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ORIGINAL ARTICLE

Molecular association between β-catenin degradation complex and Rac guanine exchange factor DOCK4 is essential for Wnt/β-catenin signaling

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The canonical Wnt/β-catenin pathway is a highly conserved signaling cascade that is involved in development and stem cell renewal. The deregulation of this pathway is often associated with increased cell growth and neoplasia. The small GTPase Rac has been shown to influence canonical Wnt signaling by regulating β-catenin stability through an unknown mechanism. We report that DOCK4, a guanine nucleotide exchange factor (GEF) for Rac and a member of the CDM family of unconventional GEFs, mediates Wnt-induced Rac activation in the canonical Wnt/β-catenin pathway. DOCK4 expression regulates cellular β-catenin levels in response to the Wnt signal, in vitro. Biochemical studies demonstrate that DOCK4 interacts with the β-catenin degradation complex, consisting of the proteins adenomatosis polyposis coli, Axin and glycogen synthase kinase 3β (GSK3β). This molecular interaction enhances β-catenin stability and Axin degradation. Furthermore, we observe that DOCK4 is phosphorylated by GSK3β, which enhances Wnt-induced Rac activation. Using a T-cell factor reporter zebrafish we confirm that DOCK4 is required for Wnt/β-catenin activity, in vivo. These results elucidate a novel intracellular signaling mechanism in which a Rac GEF, DOCK4 acts as a scaffold protein in the Wnt/β-catenin pathway.

Introduction

Wnts are extracellular glycoproteins that bind and activate cell-surface receptors initiating a signaling cascade that is important in development and disease (Clevers, 2006). Depending on the receptor repertoire of the cell, Wnts induce either β-catenin-dependent (canonical) or β-catenin-independent (noncanonical) signaling cascades (Mikels and Nusse, 2006). In canonical Wnt/β-catenin pathway, Wnt ligand Wnt3A interacts with cell-surface receptors leading to stabilization of β-catenin in the cytosol, followed by nuclear translocation where it acts as a transcriptional coactivator. In the absence of canonical Wnt signals, β-catenin is bound to a multi-protein degradation complex consisted of the tumor suppressor protein, adenomatosis polyposis coli (APC), the signaling scaffold protein, Axin and the serine-threonine kinase, glycogen synthase kinase 3β (GSK3β) (Moon et al., 2002; Kimelman and Xu, 2006). When bound to this complex, β-catenin is constitutively phosphorylated by GSK3β, which serves as an earmark for its ubiquitin-mediated degradation (Yost et al., 1996; Aberle et al., 1997; Willert et al., 1999; Ha et al., 2004). Wnt stimulation results in the inhibition of GSK3β-mediated β-catenin phosphorylation and activation of GSK3β-mediated phosphorylation of Axin and the Wnt co-receptor LRP5/6 (Zeng et al., 2005). Axin phosphorylation triggers its membrane translocation and subsequent degradation (Zeng et al., 2005). These molecular events ultimately result in the disassembly of the β-catenin degradation complex and an increase in β-catenin levels in the cytosol. The formation and disassembly of the β-catenin degradation complex is a tightly controlled process and several regulators of this process have been described such as disheveled, protein phosphatase 1, presenilin and casein kinase1 (Amit et al., 2002; Kang and Massague, 2004; Luo et al., 2007; Macdonald et al., 2007).

The small GTPase Rac, a regulator of actin cytoskeleton, has been recently shown to be important for nuclear translocation of β-catenin and T-cell factor (TCF) activation (Esufali and Bapat, 2004; Wu et al., 2008). Furthermore, the neurotropic JC virus T antigen has been shown to recruit Rac to stabilize β-catenin (Bhattacharyya et al., 2007). Prior to these reports, Rac was believed to be a critical regulator of β-catenin-independent, noncanonical signaling (Habas et al., 2003; Veeman et al., 2003).

In cell signaling, Rac is activated by regulatory proteins called guanine exchange factors or GEFs (Burridge and Wennerberg, 2004). We previously identified and cloned DOCK4, a member of DOCK

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family of GEFs, which regulates β-catenin at adherence junctions by Rap activation (Yajnik et al., 2003). DOCK4 is a multi-domain protein, which is a part of the DOCK superfamily of 11 unconventional GEFs, characterized by the presence of a DHR1 and DHR2 (DOCK homology regions 1 and 2) domains (Brugnera et al., 2002; Cote and Vuori, 2002). The DHR1 domain binds phospholipid PIP3 and the DHR2 domain activates Rac and together regulate protein function in cell migration (Cote et al., 2005; Lu et al., 2005). The precise mechanism of Rap1 regulation by DOCK4 remains presently unclear but we demonstrated that like other DOCK family members, DOCK4 activates Rac1 through its DHR2 domain (Lu et al., 2005; Yan et al., 2006). In light of the fact that Rac regulates β-catenin, we hypothesized that DOCK4 regulates the cytosolic pool of β-catenin by Rac activation in canonical Wnt signaling. Here we report that DOCK4 is required for Wnt-induced Rac activation, TCF transcription and cell migration. Furthermore, we illustrate the molecular mechanism of DOCK4 action in canonical Wnt signaling.

Results

DOCK4 enhances Wnt/β-catenin signaling

To determine whether DOCK4 influences Wnt/β-catenin signaling, we used HEK293 cells, which have an intact Wnt pathway (Liu et al., 2007). HEK293 cells were transfected with a constant amount of Flag β-catenin with increasing concentration of Flag-DOCK4. TopFlash reporter assays revealed that β-catenin-induced TCF activation was enhanced 2- to 5-fold by DOCK4 in a dose-dependent manner (Figure 1A, a). In parallel, western blot analysis of lysates showed that steady-state levels of β-catenin were also increased (2- to 5-fold) upon DOCK4 expression (Figure 1A, b). Thus, ectopic coexpression of DOCK4 with β-catenin enhances β-catenin stability and TCF activation, suggesting that DOCK4 may be an intracellular regulator of canonical Wnt signaling.

