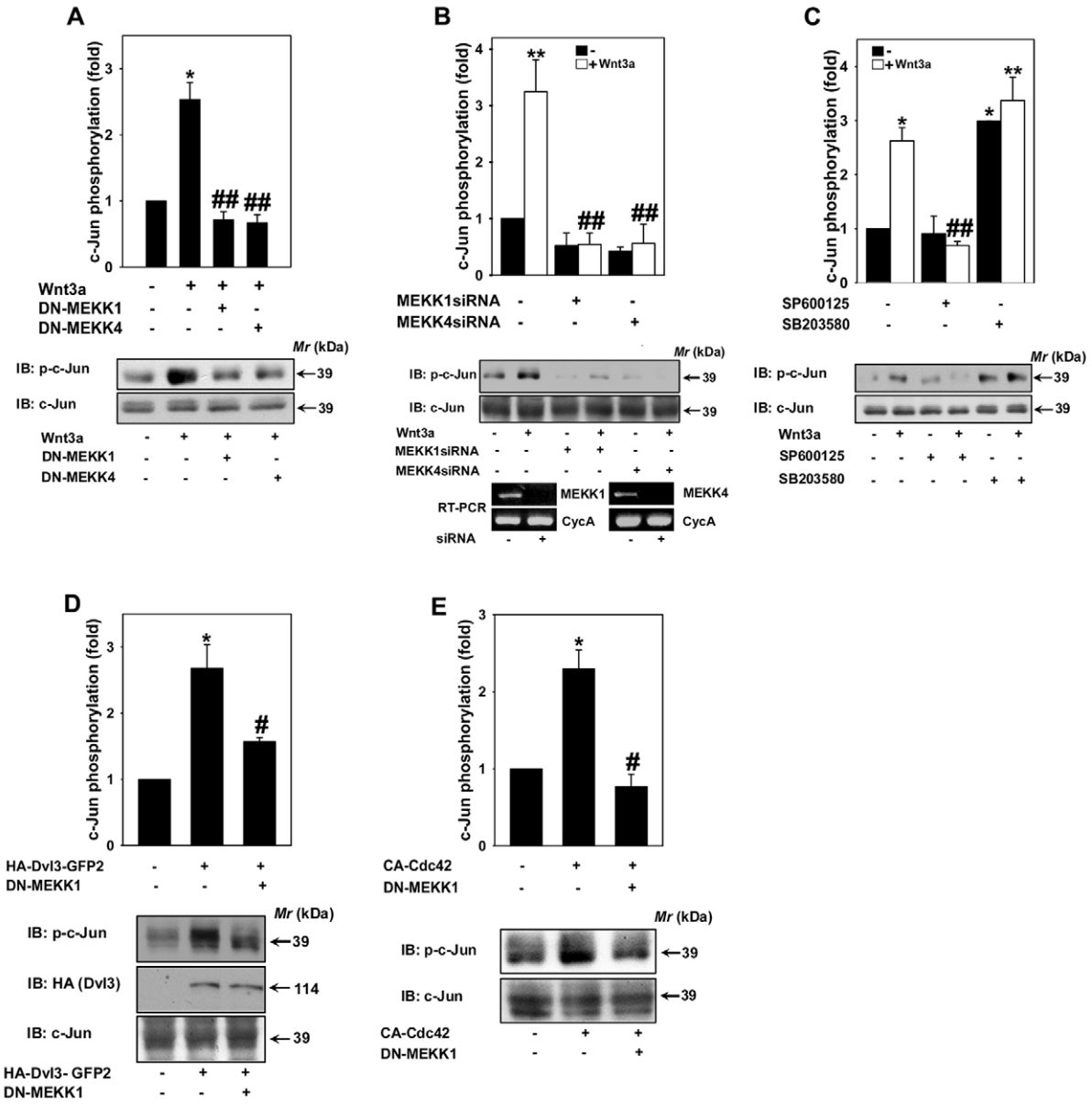

Fig. 6. Expression of dominant-negative (DN) versions of either MEKK 1 or MEKK 4 as well as use of siRNAs specifically targeted to MEKK 1/MEKK 4 blocks the activation of JNK in response to WNT3a stimulation. (A,B) F9 cells expressing rFZ1 were transfected (1 μg/well in a 12-well plate) with an expression vector harboring either DN-MEKK 1 or DN-MEKK 4 (A) or treated with siRNAs specific to either MEKK 1 or MEKK 4 (B) for 24 hours, and JNK activity was determined. (C) Confluent F9 cells expressing rFZ1 were treated with or without inhibitors of JNK (SP600125, 0.4 μM) or p38 (SB203580, 6 μM) for 1 hour before stimulation with WNT3a, followed by determination of JNK activity. (D) F9 cells stably expressing rFZ1 were transfected with DVL3-GFP2 either alone or together with DN-MEKK 1 for 24 hours, followed by determination of JNK activity. (E) F9 cells stably expressing rFZ1 were transfected with constitutively active (CA)-Cdc42 either alone or together with DN-MEKK 1 for 24 hours, followed by determination of JNK activity. The upper panel displays mean values±s.e.m. obtained from three independent experiments; the lower panel displays the corresponding representative blots. The DVL3–DN-MEKK 1 and CA-Cdc42–DN-MEKK 1 epistasis experiments are representative of two independent experiments whose results were in strong agreement. *P<0.05; **P<0.01; versus –WNT3a control; $^{\#}$, P<0.05; $^{\#\#}$, P<0.01 versus +WNT3a control.

sensitive transcription by approximately 50% (Fig. 7C). The inhibition of p38 kinase with SB203580 provokes activation of JNK in the absence of WNT3a (Fig. 6C), while attenuating WNT3a-stimulated activation of LEF/TCF-sensitive transcription by more than a factor of three (Fig. 7C).

