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Rac1 Activation Controls Nuclear Localization of β-catenin during Canonical Wnt Signaling

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SUMMARY

Canonical Wnt signaling critically regulates cell fate and proliferation in development and disease. Nuclear localization of β-catenin is indispensable for canonical Wnt signaling; however, the mechanisms governing β-catenin nuclear localization are not well understood. Here we demonstrate that nuclear accumulation of β-catenin in response to Wnt requires Rac1 activation. The role of Rac1 depends on phosphorylation of β-catenin at Ser191 and Ser605, which is mediated by JNK2 kinase. Mutations of these residues significantly affect Wnt-induced β-catenin nuclear accumulation. Genetic ablation of *Rac1* in the mouse embryonic limb bud ectoderm disrupts canonical Wnt signaling and phenocopies deletion of *β-catenin* in causing severe truncations of the limb. Finally, *Rac1* interacts genetically with *β-catenin* and *Dkk1* in controlling limb outgrowth. Together these results uncover Rac1 activation and subsequent β-catenin phosphorylation as a hitherto uncharacterized mechanism controlling canonical Wnt signaling and may provide additional targets for therapeutic intervention of this important pathway.

INTRODUCTION

Wnt signaling is critical for normal development of multicellular organisms via regulation of cell fate, proliferation, and behavior. In the canonical Wnt pathway, Wnt binding to Frizzled receptors and the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) activates the cytoplasmic signaling protein Dishevelled (Dvl) to stabilize cytosolic β-catenin; β-catenin, upon entering the nucleus, in turn activates transcription of downstream target genes via lymphoid enhancer-binding factor 1 (Lef1) and T cell factors (Tcf1, 3, 4) (Huelsken and Birchmeier, 2001). The amplitude of signaling is fine-tuned in part via negative feedback mechanisms that include the secreted molecule Dickkopf 1 (Dkk1) (Glinka et al., 1998), which antagonizes the pathway by interfering with LRP5/6-Wnt interactions (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001).

Although nuclear localization of β-catenin in response to Wnt is essential for canonical signaling, mechanisms controlling this process are not well understood. Previous reports suggested that BCL9 (Townsley et al., 2004) may actively import β-catenin to the nucleus whereas APC (Henderson, 2000; Neufeld et al., 2000) and Axin (Cong and Varmus, 2004) may export it to the cytoplasm; however, a recent study indicated that these molecules function mainly by retaining β-catenin in either the nucleus or the cytoplasm (Krieghoff et al., 2006).

The Rho family of small GTPases regulates cytoskeleton and transcription by virtue of cycling between inactive GDP-bound and active GTP-bound forms (Hall, 1998). Members of the family, including RhoA, Rac1, and Cdc42, have been shown to participate in noncanonical Wnt signaling pathways that control planar cell polarity (PCP) in *Drosophila* (Eaton et al., 1996; Fanto et al., 2000; Strutt et al., 1997) or convergent extension (CE) in *Xenopus* (Choi and Han, 2002; Habas et al., 2001, 2003; Penzo-Mendez et al., 2003). Moreover, Rac1 may function in part by activating c-Jun NH2-terminal kinase (JNK) (Habas et al., 2003), itself important for both *Drosophila* PCP (Boutros et al., 1998) and *Xenopus* CE (Yamanaka et al., 2002).

Here we report that Rac1 activation is a critical component of canonical Wnt signaling. Specifically, we show that Rac1 activates JNK2 that in turn phosphorylates β-catenin on critical residues and controls its nuclear translocation. Moreover, we present evidence that Rac1 interacts genetically with β-catenin and Dkk1 in controlling limb outgrowth in mouse embryos.

RESULTS

Rac1 Activation by Wnt3a via $G\alpha_{q/11}\beta\gamma$ and PI3K Is Required for β-catenin Signaling

We have studied the potential role of Rho small GTPases in Wnt signaling during osteoblast differentiation. The murine bone marrow-derived stromal cell line ST2 undergoes robust osteoblastogenesis in response to Wnt (Tu et al., 2007). We used an established binding assay to determine whether the GTP-bound