DOCK4 is required for cytosolic accumulation of β-catenin

To test whether DOCK4 is required in Wnt/β-catenin signaling, we used NIH3T3 cells, which also have an intact Wnt pathway (Hocevar et al., 2003). NIH3T3 cells were transfected with DOCK4 SiRNA and treated with either Wnt3A (canonical ligand) or Wnt5A (noncanonical ligand). Transient transfection with DOCK4 SiRNA reduced DOCK4 expression by over 90% (Figure 1B, a , inset) without affecting cell viability (Supplementary Figure 1A). A significant inhibition (fivefold) in the Wnt3A-induced TCF activation was observed when cells were treated with DOCK4 SiRNA using TopFlash reporter assays (Figure 1B, a). In addition, two commercially available SiRNA for DOCK4 (Dharmacon Technologies, Lafayette, CO, USA) were tested for Wnt3A-induced TCF activation.
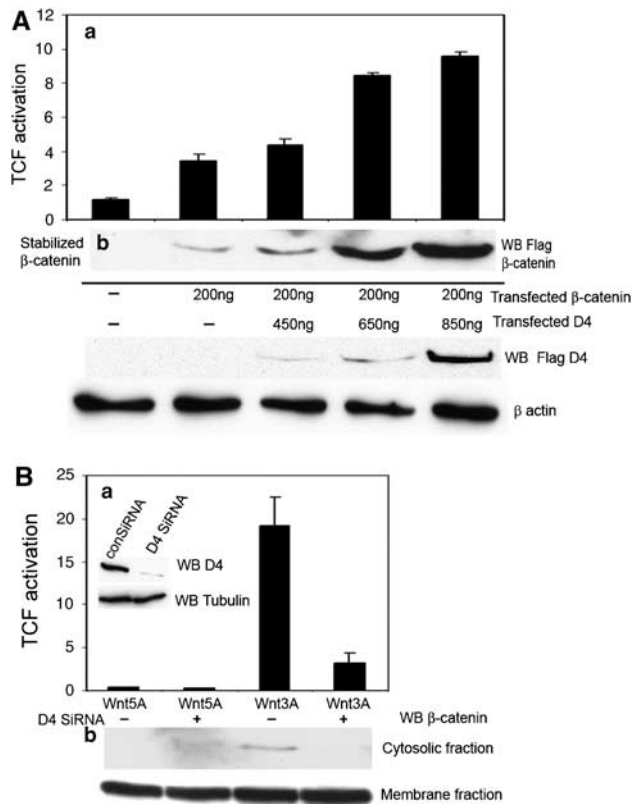

Figure 1 DOCK4 is required for a transcriptionally active pool of β-catenin. (**A**) DOCK4 enhances β-catenin-induced T-cell factor (TCF) transcription. β-Catenin (200 ng) was co-transfected with increasing amounts of DOCK4 for relative TCF reporter assay (a), western blot analysis performed in parallel demonstrates DOCK4-dependent increase in the total levels of β-catenin but not actin (b). (**B**) Silencing of DOCK4 abrogates Wnt3A-induced TCF activation demonstrated by relative TCF reporter assay (a) cytosolic and membrane fractions were isolated in parallel from these samples. Western blot analysis of these fractions showed that Wnt3A but not Wnt5A treatment leads to cytosolic accumulation of free β-catenin, which is inhibited in the presence of DOCK4 SiRNA (b). DOCK4 is abbreviated as D4 in the figure.

These SiRNAs showed a greater than 50% reduction in DOCK4 expression and threefold reduction in TCF activation, further confirming that DOCK4 expression is required for TCF activation. Cells transfected with the SH3-domain-deleted DOCK4 are resistant to down-regulation by the DOCK4 SiRNA as it targets the nucleotides within the SH3-domain-coding segment of the DOCK4 mRNA (Yajnik et al., 2003). Thus, co-transfection of SH3-domain-deleted DOCK4 along with DOCK4 SiRNA rescued the Wnt3A-mediated TCF activation (Supplementary Figure1B).

To confirm whether DOCK4 expression is required for TCF activation we tested a panel of cell lines including mouse intestinal epithelial cell IEC6 and colon cancer cell lines with constitutively activated Wnt signaling that included Hct116, SW480, DLD-1 and HT29. TopFlash reporter studies revealed that silencing DOCK4 expression reduced Wnt3A-induced TCF activation fivefold in the IEC6 cell line ($P < 0.001$). Among colon cancer cell lines, we found inhibition of

TCF activity in DLD-1 and HT29 cells but not in Hct116 and SW480 cells (Supplementary Table 1). DLD-1 and HT-29 cells contain an active cellular machinery for ubiquitin-mediated degradation of β-catenin and this mechanism is nonfunctional in HCT116 and SW480 cell lines (Ilyas *et al.*, 1997; Yang *et al.*, 2006). In summary, together these results demonstrate that DOCK4 is required for Wnt3A-induced TCF activation, *in vitro*. Results from colon cancer cell lines suggest that DOCK4 can regulate β-catenin-mediated TCF activation by inhibiting the β-catenin degradation complex.

To understand the underlying mechanism of DOCK4 in TCF activation, we treated NIH3T3 cells with Wnt3A or Wnt5A for 1 h and isolated the free cytosolic and membrane protein fractions from total cytosolic, post-nuclear cellular lysates. As shown in literature, western blot analysis showed that Wnt3A, but not Wnt5A, results in the accumulation of β-catenin in the post-nuclear 'free' cytosolic fraction. Interestingly, the Wnt3A-induced cytosolic accumulation of β-catenin was inhibited in DOCK4 SiRNA cells without affecting the membrane-bound pool of β-catenin (Figure 1 B, b).

We next tested the nuclear translocation of β-catenin by immunofluorescence (IF). Cells were transfected with either control or DOCK4 SiRNA, then treated with Wnt3A-conditioned media and labeled with anti-DOCK4 and anti-β-catenin antibodies. The IF revealed that DOCK4 expression is required for nuclear translocation of β-catenin (Supplementary Figure 1C), which could be a consequence of decreased levels of free cytosolic β-catenin in the DOCK4 SiRNA cells. Moreover, dual IF showed that DOCK4 does not colocalize with β-catenin (Supplementary Figure 1C, upper panel, merge), suggesting that DOCK4 does not directly interact with β-catenin. To test the direct interaction of DOCK4 with β-catenin, Flag-tagged β-catenin was coexpressed with green fluorescent protein (GFP)-tagged DOCK4. Coimmunoprecipitation (Co-IP) was performed using anti-Flag antibody that showed that β-catenin did not co-precipitate with DOCK4 (Supplementary Figure 1D).

Taken together, these results demonstrate that DOCK4 is required for cytosolic release and subsequent nuclear translocation of free β-catenin after Wnt3A stimulation but without a direct molecular interaction with β-catenin.

Endogenous and in vitro *interactions between DOCK4 and the β-catenin degradation complex in Wnt/β-catenin signaling*
We hypothesized that DOCK4 interacts with key members of the β-catenin degradation complex namely APC, Axin and GSK3β to regulate β-catenin. For this purpose epitope (Flag)-tagged DOCK4 and its domain deletion constructs (diagram of constructs shown in Figure 2A) were coexpressed with epitope-tagged GFP-APC, Myc-Axin and HA-GSK3β. Results from Co-IP studies performed using anti-Flag monoclonal antibody are shown in Figure 2B, a.