Finally, we probed the role of the WNT3a-JNK pathway on the ability of WNT3a to stimulate formation of primitive endoderm in F9 cells. F9 cells stably expressing rFZ1 were either transfected with DN-RhoA/DN-MEKK 1 or treated with a JNK inhibitor (SP600125, 0.4 μM), and the ability of WNT3a to promote formation of primitive endoderm was determined by positive staining of cytokeratin endoA, a hallmark protein for primitive endoderm, with the TROMA-1 antibody (Liu et al., 2001). Interestingly, treatment of cells with SP600125 that specifically blocks signaling downstream of JNK has no impact on WNT3a-induced formation of primitive endoderm (Fig. 8). Furthermore, expression of either DN-RhoA or DN-MEKK 1, which were indispensable for the WNT3a-JNK pathway but not for the

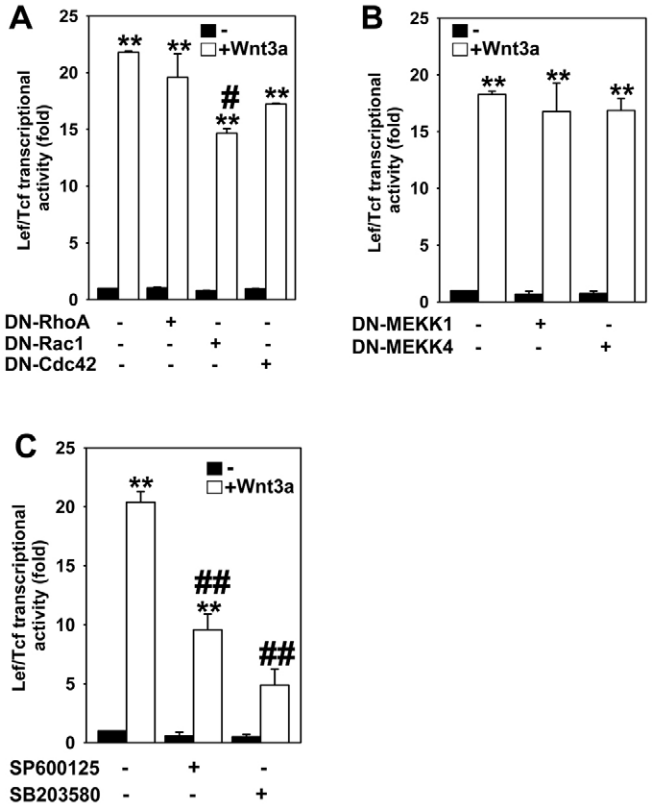

Fig. 7. A JNK inhibitor blocks WNT3a-stimulated LEF/TCF-sensitive transcription. (A,B) F9 cells stably expressing rFZ1 and pTOPFLASH luciferase reporter were transfected with dominant-negative (DN) versions of either RhoA, Rac1 or Cdc42 (A) and either DN-MEKK 1 or DN-MEKK 4 (B) for 24 hours, followed by overnight serum starvation. The cells then were treated with or without WNT3a for 7 hours and luciferase gene reporter assays were performed. (C) Confluent F9 cells stably expressing rFZ1 and pTOPFLASH luciferase reporter were serum-starved overnight and treated with JNK (SP600125, 0.4 μM) or p38 (SB203580, 6 μM) inhibitors for 1 hour, followed by treatment with or without WNT3a for 7 hours. Lysates were collected and a luciferase assay was performed, as described in the Materials and Methods. The data represent mean values±s.e.m. from a single experiment performed in triplicate and are representative of three separate experiments whose results were in strong agreement. $^{**}P<0.01$; versus –WNT3a control; $^{\#}$, $P<0.05$; $^{\#\#}$, $P<0.01$ versus +WNT3a control.

canonical pathway, also shows no effect on the ability of WNT3a to induce formation of primitive endoderm (Fig. 8).

Discussion

Genetic studies in *Drosophila* (Katanaev et al., 2005; Katanaev and Tomlinson, 2006), *Caenorhabditis elegans* (Gotta and Ahringer, 2001), *Xenopus* (Sheldahl et al., 1999) and zebrafish (Slusarski et al., 1997) demonstrate an obligate role for G-proteins in signaling pathways controlling development (Malbon, 2005). More recently, $G\alpha_o$ has been implicated also in the PCP pathway in *Drosophila* (Katanaev et al., 2005; Katanaev and Tomlinson, 2006). In the present study, utilizing pertussis toxin as well as siRNAs, we show that $G\alpha_o$ is obligate for WNT3a signaling to the MAP kinase JNK (Fig. 9). By contrast, the $G\alpha_q$ G-protein, which is obligate for WNT-stimulated canonical signaling for LEF/TCF-sensitive transcriptional activation (Liu et al., 2001; Liu et al., 1999), was found to be dispensable for WNT3a-JNK signaling, demonstrating divergence of the two pathways at the step proximal to FZ1.

Expression of constitutively active Q205LGα_o stimulates JNK, mimicking the effect of WNT3a. Furthermore, the Q205LGα_o-stimulated activation of JNK was found to be sensitive to the expression of Dapper1. This ability of Dapper1, a known antagonist of DVL function, to block the response to Q205LGα_o places DVL proteins downstream of G-proteins in WNT-JNK signaling and confirms genetic epistasis experiments performed in the fly (Katanaev et al., 2005). The role of DVL proteins in mammals is more complex than that in the fly as three genes encoding DVL (rather than a single gene as in fly) are present in mammals (*Dvl1*, *Dvl2* and *Dvl3*), and all three are expressed in mouse F9 cells. Suppression of expression of either DVL1 or DVL3, but not DVL2, completely abolishes the ability of WNT3a to stimulate JNK activation. It was unexpected that knockdown of either DVL1 or DVL3 individually would abolish WNT3a-stimulated activation of JNK. To probe for possible off-target effects of the siRNA reagents employed to knockdown the expression of DVL proteins, we performed rescue experiments in cells in which expression of the DVL protein of interest had been knocked-down by treatment with siRNA. The results of these experiments demonstrate clearly that the effects observed in siRNA-treated cells reflect the knock-down of the intended target as exogenous expression of an siRNA-resistant form rescued the lost response to WNT3a. The effective, selective use of siRNAs to knock-down DVL protein expression has been reported in other studies (Endo et al., 2005; Zhong et al., 2006).