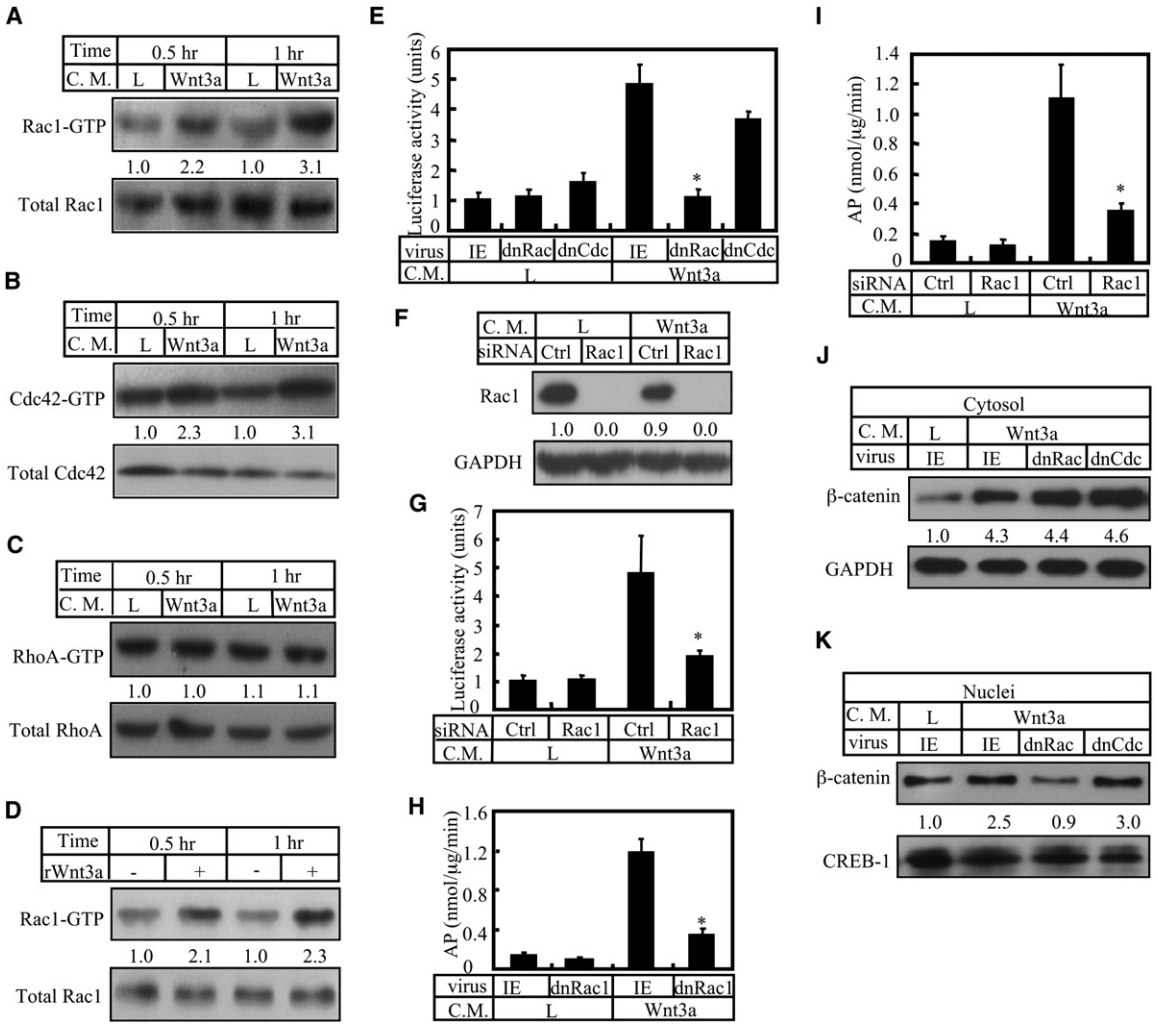

Figure 1. Rac1 Activation Is Required for Canonical Wnt Signaling

(A–C) Western analyses to detect activation of Rac1, Cdc42, and RhoA in ST2 cells cultured in Wnt3a versus L conditioned medium (C.M.). The relative amount of the GTP-bound form normalized to the total amount, in L medium, is designated 1.0.

(D) Rac1 activation by purified recombinant Wnt3a protein (rWnt3a) at 50 ng/ml.

(E) Expression of *Lef1-luciferase* in cells infected with a control virus (IE, expressing GFP) or viruses expressing dnRac1 or dnCdc42.

(F) Western analyses of Rac1 in cells at ~96 hr after transfection with control (Ctrl) or Rac1 siRNA.

(G) Expression of *Lef1-luciferase* following siRNA transfections.

(H and I) AP activity assay following viral infection (H) or siRNA transfection (I).