These results indicate that GFP-APC binds to full-length DOCK4, the SH3-domain-deleted DOCK4

(ΔSH3 DOCK4), and to C-terminal-deleted DOCK4 (ΔC-term DOCK4); whereas, it did not bind to DHR2-domain-deleted DOCK4 (ΔDHR2 DOCK4; Figure 2B, b). To determine whether the DHR2 domain is not only necessary but also sufficient to bind APC, a glutathione S-transferase (GST) fusion of DHR2 domain and GST fusion of C-terminal of DOCK4 was coexpressed with GFP-APC. GST pull-down experiments showed that only DHR2 domain but not C-terminal of DOCK4 co-precipitated with APC (Supplementary Figure 2). Collectively, these findings suggest that APC binds to DHR2 domain of DOCK4.

The DOCK4 interaction to Axin was tested in a similar approach and demonstrated that DOCK4 binds to Axin as well. The Co-IP experiment demonstrated that Myc-Axin co-precipitates with all DOCK4 variants except the ΔC-term DOCK4 (Figure 2B, c). In GST pull-down experiments, the C-terminus proved to be sufficient to co-precipitate Axin, suggesting that these two proteins specifically interact via the C-terminus of DOCK4 (shown in Figure 3). Further Co-IP experiments with DOCK4 and HA-GSK3β revealed that DOCK4 binds to GSK3β by its C terminus (Figure 2B, d). This result was further evaluated and confirmed by GST pull-down assays (shown in Figure 3). On the basis of these results, a composite interaction map of DOCK4 with the β-catenin degradation complex is shown (Figure 2C).

To evaluate the functional consequences of these interactions, the deletion constructs of DOCK4 were analysed for their impact on β-catenin-induced TCF activation. TCF reporter assays revealed that full-length DOCK4 synergistically increases TCF activation due to β-catenin overexpression, whereas ΔSH3 DOCK4 enhances the TCF activation even further. The ΔDHR2 DOCK4 and ΔC-term DOCK4 abolished the TCF activation (Figure 2D). These results showed that the domains of DOCK4, which are required in interaction with APC/Axin/GSK3β, are also required for β-catenin-induced TCF activation. Interestingly, overexpression of ΔSH3 DOCK4 showed the maximum effect on β-catenin-induced TCF activation that could be related to the fact ΔSH3 DOCK4 has an enhanced Rac activation (Lu *et al.*, 2005).