Recently, it has been shown that specific suppression of expression of DVL1, DVL2 and DVL3 inhibits RCJ-chondrocyte cell proliferation and differentiation (Zhong et al., 2006). Furthermore, among the three forms, suppression of DVL2 and DVL3 showed greater inhibition of chondrocyte differentiation than suppression of DVL1 (Zhong et al., 2006). In another recent study, by using siRNA-mediated silencing in Chinese hamster ovary cells in culture, it was shown that specific depletion of DVL2 reduces WNT3a-dependent changes in cell shape and movement (Endo et al., 2005). These existing data, when taken together with the data from the current study, suggest clearly that the DVL isoforms display some level of functional redundancy. Sequence analyses of the three mouse DVL proteins reveal no major differences in the DIX, PDZ or DEP domain regions but show considerable differences in the inter-domain regions. Unique stretches of sequences can be found in the C-terminal regions of each of the DVL proteins that could account for differential roles in the three distinct WNT pathways. In addition, specific Dishevelled-associated proteins (DAPs) have been identified that regulate the function of DVL proteins and allow proper signal transduction (Wharton, Jr, 2003). DVL isoforms might utilize different DAP proteins to uniquely affect cellular physiology. It seems likely that, through their DIX domains, DVL proteins form functional oligomers/polymers that transduce WNT signaling (Schwarz-Romond et al., 2007). Previously, it was shown that the DIX domain of DVL1 interacts with DVL3 and can form also homo-oligomers (Kishida et al., 1999). Taken together, it is possible that the DVL1, DVL2 and DVL3 operating in unison might form functional complexes that transduce WNT signaling. Hence, removing one of the isoforms from the dynamic complex environment (or changing its stoichiometry with respect to the other isoforms in the complex) might disrupt the ability of the DVL-based signaling complexes to transduce WNT signals downstream.

Members of the Rho family – RhoA, Rac1 and Cdc42 – have been implicated in the PCP pathway of the fly (Strutt et al., 1997)

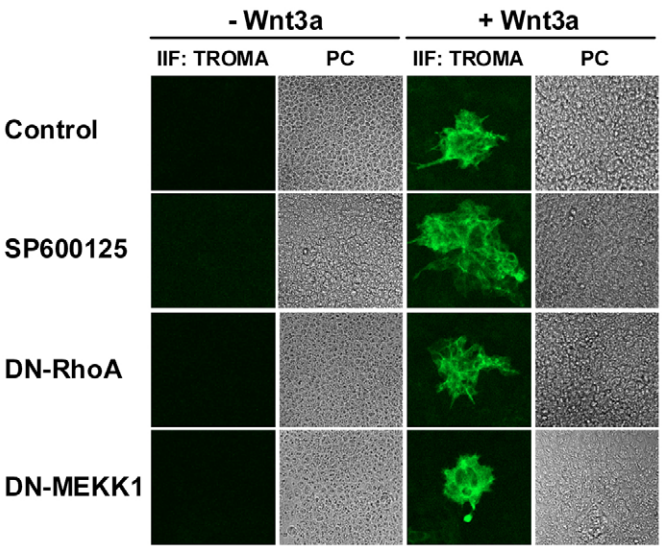

Fig. 8. Activation of the JNK pathway by WNT3a is not obligate for formation of primitive endoderm. F9 cells stably expressing rFZ1 were treated with a JNK inhibitor (0.4 μM SP600125) or transiently transfected with DN-RhoA or DN-MEKK 1 before stimulation with WNT3a for 4 days. Subsequently, the cells were prepared for immunocytochemistry and stained with a monoclonal antibody against the cytokeratin endo A (TROMA-1), a marker protein for primitive endoderm. Alexa-Fluor-488-conjugated secondary antibodies were employed together with indirect epifluorescence to detect the immune complexes. Typical phase-contrast images (PC) and the indirect immunofluorescence images (IIF) are shown from a single experiment, representative of three independent experiments.

and convergent extension movements during gastrulation (Habas et al., 2003; Kim and Han, 2005). We show that WNT3a signaling to JNK in mammalian cells involves all three small GTPases. Expression of constitutively active mutants of RhoA, Rac1 or Cdc42 alone activates JNK, mimicking the effects of WNT3a (Fig. 9). Expression of dominant-negative mutant forms of these small-molecular-weight GTPases blocks JNK activation by WNT3a, but not the activation of LEF/TCF-sensitive transcription. It has been demonstrated also in the fly that RhoA and Rac1 are required for the establishment of epithelial planar polarity in the eye, where RhoA acts downstream or in parallel to Rac1 (Fanto et al., 2000). In agreement with the data from the current study, expression of DN-RhoA, DN-Rac1 or DN-Cdc42 individually has been shown to block retinoic-acid-induced and $G\alpha_{13}$-induced JNK activation in embryonal P19 cells (Lee et al., 2004). Similarly, expression of dominant-negative mutants of these GTPases also blocks angiotensin-II-stimulated activation of JNK in cardiac fibroblasts prepared from 1- to 2-day-old Wistar rats (Murasawa et al., 2000). Cell-type-dependent differences add further possible complexity to these signaling pathways (Jaffe and Hall, 2005). Scaffold proteins also play a major role in controlling the signaling events of the small-molecular-weight GTPases. MEKK 4 interacts with Rac1 and Cdc42, whereas MEKK 1 interacts with RhoA, Rac1 and Cdc42 (Burbelo et al., 1995; Fanger et al., 1997; Gallagher et al., 2004; Teramoto et al., 1996). Downstream of small-molecular-weight GTPases, MAP kinase cascades operate, demonstrated at the level of the MEKK to the level of JNK. In P19 cells, either MEKK 1 or MEKK 4 can mediate $G\alpha_{13}$-induced activation of JNK and formation of primitive endoderm (Wang et al., 2002). We tested a similar hypothesis directly, showing that expression of either DN-MEKK1 or DN-MEKK 4 blocks the WNT3a-stimulated activation of JNK, while having no effect on the ability of WNT3a to activate LEF/TCF-sensitive transcription (i.e. the WNT canonical pathway). Studies utilizing the expression of dominant-negative mutant forms of MEKK 1 and MEKK 4 as well as those making use of siRNAs to suppress either MEKK 1 and/or MEKK 4 reported results in good agreement with those shown herein (Lee et al., 2004; Song and Lee, 2007; Wang et al., 2002; Yamauchi et al., 2007; Zou et al., 2007). Of interest, chemical inhibition of JNK blocks the most distal element in WNT3a-JNK signaling and also attenuates the activation of the WNT3a canonical pathway.