(J and K) Western analyses of β-catenin in cytosolic (J) or nuclear (K) fractions of cells cultured in L or Wnt3a medium for 1 hr following viral infections. Cytosolic and nuclear signals were normalized to GAPDH and CREB-1, respectively. Error bars: SD, *$p < 0.05$, $n = 3$.

(active) forms of Rho GTPases were increased upon Wnt signaling (see Experimental Procedures). Wnt3a consistently activated Rac1 by 2- to 3-fold over the control at 30 and 60 min after stimulation (average fold change at 60 min: 2.8 ± 0.7, n = 7) (Figure 1A). Wnt3a activated Cdc42 to a similar extend but did not significantly affect RhoA (Figures 1B and 1C). We confirmed the activation of Rac1 with purified recombinant Wnt3a protein (Figure 1D). To examine whether Rac1 or Cdc42 participate in canonical Wnt signaling, ST2 cells were infected with retroviruses expressing a dominant-negative form of each molecule (N17Rac1 or N17Cdc42) and assayed for their responses to Wnt3a in upregulating expression of a *Lef1-luciferase* reporter. The Rac1 mutant (dnRac1) completely abolished the induction by Wnt3a, whereas dnCdc42 did not have a significant effect (Figure 1E). The specificity of dnRac1 was confirmed by Rac1 siRNA, which reduced Rac1 protein to an undetectable level and significantly diminished *Lef1-luciferase* induction by Wnt3a, whereas the scrambled control RNA did not have any effect (Figures 1F and 1G). To confirm the biological relevance of Rac1 activity in Wnt signaling, ST2 cells either transfected with Rac1 siRNA or expressing dnRac1 were examined for their ability to undergo osteoblast differentiation in response to Wnt3a. Disruption of Rac1 activity by either means reduced approximately 70% of Wnt3a-induced expression of alkaline phosphatase