To determine the regulation of DOCK4 on APC/Axin/GSK3β in the context of canonical Wnt3A signaling, we extended our studies on endogenous proteins. HEK 293 cells were treated with Wnt3A-conditioned media for indicated time points. Endogenous DOCK4 was immunoprecipitated and analysed for co-precipitation with endogenous APC, Axin and GSK3β proteins. This experiment demonstrated that indeed DOCK4 binding to APC/Axin/GSK3β is regulated by Wnt signaling (Figure 2E). Upon Wnt3A stimulation DOCK4 binding to APC/Axin/GSK3β is increased 2- to 3-fold (Figure 2E). Interestingly, DOCK4 specifically binds to phosphorylated APC (appeared as a slow migrating band (arrows), top panel, Figure 2E) at 10 min of Wnt stimulation. The endogenous protein interaction data suggest that in response to Wnt signaling, DOCK4 sequesters the β-catenin
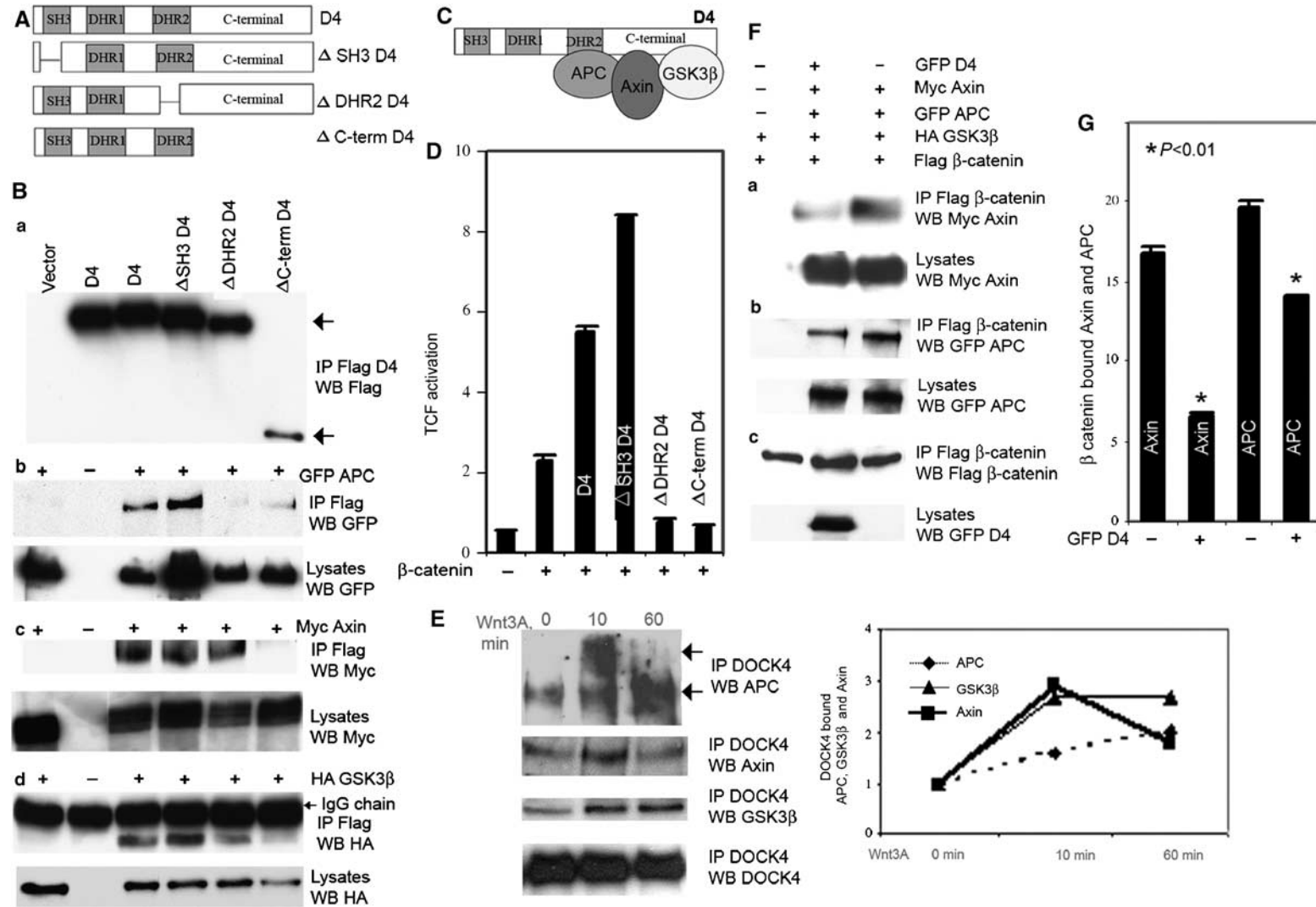
Figure 2 Interaction of DOCK4 with β-catenin degradation complex enhances relative T-cell factor (TCF) activation and the release of β-catenin from adenomatosis polyposis coli (APC)–Axin complex. (**A**) Diagram of Flag-tagged full-length and deletion constructs of DOCK4. (**B**) These constructs were co-transfected with epitope-tagged APC, Axin or glycogen synthase kinase 3β (GSK3β). Coimmunoprecipitation was performed using monoclonal Flag antibody and analysed by western blot using monoclonal antibodies against specified epitopes. These results show that APC binds to DHR2 domain of DOCK4 (b), whereas Axin (c) and GSK3β (d) bind to C terminus of DOCK4. (**C**) Composite interaction map is shown. (**D**) Full-length and deletion constructs of DOCK4 were co-transfected with β-catenin for relative TCF reporter assay. The β-catenin-induced TCF activation was enhanced in the presence of full-length DOCK4, which was further increased in the presence of Δ SH3 DOCK4, whereas Δ DHR2 and Δ C-term DOCK4 abolished β-catenin-induced TCF activation. (**E**) Endogenous DOCK4 interacts with APC, Axin and GSK3β in HEK-293 cells treated with Wnt3A-conditioned media for 10 min and 1 h. DOCK4 binding to APC, Axin and GSK3β is increased in time-dependent manner. The graph to the right of the blot shows the fold change in binding by densitometry. (**F**) Flag-tagged β-catenin was co-transfected with APC, Axin and GSK3β with or without DOCK4. Western blot analysis of Flag β-catenin immunoprecipitation showed that β-catenin binding to APC and Axin is decreased in the presence of DOCK4. (**G**) The western blots for three different experiments were quantified and data presented in a bar graph. DOCK4 is abbreviated as D4 in the figure and *denotes $P < 0.01$ by Student's t-test.
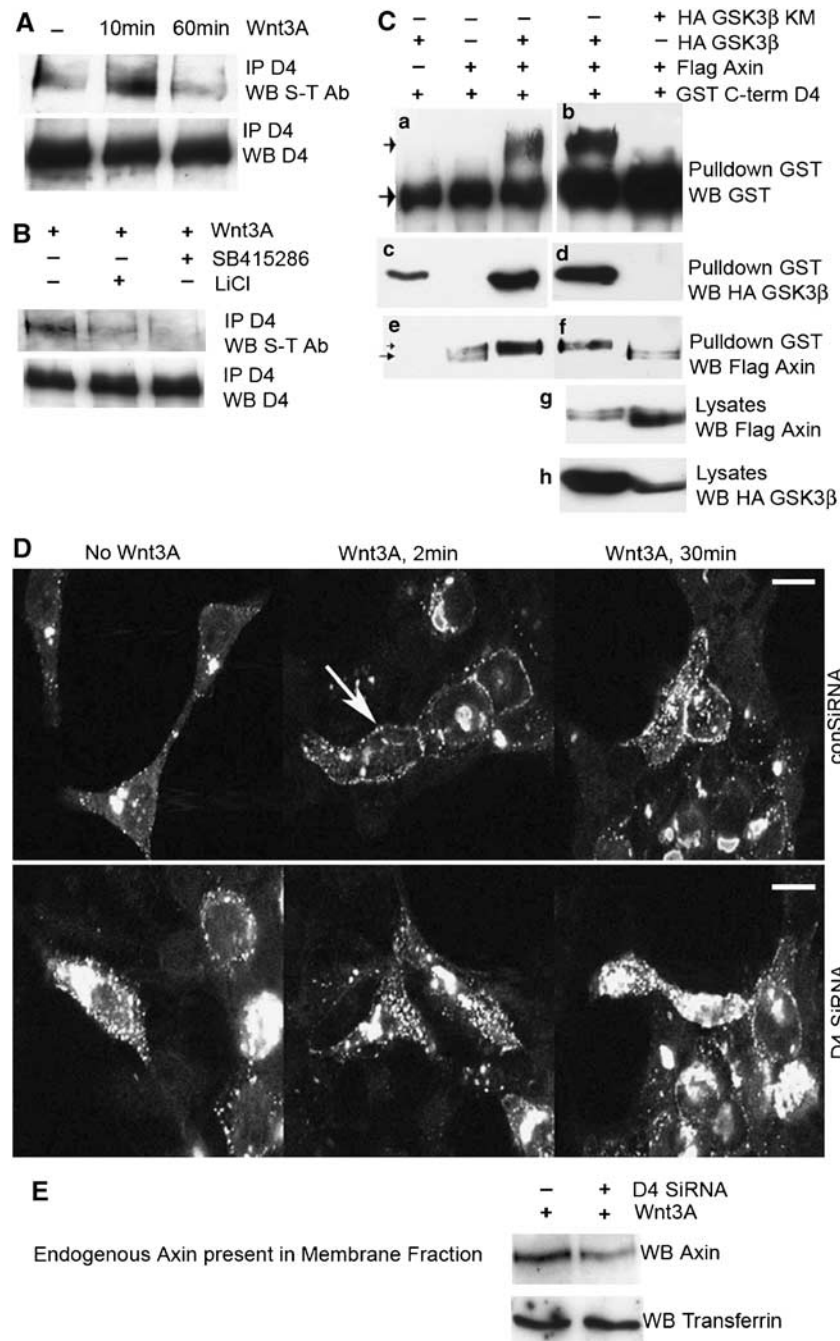
Figure 3 DOCK4 is phosphorylated by glycogen synthase kinase 3β (GSK3β) and DOCK4 enhances the destabilization of Axin. (**A**) DOCK4 phosphorylation is observed when endogenous DOCK4 is immunoprecipitated from HEK293 cells and analysed by phospho-serine/threonine antibody. Phosphorylation is increased 10 min after Wnt3A stimulation. (**B**) Treatment with GSK3β kinase inhibitors SB415286 or LiCl inhibits phosphorylation of DOCK4. (**C**) Co-transfection studies with glutathione S-transferase (GST)-tagged C-terminal domain of DOCK4 demonstrates a mobility shift, left arrows, in the simultaneous presence of Axin and GSK3β (a) the mobility shift is phosphorylation specific as it disappears when kinase mutant GSK3β is coexpressed (b). The wild-type (WT) GSK3β binding to C-term of DOCK4 is increased in the presence of Axin (c), whereas the KM GSK3β does not bind to C-term of DOCK4 (d). Expression of WT and kinase mutant GSK3β in total lysate is shown (h). Note that the slow migrating, phosphorylated Axin binds to C-term of DOCK4 in the presence of WT GSK3β (e). The binding of unphosphorylated Axin (appeared as the fast migrating band) binds to C-term of DOCK4 in the presence of KM GSK3β is significantly lower (f), despite adequate expression in total lysates (g). (**D**) Flag-tagged Axin translocates to plasma membrane (arrow) upon 2 min of Wnt3A stimulation in control but not DOCK4 SiRNA cells. This effect was observed in >90% transfected cells. The scale bar in the figure is at 20 μm. (**E**) Membrane fraction of Wnt3A-treated control and DOCK4-silenced HEK293 cells shows low levels of endogenous Axin in DOCK4 SiRNA cells. DOCK4 is abbreviated as D4 in the figure.

degradation complex APC/Axin/GSK3β to facilitate the release and accumulation of β-catenin in the cytosol.