It is well known that the FZ-JNK pathway regulates PCP in the fly or convergent extension (CE) movements during gastrulation in *Xenopus* and zebrafish; however, no such phenotype has been identified in mammalian cells to date. Previous studies reveal that activation of the canonical pathway leads to formation of primitive endoderm in F9 cells (Liu et al., 2002; Liu et al., 1999). In the present study, experiments were performed to determine whether the WNT3a-JNK pathway also participates in the formation of primitive endoderm by F9 cells. Interestingly, specific inhibition of the WNT3a-FZ1-JNK pathway

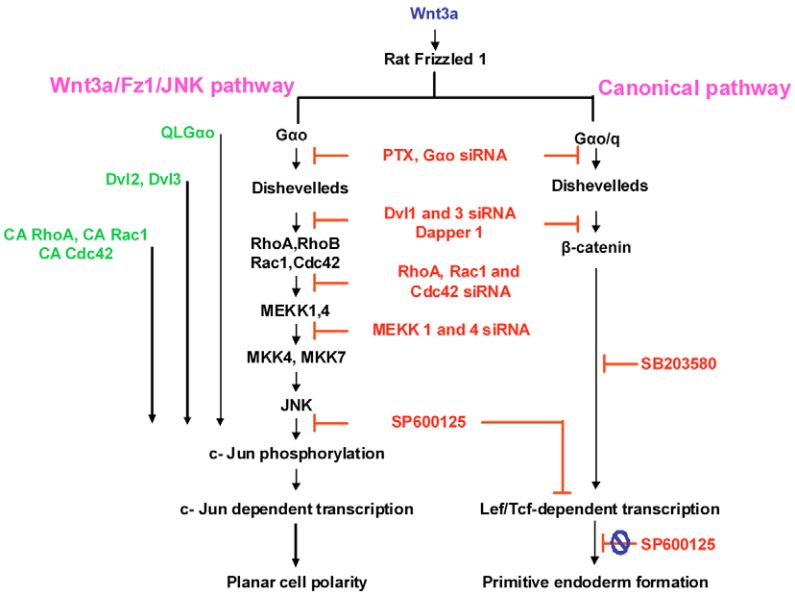

Fig. 9. Schematic representation of mammalian WNT3a-sensitive signaling pathways. The scheme represents a work-in-progress of our understanding of WNT3a-stimulated pathways in mammalian cells. JNK activation, crosstalk between the WNT3a–β-catenin canonical and WNT3a-JNK pathways in mouse F9 embryonal stem cells are highlighted. Constitutively active mutants of signaling molecules whose expression provokes activation of JNK in the absence of WNT3a are shown in green. Dominant-negative versions of signaling molecules, siRNAs and inhibitors that can effectively attenuate or block WNT3a-induced JNK activation or WNT3a-stimulated LEF/TCF-sensitive transcription are shown in red. The vectoral flow of information from WNT3a to JNK activation and from WNT3a to LEF/TCF transcription is displayed with arrows.

by use of SP600125 or DN-RhoA or DN-MEKK 1 has no effect on WNT3a-stimulated formation of primitive endoderm. Furthermore, a specific JNK inhibitor, while partially attenuating WNT3a-induced LEF/TCF transcription, shows no effect on formation of primitive endoderm. Earlier studies demonstrate that blocking signaling at the level of LEF/TCF in the canonical pathway by expression of dominant-negative TCF4 also blocks formation of primitive endoderm (Li et al., 2004). The treatment with JNK inhibitor SP600125 attenuates the ability of WNT3a to activate LEF/TCF-dependent gene transcription but appears to allow sufficient signal to promote formation of primitive endoderm. These observations suggest that there is some level of crosstalk between the WNT3a-JNK pathway and the canonical pathway, but apparently none vice versa with respect to the read-out of formation of primitive endoderm. The mechanism by which chemical inhibition of JNK attenuates LEF/TCF transcription remains unclear. Previous studies reported that phosphorylated Jun interacts with the HMG-box transcription factor TCF4 (Nateri et al., 2005), and the AP-1 complex interacts with LEF1 (Rivat et al., 2003). Thus, it can be hypothesized that the phosphorylated Jun and AP-1 complexes also induce LEF/TCF-dependent transcription together with β-catenin, and the signal from this pool is attenuated upon chemical inhibition of JNK with SP600125. Divergence of WNT3a signaling downstream of FZ1 has been established at several key points in the WNT3a-induced stimulation of JNK activation in comparison with LEF/TCF-sensitive gene activation: (i) $G\alpha_o$ is obligate and sufficient for JNK activation, whereas the WNT3a–β-catenin response requires both $G\alpha_o$ as well as $G\alpha_q$; (ii) members of the Rho family of GTPases are necessary for the JNK pathway, but not for the canonical pathway; (iii) MEKK 1/MEKK 4 can transduce signals from WNT3a to JNK but appear to contribute little to the canonical pathway; and, (iv) chemical inhibition of the p38 MAP kinase potentiates the JNK response while suppressing the canonical response. Crosstalk between the two WNT3a-sensitive pathways can occur at the level of heterotrimeric G-proteins (which can share common G β/γ subunits), DVL proteins (which are downstream of all known WNT-sensitive pathways – for example, the canonical, WNT-JNK and cGMP-Ca^{2+} pathways) and MAP kinases (as observed by the effects of chemical inhibitors of these kinases) (Fig. 9). Thus, the signal transduced might bifurcate at the level of G-proteins or of DVL proteins. In embryonal mouse cells, $G\alpha_{13}$ mediates activation of JNK through p115RhoGEF, RhoA, MEKK 1/MEKK 4 and MKK (Lee et al., 2004) and herein we report that $G\alpha_o$ mediates activation of JNK through DVL, RhoA family members and MEKK 1/MEKK 4 (Fig. 9). DVL proteins, presumably acting through a DEP domain (Li et al., 1999; Poliakov and Wilkinson, 2006) (conserved in fly DSH, as well as in all the mouse DVL proteins – DVL1, DVL2 and DVL3), probably bind and thereby regulate members of the Rho family that operate in a WNT3a-sensitive $G\alpha_o$-mediated pathway, at a level similar to that of the GDP-GTP exchange factor p115RhoGEF operating in $G\alpha_{13}$-mediated signaling.

This study reveals downstream signaling cascades from WNT3a, through FZ1 to activation of JNK. Our results show clearly the differential roles of DVL1, DVL2 and DVL3, divergence between the WNT3a signaling pathway controlling β-catenin accumulation/LEF-TCF-sensitive transcription and that regulating the activation of JNK, and also the existence of crosstalk from JNK to the WNT–β-catenin signaling pathway. The current work leaves unresolved several key questions, including the nature of specific DVL-associated proteins that might direct signaling through canonical versus non-canonical pathways. In addition, how JNK functions in the WNT3a-sensitive canonical signaling pathway in molecular terms awaits further study.

Materials and Methods

Cell culture

Mouse F9 teratocarcinoma cell stocks were obtained from the ATCC (Manassas, VA) and the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated fetal bovine serum (Hyclone, South Logan, UT) at 37°C in a 5% CO_2 incubator. For generation of F9 clones stably expressing rFZ1, cells were transfected with the pcDNA3.1-rFZ1 plasmid using Lipofectamine and Plus reagents (Invitrogen, Carlsbad, CA). Two days after transfection, cells stably expressing rFZ1 were selected in culture medium supplemented with 1 mg/ml of G418 (Invitrogen, Carlsbad, CA). Fifteen independent clones resistant to G418 were isolated, propagated and the level of expression of rFZ1 mRNA was measured indirectly by reverse-transcription polymerase chain reaction (RT-PCR). The clone expressing the highest level of rFZ1 mRNA was propagated in culture medium supplemented with 100 μg/ml of G418 and used for all the studies reported herein (rFZ1). F9 cells stably expressing rFZ1 and the pTOPFLASH luciferase reporter were generated in a manner similar to that described above.

Plasmids and transfections

The constitutively active (Q205L) form of $G\alpha_{oA}$ was procured from the UMR cDNA resource center (www.cdna.org). Expressed proteins were epitope tagged with either hemagglutinin antigen (HA-tagged) or with Myc (Myc-tagged). Mouse DVL proteins 1-3 were engineered in-house with GFP2 and HA tags. Mouse Dapper1 in $pCS2^+$ vector, hDVL1-EGFP, hDVL3-EGFP and AP-1-luciferase gene reporter constructs were a gift from Randall T. Moon (University of Washington, Seattle, WA). Dapper1 was shuttled subsequently into pCMV-Myc plasmid (Myc-Dapper1). The HA-tagged versions of constitutively active Cdc42 [pCDNA3-HA-Cdc42Hs (Q61L)] and dominant-negative Cdc42 [pCDNA3-HA-Cdc42Hs (T17N)] as well as constitutively active Rac1 [pCDNA3-HA-Rac1(Q61L)] and dominant-negative Rac1 [pCDNA3-HA-Rac1(T17N)] plasmids were a gift from Richard A. Cerione (Department of Chemical Biology, Cornell University). The Myc-tagged version of the dominant-negative RhoA [pCDNA3-Myc-RhoA (T19N)] plasmid was a gift from Dafna Bar-Sagi (Department of Biochemistry, New York University) and subsequently was engineered with three HA tags to replace the Myc tag. The constitutively active version of RhoA [pCDNA3-Myc-RhoA (Q63L)] plasmid was a gift from Alan Hall (CRC Oncogene and Signal Transduction Group, Medical Research Council Laboratory for Molecular Cell Biology, University College London, UK). The wild-type HA-MEKK 1 and HA-MEKK 4 and dominant-negative forms of HA-MEKK 1 and HA-MEKK 4 were provided generously by Gary L. Johnson (Department of Pharmacology, University of North Carolina-Chapel Hill). The assays of activated GTPases make use of 'pull-down' assays using GST-fusion proteins that contain domains that can bind only to the activated forms of each small-molecular-weight GTPase. The plasmids employed were pGEX-GST-PAK1CRIB (harboring the Rac1/Cdc42-binding domain, residues 70-149) and pGEX-GST-RBD (harboring the RhoA-binding domain of Rokα, residues 809-1062). These constructs were gifts from Lu-Hai Wang (Department of Microbiology, Mount Sinai School of Medicine, New York). Cells stably expressing rFZ1 were transfected with one or more plasmids or empty vectors using Lipofectamine reagent.

JNK activity assays

F9 cells expressing rFZ1 were grown to confluence in 12-well plates. The cells were then serum starved by reducing the FBS supplement to 0.5% for 8 hours followed by reduction to 0.005% for a further 16 hours. The cells were then exposed to recombinant WNT3a (20 ng/ml, R&D Systems, Minneapolis, MN) for 15 minutes at 37°C. The cells were lysed in lysis buffer (25 mM Tris-HCl pH 6.8, 1% SDS, 1 mM sodium orthovanadate, 200 μM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotonin), boiled for 5 minutes and stored at –80°C until use. Total lysates (30-60 μg) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred electrophoretically to nitrocellulose membranes. The blots were then probed with antibodies specific for phospho-Jun (KM1, Ser 63, Santa Cruz Biotechnology, Santa Cruz, CA). All of the blots were re-probed with antibodies against Jun for normalization.