DOCK4 enhances the release of β-catenin from APC and Axin

Sequestration of β-catenin degradation complex by DOCK4 should result in a corresponding decrease in the binding of β-catenin with APC and Axin. To test this possibility, β-catenin was coexpressed with Axin, APC and GSK3β with or without DOCK4. We observed that β-catenin binding to Axin and APC decreased significantly ($P < 0.005$) in the presence of DOCK4 (Figure 2F; a, b). The decreased binding of β-catenin to its degradation complex in the presence of DOCK4 leads to an increased cellular level of β-catenin (Figure 2F, c). These results also explain the reduced free β-catenin in the cytosol after Wnt3A stimulation in DOCK4-silenced cells (shown in Figure 1B, b).

DOCK4 is phosphorylated by GSK3β

DOCK4 binds to the serine-threonine kinase GSK3β through its serine-proline-rich C terminus. Conceivably, this binding could represent an enzyme/substrate interaction. To test whether DOCK4 is phosphorylated during Wnt/β-catenin signaling, endogenous DOCK4 was immunoprecipitated and analysed by western blot using a commercially available, phospho-serine/threonine antibody. DOCK4 phosphorylation is observed within 10 min of Wnt3A stimulation (Figure 3A; Supplementary Figure 3A). Prior treatment with GSK3β kinase inhibitors LiCl or SB415286 resulted in abrogation of Wnt3A-induced phosphorylation of DOCK4 (Figure 3B). These data indicate that DOCK4 is phosphorylated by GSK3β during Wnt/β-catenin signaling.

To investigate the mechanism of GSK3β-mediated phosphorylation of DOCK4, we utilized C terminus of DOCK4, which interacts with GSK3β and Axin (see Figure 2C). Axin has been shown to function as scaffold in APC phosphorylation by CK1 and GSK3β (Hart et al., 1998; Ha et al., 2004). To test if Axin is a scaffold for DOCK4 phosphorylation by GSK3β, GST-fused C terminus of DOCK4 was coexpressed with GSK3β in the presence or absence of Axin. Indeed, a slow migrating band (indicative of phosphorylation) of the C terminus of DOCK4 appeared exclusively in the presence of both Axin and GSK3β (Figure 3C, a). This band disappeared upon replacing GSK3β with a kinase-deficient mutant, KM-GSK3β or by removing Axin (Figure 3C, b). To further confirm that the slow migration of the above-mentioned band is due to phosphorylation, we excised it from the gel and analysed it by mass spectrometry. This analysis allowed us to map 10 specific residues (nine serines and one threonine) in the C terminus of DOCK4 that are phosphorylated by GSK3β (Supplementary Figure 3B, C). In agreement with the increased phosphorylation of DOCK4, we observed that GSK3β binding to the C terminus of DOCK4 is increased in the presence of Axin (Figure 3C, c). In contrast, KM-GSK3β did not bind to DOCK4,

whether or not Axin was coexpressed (Figure 3C, d). Taken together, these data provide biochemical evidence that DOCK4 is phosphorylated by GSK3β in the presence of Axin in Wnt/β-catenin signaling.

DOCK4 is required for Axin translocation upon Wnt3A stimulation

Axin phosphorylation by GSK3β has also been reported in Wnt/β-catenin signaling (Zeng et al., 2005). In addition, we know that the C terminus of DOCK4 binds preferentially to the slow migrating phosphorylated form of Axin (Figure 3C, e, f and g). Following phosphorylation, Axin translocates to the membrane, which is a prerequisite for its degradation (Zeng et al., 2005). As DOCK4 binds preferentially to phosphorylated Axin, we tested whether DOCK4 is required for membrane translocation of Axin. Confocal imaging demonstrated that Wnt3A-induced membrane translocation of transfected Axin in DOCK4 SiRNA cells was inhibited despite high intracellular levels (Figure 3D). The membrane translocation of endogenous Axin was tested in the membrane fractions prepared from control and DOCK4 SiRNA cells treated with Wnt3A. A decreased level of endogenous Axin in membrane fraction was observed in DOCK4 SiRNA cells when compared to control (Figure 3E), suggesting that DOCK4 is required for Axin translocation.

DOCK4 is required for Wnt-induced Rac activation

Small deletions in individual DOCK4 domains can dramatically affect its Rac GEF function. For example, in comparison to wild-type DOCK4, the ΔDHR2 DOCK4 mutant has been shown to be dominant negative whereas ΔSH3 DOCK4 has been shown to be more active (Lu et al., 2005). Curiously, the ΔDHR2 DOCK4 inhibited whereas ΔSH3 DOCK4 enhanced β-catenin-induced signaling (see Figure 2), suggesting that DOCK4 Rac1 activation was required for its regulation in Wnt signaling. Furthermore, TCF reporter assay revealed that the cells transfected with wild-type Rac, dominant-negative Rac (N17) and constitutively active Rac (V12) showed that wild-type Rac synergistically activated, RacN17 abolished and Rac V12 had the maximum effect on TCF activation induced by Wnt3A (Supplementary Figure 4A). To test for the specificity for Rac1, we used active mutants of other GTPases and observed that only Rac1 cooperates in Wnt3A-mediated TCF activation (Supplementary Figure 4A). These results suggested that Rac1 signaling positively affects canonical Wnt signaling, which is in agreement with earlier studies (Esufali and Bapat, 2004; Wu et al., 2008).

Next, we asked whether DOCK4 is required for Wnt-induced Rac activation. For this purpose, we used the GST-fused PBD (PAK-binding domain) that binds preferentially to GTP-bound Rac in cell lysates. In each sample, the GTP-Rac detected by western blots was normalized to total cellular Rac1 as described (Yajnik et al., 2003). Measurement of GTP-bound Rac levels in HEK293 cells showed that both Wnt3A and Wnt5A

effectively activate endogenous Rac1. The DOCK4 SiRNA inhibited this Rac activation without affecting the basal GTP-Rac levels (Figure 4A). Densitometry studies revealed that DOCK4 SiRNA inhibited both Wnt3A- (twofold) and Wnt5A (threefold)-induced Rac activation (Figure 4B), suggesting that DOCK4 is required for Rac activation in both canonical and noncanonical Wnt signaling.