Knockdown protocol

Sense and antisense oligonucleotides were designed and synthesized targeting each of the following molecules: mouse DVL1 (NM_010091), mouse DVL2 (NM_007888) and mouse DVL3 (NM_007889). The oligo pairs were annealed and stored as recommended by the commercial supplier. The sequence of each siRNA is as follows: the siRNA oligos CCAGGAUAUUGGCUUGACAtt and UGUCAAGCCAAUAUCCUGGtg target mouse DVL1, GGAAGAGAUCUC-

CGAUGACtt and GUCAUCGGAGAUCUCUUCCtt target mouse DVL2, and GGAAGAGAUCUCGGACGACtt and GUCGUCCGAGAUCUCUUCCtt target mouse DVL3. The siRNAs targeting Gα subunits $G\alpha_o$ (sc-37256), $G\alpha_q$ (sc-35430), $G\alpha_{11}$ (sc-41741), RhoA (sc-29471), Rac1 (sc-36352), Cdc42 (sc-29257), RhoB (sc-36415), RhoC (sc-41888), MEKK 1(m) (sc-35899), MEKK 4(m) (sc-35903), MEKK 1 (h) (sc-35898), MEKK 4 (h) (sc-35902) and the control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. F9 cells expressing rFZ1 were treated with 100 nM siRNAs by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, siRNAs were incubated with 5 μl Lipofectamine 2000 for 20 minutes in 200 μl Optimem medium (Invitrogen, Carlsbad, CA), and then the mixture was added into 1 ml of growth medium in a 12-well plate in which F9 cells expressing rFZ1 were cultured to 80% confluency. After siRNA treatment for 48 hours, the cells were starved and the JNK assay was performed as described above.

Rescue experiments

Rescue experiments were performed by transfecting into rFZ1-expressing F9 cells expression vectors harboring either siRNA-resistant hDVL1-EGFP or hDVL3-EGFP. Briefly, F9 cells were seeded onto 12-well plates and 24 hours later transfected with siRNA-lipofectamine 2000 complexes to achieve the knockdown of the targeted endogenously expressed molecules, as described above. For rescue, 24 hours after siRNA treatment, the cells were transfected with the plasmids (0.5 μg/well for DVL proteins) containing expression vectors for siRNA-resistant forms for another 24 hours. The JNK assay was performed as described above.

Immunoblotting

Total lysates (30-60 μg of protein/lane) were subjected to electrophoresis in 10-12% SDS-PAGE gels. The resolved proteins were transferred electrophoretically to nitrocellulose membrane 'blots'. The blots were incubated with primary antibodies overnight at 4°C and the immunocomplexes were made visible by use of a secondary antibody coupled to horseradish peroxidase and developed using the enhanced chemiluminescence method. The antibodies were purchased from the following sources: anti-phospho-Jun Ser 63 (KM1, sc-822), anti-Jun (H-79, sc-1694), anti-DVL1 (3F12, sc-8025), anti-DVL2 (10B5, sc-8026), anti-DVL3 (4D3, sc-8027), anti-$G\alpha_o$ (A2, sc-13532), anti-$G\alpha_{11}$ (D17, sc-394) and anti-Cdc42 (sc-87) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-$G\alpha_q$ (BD Transduction Laboratories, San Jose, CA), anti-HA high-affinity antibody (Roche Applied Science, Indianapolis, IN), anti-Myc (9E10) and anti-β-actin antibodies were from Sigma-Aldrich (St Louis, MO). Anti-RhoA (C55, 05-778) and anti-Rac1 (23A8, 05-389) antibodies were purchased from Upstate Cell Signaling Solutions (Lake Placid, NY).

Gene transcription assay

F9 cells stably expressing rFZ1 and the pTOPFLASH luciferase reporter were seeded into 12-well plates and transiently transfected with or without dominant-negative constructs of small-molecular-weight GTPases or MEKK proteins. Following incubation for 24 hours at 37°C, the cells were serum-starved overnight, followed by treatment with or without purified WNT3a for 7 hours. The cells were then directly lysed on the plates by addition of 1× Cell Culture Lysis Reagent (Promega, Madison, WI). Lysates were collected into chilled microfuge tubes on ice and centrifuged at 15,000 ***g*** for 5 minutes. The supernatant was transferred into a new tube and directly assayed as described below. 20 μl of the lysate was added to 100 μl of luciferase assay buffer (20 mM Tricine, pH 7.8, 1.1 mM $MgCO_3$, 4 mM $MgSO_4$, 0.1 mM EDTA, 0.27 mM coenzyme A, 0.67 mM luciferin, 33 mM DTT and 0.6 mM ATP), and the luciferase activity was measured by use of a luminometer (Berthold Lumat LB 9507).

RhoA, Rac1 and Cdc42 activation assays

The assay was performed as described previously (van Triest and Bos, 2004). The synthesis of fusion polypeptides of GST-RBD and GST-PAK1CRIB were induced in *Escherichia coli* with 0.1 mM isopropyl-β-galactopyranoside at 30°C for 4 hours and affinity purified using 50% slurry of glutathione-sepharose 4B (Amersham Biosciences) according to the batch purification method suggested by the manufacturer.

RNA isolation and RT-PCR analysis

After siRNA treatment for 24 hours, total RNA was extracted from the cultures by using RNA STAT-60 reagent (Tel-test, Friendswood, TX) according to the manufacturer's instructions. Primer pairs for mouse MEKK 1, MEKK 4 and cyclophilin A were as follows: (5′) primer 5′-ACGGAGCTTTCAAGGAGTCA-3′ and (3′) primer 5′-GGATGGACGGTGCAGTAGTT-3′ for MEKK 1; (5′) primer 5′-GACTGTACGCCATGAGCAGA-3′ and (3′) primer 5′-GGGCTTGCAAACTCAAGAAG-3′ for MEKK 4; (5′) primer 5′-AGCACTGGAGAGAAAGGATTTG-3′ and (3′) primer 5′-CACAATGTTCATGCCTTCTTTC-3′ for cyclophilin A. The following primer pairs were used in HEK 293 cells: (5′) primer 5′-GTGAGCCGACCAAGGTGTAT-3′ and (3′) primer 5′-CAGCCGGACAAGAAGATGAT-3′ for FZ1; (5′) primer 5′-GCGTCTTCTCCGTGCTCTAC-3′ and (3′) primer 5′-CTGTTGGTGAGGCGAGTGTA-3′ for FZ2; (5′) primer 5′-TGAGTGTTCGAAGCTCATGG-3′ and (3′) primer 5′-ATCACGCACATGCAGAAAAG-3′ for FZ3; (5′) primer 5′-AACCTCGGCTACAACGTGAC-3′ and (3′) primer 5′-GTTGTGGTCGTTCTGTGGTG-3′ for FZ4; (5′) primer 5′-AAGGAGTCACACCCGCTCTA-3′ and (3′) primer 5′-CGTAGTGGATGTGGTTGTGC-3′ for FZ5; (5′) primer 5′-ATTTTGGTGTCCAAGGCATC-3′ and (3′) primer 5′-TATTGCAGGCTGTGCTATCG-5′ for FZ6; (5′) primer 5′-GTGCAGTGTTCTCCCGAACT-3′ and (3′) primer 5′-GAACGGTAAAGAGCGTCGAG-3′ for FZ7; (5′) primer 5′-TCTACAACCGCGTCAAGACA-3′ and (3′) primer 5′-GAACCATGTGAGCGACAAGA-3′ for FZ8; (5′) primer 5′-CGCTGGTCTTCCTACTGCTC-3′ and (3′) primer 5′-AGAAGACCCCGATCTTGACC-3′ for FZ9; (5′) primer 5′-CAACAAGAACGACCCCAACT-3′ and (3′) primer 5′-GACGCAGTAGCACATGGAGA-3′ for FZ10. PCR amplifications were performed using the following program: 94°C for 5 minutes followed by 25 cycles of 94°C for 30 seconds/60°C for 30 seconds/72°C for 1 minute followed by a final extension at 72°C for 5 minutes. PCR products were made visible on 1.5% agarose gels stained by ethidium bromide and UV-transillumination.