DOCK4-mediated Rac activation is enhanced by its phosphorylation

Next, we tested whether DOCK4-mediated Rac activation is enhanced by its phosphorylation. Using the GST PBD pull-down assay on cells stimulated with Wnt3A for 10 min, we observed that expression of DOCK4 alone increases Rac activation (Yan et al., 2006). The highest GTP-Rac1 levels are observed when DOCK4 is coexpressed with GSK3β, Axin and APC, the members of the β-catenin degradation complex (Figure 4C). Consistent with the requirement of DOCK4 phosphorylation, the combination of DOCK4 with GSK3β and Axin also results in a modest increase in Rac activation as compared to DOCK4 alone. Although, APC increased steady-state levels of DOCK4, it did not increase DOCK4-mediated Rac activation (Figure 4C, Lysates, WB, Flag, DOCK4). Together these observations suggest that phosphorylation by GSK3β regulates DOCK4-mediated Rac1 activation. To confirm the absolute requirement of phosphorylation in regulation
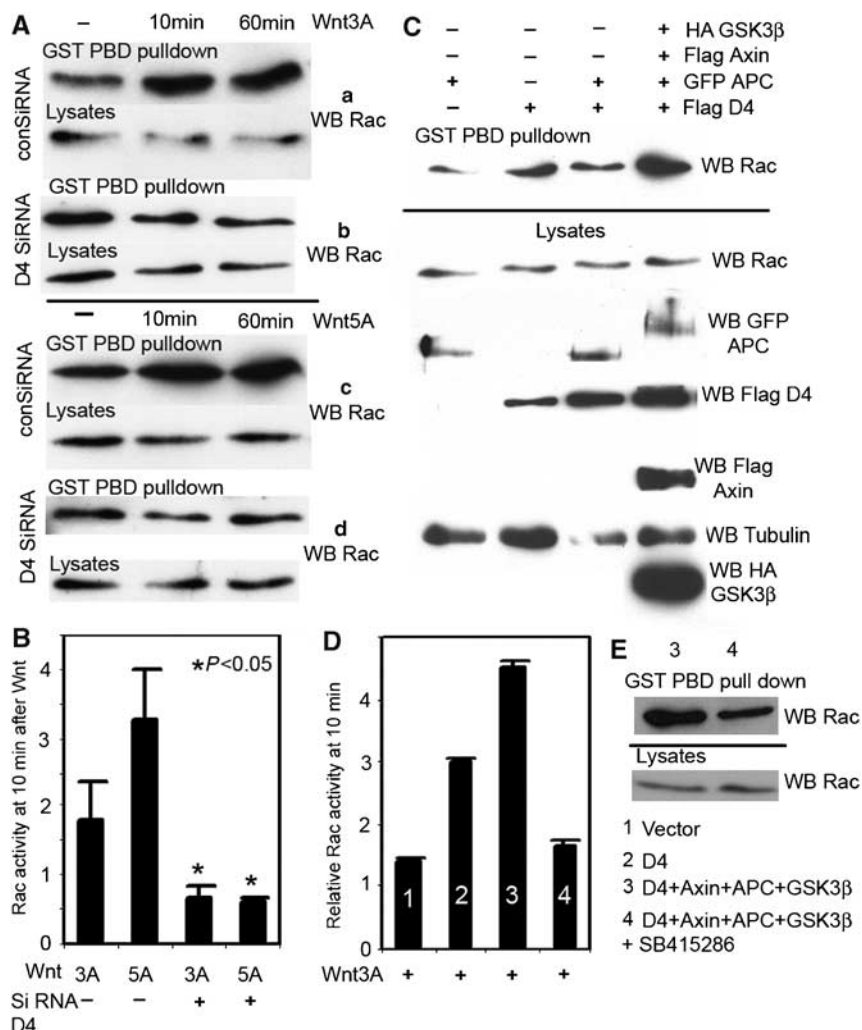

Figure 4 DOCK4 is required for Wnt-induced Rac activation and is enhanced upon phosphorylation of DOCK4. Levels of GTP-bound and total Rac were determined in Wnt3A-stimulated control and DOCK4-silenced HEK293 cells. (**A**) Wnt3A treatment induces Rac activation (a), which is diminished in the presence of DOCK4 SiRNA (b). Wnt 5A treatment also induces Rac activation (c), which is diminished in presence of DOCK4 SiRNA (d). (**B**) Densitometry from five such experiments revealed that DOCK4 SiRNA significantly ($P < 0.05$) reduces Wnt-induced Rac activation. (**C**) Overexpression of DOCK4 enhances Wnt3A-induced Rac activation, which is further increased by coexpression of adenomatosis polyposis coli (APC), Axin and glycogen synthase kinase 3β (GSK3β). Expression levels of the various proteins in the lysates are shown along with the control, tubulin. (**D**) Densitometry showed that DOCK4-induced Rac activation in cooperation with APC, Axin and GSK3β is reduced when cells were treated with GSK3β kinase inhibitor SB415286. (**E**) Western blot of Rac activation in cells coexpressing DOCK4, APC, Axin, GSK3β with (4) or without SB415286 (3). DOCK4 is abbreviated as D4 in the figure and *denotes $P < 0.05$ by Student's t-test.

of DOCK4 GEF function, cells were treated with GSK3β inhibitor SB415286. Rac activation with treatment was reduced to basal levels as determined by densitometry (Figure 4D, E).

DOCK4 is required for Wnt-induced cell migration in vitro

In addition to β-catenin-induced gene transcription leading to cellular proliferation, Wnt ligands also regulate cell migration (Wu et al., 2007). Members of the CDM family proteins are critical intracellular regulators of cell migration (Grimsley et al., 2003). To test whether DOCK4 is required for Wnt-induced cell migration, Wnt3A- and Wnt5A-conditioned media were used as chemoattractants in Transwell chamber migration assay. We observed that both Wnt3A and Wnt5A facilitate cell migration, with Wnt5A, having a more pronounced effect (Figure 5A, upper panel). The increased migration associated with Wnt5A could

potentially be a result of higher Rac activation by Wnt5A (see Figure 4B). Both Wnt3A- and Wnt5A-induced migration was inhibited in cells transfected with DOCK4 SiRNA (Figure 5A, lower panel), which leads to the conclusion that DOCK4-mediated Rac activation could be required for Wnt-induced cell migration in vitro.