Indirect immunofluorescence studies

F9 cells stably expressing rFZ1 cultured in 24-well plates were fixed with 3% paraformaldehyde at room temperature for 5 minutes, followed by three washes with MSM-PIPES buffer (18 mM $MgSO_4$, 5 mM $CaCl_2$, 40 mM KCl, 24 mM NaCl, 5 mM PIPES, 0.5% Triton X-100, 0.5% NP40). The cells were then incubated with the TROMA-1 antibody at 37°C for 30 minutes. After three washes with MSM-PIPES buffer, the cells were incubated with an anti-mouse antibody coupled to Alexa Fluor 488 (Invitrogen) at 37°C for 30 minutes. Finally, the cells were washed in blotting buffer (560 mM NaCl, 10 mM KH_2PO_4, 0.1% Triton X-100, 0.02% SDS), and images were captured using a Zeiss LSM510 inverted fluorescence microscope.

Data analysis

For all of the experiments, data were compiled from at least three independent, replicate experiments performed on separate cultures on separate occasions. All of the blots were scanned and the band intensities were normalized for Jun band intensities. In all cases, the fold-changes over the untreated control (set to 1.0) were calculated and displayed. Comparisons of data among groups were performed using one-way analysis of variance (ANOVA) or *t* test. Statistical significance (P value of <0.05) is denoted with either an asterisk (*) or a hash ($^{\#}$) symbol. The indirect immunofluorescence and phase-contrast images are of representative fields of interest.

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References

Axelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T. and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. *Genes Dev.* **12**, 2610-2622.

Barrow, J. R. (2006). Wnt/PCP signaling: a veritable polar star in establishing patterns of polarity in embryonic tissues. *Semin. Cell Dev. Biol.* **17**, 185-193.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**, 638-642.

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. and Nusse, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature* **382**, 225-230.

Boutros, M., Paricio, N., Strutt, D. I. and Mlodzik, M. (1998). Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell* **94**, 109-118.

Burbelo, P. D., Drechsel, D. and Hall, A. (1995). A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J. Biol. Chem.* **270**, 29071-29074.

Cheyette, B. N., Waxman, J. S., Miller, J. R., Takemaru, K., Sheldahl, L. C., Khlebtsova, N., Fox, E. P., Earnest, T. and Moon, R. T. (2002). Dapper, a Dishevelled-associated antagonist of beta-catenin and JNK signaling, is required for notochord formation. *Dev. Cell* **2**, 449-461.

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.

Fanger, G. R., Johnson, N. L. and Johnson, G. L. (1997). MEK kinases are regulated by EGF and selectively interact with Rac/Cdc42. *EMBO J.* **16**, 4961-4972.

Fanto, M., Weber, U., Strutt, D. I. and Mlodzik, M. (2000). Nuclear signaling by Rac and Rho GTPases is required in the establishment of epithelial planar polarity in the Drosophila eye. *Curr. Biol.* **10**, 979-988.

Gallagher, E. D., Gutowski, S., Sternweis, P. C. and Cobb, M. H. (2004). RhoA binds to the amino terminus of MEKK1 and regulates its kinase activity. *J. Biol. Chem.* **279**, 1872-1877.

Gotta, M. and Ahringer, J. (2001). Distinct roles for Galpha and Gbetagamma in regulating spindle position and orientation in Caenorhabditis elegans embryos. *Nat. Cell Biol.* **3**, 297-300.

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.

Heisenberg, C. P., Tada, M., Rauch, G. J., Saude, L., Concha, M. L., Geisler, R., Stemple, D. L., Smith, J. C. and Wilson, S. W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature* **405**, 76-81.

Jaffe, A. B. and Hall, A. (2005). Rho GTPases: biochemistry and biology. *Annu. Rev. Cell Dev. Biol.* **21**, 247-269.

Katanaev, V. L. and Tomlinson, A. (2006). Multiple roles of a trimeric G protein in Drosophila cell polarization. *Cell Cycle* **5**, 2464-2472.

Katanaev, V. L., Ponzielli, R., Semeriva, M. and Tomlinson, A. (2005). Trimeric G protein-dependent frizzled signaling in Drosophila. *Cell* **120**, 111-122.

Kim, G. H. and Han, J. K. (2005). JNK and ROKalpha function in the noncanonical Wnt/RhoA signaling pathway to regulate Xenopus convergent extension movements. *Dev. Dyn.* **232**, 958-968.

Kishida, S., Yamamoto, H., Hino, S., Ikeda, S., Kishida, M. and Kikuchi, A. (1999). DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. *Mol. Cell. Biol.* **19**, 4414-4422.

Kishida, S., Yamamoto, H. and Kikuchi, A. (2004). Wnt-3a and Dvl induce neurite retraction by activating Rho-associated kinase. *Mol. Cell. Biol.* **24**, 4487-4501.