DOCK4 is required for Wnt/β-catenin signaling in vivo

To obtain an in vivo correlation of our novel biochemical findings, we used a Top:dGFP transgenic zebrafish Wnt/β-catenin reporter line (Dorsky et al., 2002). At the protein level, the zebrafish (z) DOCK4 shares more than 80% sequence homology to human DOCK4. Using a morpholino to zDOCK4 (Supplementary Figure 5A), we were able to reduce DOCK4 expression up to 60% (Figure 5B, a). We measured GFP expression representing Wnt/β-catenin signaling activity by in situ hybridization. Embryos injected with control morpholinos
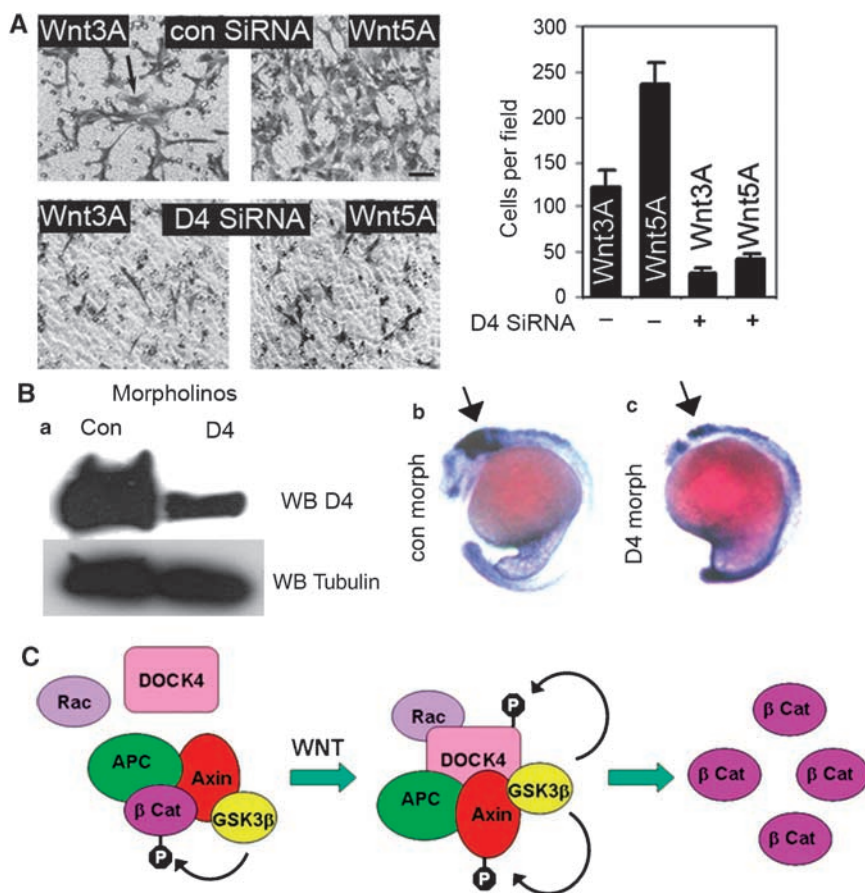

Figure 5 DOCK4 is required for Wnt-induced cell migration in vitro and Wnt/β-catenin signaling in vivo. (**A**) Wnt3A and Wnt5A both induced migration (upper panel), which was inhibited in NIH3T3 DOCK4 SiRNA cells (lower panel). Scale bar is at 3 μm, whereas the arrow shows an example of migrated cell. Quantification of migration is shown in bar graph on the right. (**B**) Top:dGFP Wnt/β-catenin reporter zebrafish (z) embryos were injected at the one-cell stage with morpholinos against mismatched control or zDOCK4 morpholino. Western blot analysis showed efficient (>60%) knockdown of zDOCK4 expression (a). In situ hybridization for green fluorescent protein (GFP, as a marker of Wnt/β-catenin signaling) at the 18-somite stage demonstrates the strong Wnt/β-catenin activity in developing embryos (b, arrow). Embryos injected with zDOCK4 morpholinos showed diminished Wnt/β-catenin activity (c, arrow). (**C**) Schematic of DOCK4 interaction with β-catenin degradation complex during Wnt signaling. DOCK4 is abbreviated as D4 in the figure.

showed Wnt/β-catenin reporter activity mainly in the developing brain region (Figure 5B, b, arrow). Embryos injected with zDOCK4 morpholinos showed diminished Wnt/β-catenin reporter activity in this area (Figure 5B, c, arrow). This effect was dose dependent and correlated well with the effects of direct β-catenin knockdown (Supplementary Figure 5B). Injection of zDOCK4 morpholinos but not control morpholinos gave rise to morphological abnormalities in terms of shortened brain and tail formation (Supplementary Figure 5C). These morphological changes have been reported with inhibition of Wnt/β-catenin signaling (Dorsky et al., 2002; Schier and Talbot, 2005; Thorpe et al., 2005). We conclude that DOCK4 is required for Wnt/β-catenin signaling, in vivo.

Discussion

How ligand-dependent Wnt signaling links to intracellular Rac1 is not understood. Here we show that Rac1 is a downstream target of both canonical Wnt3A and non-canonical Wnt5A signaling. Silencing DOCK4 expression abrogates Wnt-mediated Rac1 activation suggesting that DOCK4 links Wnt to Rac1. We confirm the functional consequence of this observation by testing Wnt-mediated cell migration. Using the Transwell chamber assay we show that DOCK4 expression is required for Wnt3A- and Wnt5A-mediated chemotaxis. Furthermore, using Wnt/β-catenin TCF reporter zebrafish, we show that DOCK4 is required for canonical Wnt signaling. Thus, we conclude that DOCK4–Rac1 pathway is a novel intracellular regulator of both canonical and non-canonical Wnt signaling.

The potential role of DOCK4–Rac1 in the regulation of β-catenin has significant implications in stem cell biology and cancer. We tested a panel of small GTPases and observed that Rac but not Rho, CDC42, Rap or RhoG facilitate Wnt3A-mediated TCF transcription. This suggests that Rac activation is essential for ligand-activated Wnt signaling and is consistent with the literature (Esufali and Bapat, 2004; Wu et al., 2008). DOCK4 is a multi-domain protein and we found that the Rac GEF domain DHR2 and the serine-proline-rich C terminus were essential for TCF activation. Corresponding studies on silencing endogenous DOCK4 and testing for Wnt3A-stimulated TCF activation showed that DOCK4 expression is required in multiple, immortalized tissue culture cell lines. This effect was sustained when different regions of DOCK4-coding sequence were targeted by the Si-RNA reagent and importantly, TCF activation was rescued by expression of silencing resistant, SH3-domain-deleted DOCK4. In the context of colon cancer cell lines, we observed that DOCK4 expression regulates TCF activation in a subset of cell lines, which have the β-catenin ubiquitination machinery intact. The cytosolic accumulation of free β-catenin has been widely accepted as mechanistic prerequisite for nuclear accumulation of β-catenin and

subsequent TCF transcription (Hagen et al., 2004; Macdonald et al., 2007). We observed that DOCK4 silencing inhibits Wnt3A-dependent cytosolic accumulation of 'free' β-catenin and therefore its subsequent nuclear translocation. Thus, we conclude that the DOCK–Rac1 pathway is a novel regulator of β-catenin stability in Wnt/β-catenin signaling in normal and tumor cells.