Lee, Y. N., Malbon, C. C. and Wang, H. Y. (2004). G alpha 13 signals via p115RhoGEF cascades regulating JNK1 and primitive endoderm formation. *J. Biol. Chem.* **279**, 54896-54904.

Li, H., Malbon, C. C. and Wang, H. Y. (2004). Gene profiling of Frizzled-1 and Frizzled-2 signaling: expression of G-protein-coupled receptor chimeras in mouse F9 teratocarcinoma embryonal cells. *Mol. Pharmacol.* **65**, 45-55.

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.

Liu, T., Liu, X., Wang, H., Moon, R. T. and Malbon, C. C. (1999). Activation of rat frizzled-1 promotes Wnt signaling and differentiation of mouse F9 teratocarcinoma cells via pathways that require Galpha (q) and Galpha (o) function. *J. Biol. Chem.* **274**, 33539-33544.

Liu, T., DeCostanzo, A. J., Liu, X., Wang, H., Hallagan, S., Moon, R. T. and Malbon, C. C. (2001). G protein signaling from activated rat frizzled-1 to the beta-catenin-Lef-Tcf pathway. *Science* **292**, 1718-1722.

Liu, T., Lee, Y. N., Malbon, C. C. and Wang, H. Y. (2002). Activation of the beta-catenin/Lef-Tcf pathway is obligate for formation of primitive endoderm by mouse F9 totipotent teratocarcinoma cells in response to retinoic acid. *J. Biol. Chem.* **277**, 30887-30891.

Malbon, C. C. (2005). G proteins in development. *Nat. Rev. Mol. Cell Biol.* **6**, 689-701.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* **86**, 391-399.

Murasawa, S., Matsubara, H., Mori, Y., Masaki, H., Tsutsumi, Y., Shibasaki, Y., Kitabayashi, I., Tanaka, Y., Fujiyama, S., Koyama, Y. et al. (2000). Angiotensin II initiates tyrosine kinase Pyk2-dependent signalings leading to activation of Rac1-mediated c-Jun NH2-terminal kinase. *J. Biol. Chem.* **275**, 26856-26863.

Nateri, A. S., Spencer-Dene, B. and Behrens, A. (2005). Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. *Nature* **437**, 281-285.

Poliakov, A. and Wilkinson, D. G. (2006). Ephrins make eyes with planar cell polarity. *Nat. Cell Biol.* **8**, 7-8.

Rivat, C., Le Floch, N., Sabbah, M., Teyrol, I., Redeuilh, G., Bruyneel, E., Mareel, M., Matrisian, L. M., Crawford, H. C., Gespach, C. et al. (2003). Synergistic cooperation between the AP-1 and LEF-1 transcription factors in activation of the matrilysin promoter by the src oncogene: implications in cellular invasion. *FASEB J.* **17**, 1721-1723.

Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P. J., Kikuchi, A., Higuchi, Y. and Bienz, M. (2007). The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. *Nat. Struct. Mol. Biol.* **14**, 484-492.

Sheldahl, L. C., Park, M., Malbon, C. C. and Moon, R. T. (1999). Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. *Curr. Biol.* **9**, 695-698.

Shi, C. S., Huang, N. N., Harrison, K., Han, S. B. and Kehrl, J. H. (2006). The mitogen-activated protein kinase kinase kinase kinase GCKR positively regulates canonical and noncanonical Wnt signaling in B lymphocytes. *Mol. Cell. Biol.* **26**, 6511-6521.

Slusarski, D. C., Corces, V. G. and Moon, R. T. (1997). Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* **390**, 410-413.

Sokol, S. Y. (1996). Analysis of Dishevelled signalling pathways during Xenopus development. *Curr. Biol.* **6**, 1456-1467.

Song, J. J. and Lee, Y. J. (2007). Differential activation of the JNK signal pathway by UV irradiation and glucose deprivation. *Cell. Signal.* **19**, 563-572.

Strutt, D. I., Weber, U. and Mlodzik, M. (1997). The role of RhoA in tissue polarity and Frizzled signalling. *Nature* **387**, 292-295.

Teramoto, H., Coso, O. A., Miyata, H., Igishi, T., Miki, T. and Gutkind, J. S. (1996). Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. *J. Biol. Chem.* **271**, 27225-27228.

van Triest, M. and Bos, J. L. (2004). Pull-down assays for guanoside 5′-triphosphate-bound Ras-like guanosine 5′-triphosphatases. *Methods Mol. Biol.* **250**, 97-102.

Wang, H. Y. and Malbon, C. C. (2003). Wnt signaling, Ca^{2+}, and cyclic GMP: visualizing Frizzled functions. *Science* **300**, 1529-1530.

Wang, H. Y., Kanungo, J. and Malbon, C. C. (2002). Expression of Galpha 13 (Q226L) induces P19 stem cells to primitive endoderm via MEKK1, 2, or 4. *J. Biol. Chem.* **277**, 3530-3536.

Wharton, K. A., Jr (2003). Runnin' with the Dvl: proteins that associate with Dsh/Dvl and their significance to Wnt signal transduction. *Dev. Biol.* **253**, 1-17.

Yamauchi, J., Miyamoto, Y., Murabe, M., Fujiwara, Y., Sanbe, A., Fujita, Y., Murase, S. and Tanoue, A. (2007). Gadd45a, the gene induced by the mood stabilizer valproic acid, regulates neurite outgrowth through JNK and the substrate paxillin in N1E-115 neuroblastoma cells. *Exp. Cell Res.* **313**, 1886-1896.

Zhang, L., Gao, X., Wen, J., Ning, Y. and Chen, Y. G. (2006). Dapper 1 antagonizes Wnt signaling by promoting dishevelled degradation. *J. Biol. Chem.* **281**, 8607-8612.

Zhong, N., Gersch, R. P. and Hadjiargyrou, M. (2006). Wnt signaling activation during bone regeneration and the role of Dishevelled in chondrocyte proliferation and differentiation. *Bone* **39**, 5-16.

Zou, H., Li, Q., Lin, S. C., Wu, Z., Han, J. and Ye, Z. (2007). Differential requirement of MKK4 and MKK7 in JNK activation by distinct scaffold proteins. *FEBS Lett.* **581**, 196-202.