The major regulator of β-catenin in the cell is its degradation complex whose activity is controlled by modulations in protein phosphorylation and ubiquitination along with dynamic changes in subcellular distribution (Liu et al., 2005; Cadigan and Liu, 2006). Our, biochemical studies show that DOCK4 binds to the members of the β-catenin degradation complex but not to β-catenin itself. We define that the DHR2 domain binds APC whereas the C terminus binds to Axin and GSK3β. However, other potential interactions cannot be completely excluded and are being investigated. Our model of DOCK4–Rac pathway in the regulation of β-catenin degradation complex is illustrated in Figure 5C. Wnt activation at the membrane increases the interaction of DOCK4 with β-catenin degradation complex. This interaction inhibits binding of β-catenin to both APC and Axin, and thus it facilitates the release and stabilization of β-catenin into the cytosol. In the literature, phosphorylated APC has been shown to be more efficient in β-catenin binding (Xing et al., 2003). Similarly, phosphorylated form of Axin has been shown to be more susceptible to membrane translocation and degradation. It is notable that DOCK4 binds to phosphorylated forms of both APC and Axin.

GEFs are regulatory nodes in intracellular signaling pathways that create a protein network between surface receptors and the nucleus by modulating GTP loading in small GTPases (Braga, 2002). The regulation of DOCK4 Rac-GEF function by the β-catenin degradation complex resulting in β-catenin stability is therefore a highly significant finding. Previously, only ELMO and RhoG have been shown to regulate DOCK protein GEF function (Katoh and Negishi, 2003; Lu et al., 2005). Our current studies demonstrate that a post-translation modification of DOCK4 in Wnt/β-catenin regulates its GEF function. GSK3β is a proline-directed, serine kinase and for many of its substrates it requires Axin as a scaffold protein (Jope and Johnson, 2004). Our mass spectroscopy data are in agreement with the published observations as proline residues are present next to the targeted serine residues. How each of these phosphomodifications contributes to DOCK4 function is currently being pursued.

Collectively, these findings identify the first clear molecular link between β-catenin degradation complex and the DOCK4–Rac1 intracellular signaling pathway. We speculate that when DOCK4 expression is enhanced, it can potentially facilitate two important biological outcomes of Wnt signaling, namely, TCF activation and cell migration. These properties make the DOCK4/Rac/Wnt/β-catenin pathway an attractive target for future cancer therapeutics.

Materials and methods

Antibodies

DOCK4 antibody was generated as described (Yan et al., 2006). Commercial antibodies used were from following sources: Axin, APC and GSK3β, Phospho (S33/37Thr41) β-catenin antibodies (Cell Signaling Technology Inc., Danvers, MA, USA), Transferrin (Zymed Laboratories, San Francisco, CA, USA), Rac and β-catenin antibodies (BD Biosciences, San Diego, CA, USA), monoclonal mouse hemagglutinin (HA), GFP, MYC antibodies (Covance Research products, Denver, PA, USA), GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Flag M2 and α-Tubulin antibodies (Sigma-Aldrich, St Louis, MO, USA).

Cell culture, plasmids, SiRNA, transfection studies

L Wnt-3A (CRL-2647), L Wnt-5A (CRL-2814) and other cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Wnt-conditioned media were prepared as described by the manufacturer. Plasmids used were provided by Dr Gottardi (Chicago, IL, USA; Flag-β-catenin and Myc-Axin); Dr Xi He (Boston, MA, USA; HA-GSK3β); Dr Chung (Boston, MA, USA; Flag-Axin and GFP-APC) and Dr Vogelstein (Baltimore, MD, USA; Top/Fop Flash). Flag DOCK4 along with its deletion constructs and GFP DOCK4 were as previously described (Yan et al., 2006). GST C terminus and GST DHR2 construct of DOCK4 were prepared in pEBG vector (Lu et al., 2005). DOCK4 SiRNA was used as described (Yajnik et al., 2003). Commercial SiRNAs control (no. sc-37007; Santa Cruz Biotechnology), DOCK4 (no. LQ-043254-01-0002; Dharmacon Technologies) and lentivirus construct (Broad Institute RNAi consortium).

Measurement of β-catenin-induced TCF activation: luciferase assay

For relative TCF reporter assays, TopFlash or FopFlash were transfected with pRL-CMV (Promega, Madison, WI, USA). Cells were harvested and the luciferase activity was determined with the Dual Luciferase Reporter Assay System from Promega according to the manufacturer protocol.

Microscopy

For IF studies, cells were fixed with methanol and permeabilized using 0.5%. Triton X-100 in phosphate-buffered saline. Confocal images acquired were at iris setup of 0.7 using an inverted fluorescence microscope (Bio-Rad, Hercules, CA, USA) using LaserSharp 2000. For chemotaxis, 1×10^5 cells were seeded in to the upper chamber of Transwell plate (Coster, Cambridge, MA, USA) with Wnt-conditioned media in the lower chamber. After 4 h, migrated cells were counted using a $\times 4$ objective.

Biochemical methods

Cells were lysed in Triton lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and 1 mM ethylene glycol tetraacetic acid, with protease and phosphatase inhibitors. For GST pull-down assay, lysates were incubated for 3 h at 4 °C with glutathione-Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ, USA). For cell fractionation, post-nuclear homogenate was spun at 300 000 g for 90 min in a Beckman tabletop ultracentrifuge. True blot system (E-bioscience, San Diego, CA, USA) was also used to eliminate the heavy chain. Levels of activated, GTP-bound Rac were determined as described (Yajnik et al., 2003). Quantification of western blot was carried out using NIH ImageJ program. Whole zebrafish extracts were prepared from pooled zebrafish embryos at 24 h postfertilization with a previously described protocol (Link et al., 2006).

Zebrafish injections and in situ hybridizations

Antisense morpholinos to zDOCK4 and mismatched control were designed GeneTools, LLC (Corvallis, OR, USA). The β-catenin morpholinos were designed as previously reported (Lyman Gingerich et al., 2005) and obtained from GeneTools, LLC. Morpholinos were injected into one-cell stage of TOP:dGFP (Wnt/β-catenin reporter) zebrafish embryos. At the 18-somite stage, embryos were fixed and subjected to in situ hybridization for GFP as described previously (Dorsky et al., 2002).

Statistical analysis

Statistical analysis was performed using the Student's t-test for unpaired data considered significant as the level of $P < 0.05$.

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