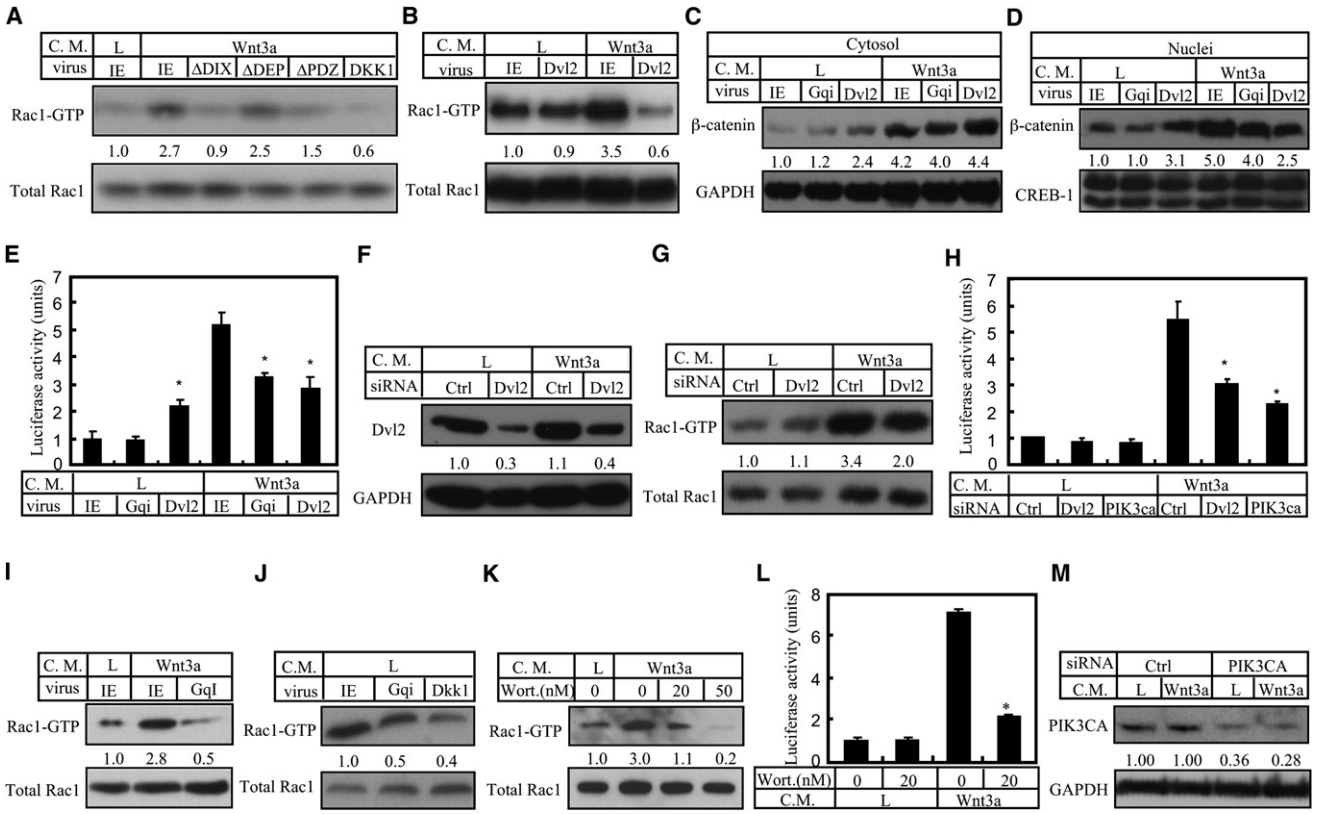

Figure 2. Mechanisms Controlling Rac1 Activation and Canonical Wnt Signaling

(A and B) Rac1 activation assays in cells cultured in L or Wnt3a medium for 1 hr following infection of indicated viruses.

(C and D) Western analyses of cytosolic (C) versus nuclear β-catenin (D) in cells cultured in L or Wnt3a medium for 1 hr following infection of indicated viruses. Cytosolic and nuclear signals were normalized to GAPDH and CREB-1, respectively.

(E) *Lef1-luciferase* expression by cells infected with viruses and cultured in L versus Wnt3a medium.

(F) Western analyses of Dvl2 in ST2 cells transfected with Dvl2-specific or control siRNA.

(G) Rac1 activation assays following Dvl2 knockdown.

(H) *Lef1-luciferase* expression by cells transfected with siRNA and cultured in L versus Wnt3a medium.

(I and J) Rac1 activation assays in cells infected with viruses and cultured in L versus Wnt3a medium.

(K) Effect of wortmannin on Rac1 activation at 1 hr following Wnt3a stimulation in ST2 cells.

(L) Effect of wortmannin (20 nM) on *Lef1-luciferase* expression in ST2 cells.

(M) Western analyses of PIK3CA in cells transfected with siRNA. Error bars: SD, *$p < 0.05$, $n = 3$.

(AP), a common osteoblast marker (Figures 1H and 1I). The remaining AP expression was likely due to differentiation induced by noncanonical Wnt signaling also activated by Wnt3a in these cells (Tu et al., 2007). Thus, Wnt3a activates Rac1, and Rac1 activity is required for canonical Wnt signaling in ST2 cells.

To explore the mechanism underlying the role of Rac1 in canonical Wnt signaling, western analyses were performed for β-catenin in cytosolic versus nuclear fractions of ST2 cells expressing dnRac1, with or without Wnt stimulation. Expression of dnRac1 did not affect β-catenin stabilization in the cytosol but completely abolished β-catenin accumulation in the nucleus in response to Wnt3a; the nuclear effect was not observed with dnCdc42 (Figures 1J and 1K). It should be noted that although dnRac1 markedly decreased β-catenin levels in the nucleus, it did not cause an obvious increase in the cytoplasm. This can be explained by the fact that the relative amount of nuclear versus cytoplasmic β-catenin is small in these cells, approximately 2% with or without Wnt3a stimulation, as per our estimation by western analyses. Finally, immunofluorescence confocal microscopy confirmed that dnRac1 indeed prevented nuclear accumulation of endogenous β-catenin in response to Wnt3a (Figure 5, compare B′ and C′). Thus, Rac1 activity is required for β-catenin nuclear localization in response to Wnt signaling.

We next investigated the molecular mechanism underlying Rac1 activation by Wnt3a. To assess the potential involvement of Dishevelled (Dvl) proteins, we first tested ST2 cells expressing Dvl2 variants individually missing each of the three conserved regions (ΔDIX, ΔDEP, and ΔPDZ) for their ability to activate Rac1 in response to Wnt3a. All three variants were previously shown to inhibit both β-catenin stabilization and PKCδ activation by Wnt3a (Tu et al., 2007) and were expressed at similar levels in these experiments (data not shown). Whereas ΔDEP had little effect, ΔDIX and ΔPDZ either abolished or significantly diminished Rac1 activation (Figure 2A). Interestingly, overexpression of the

full-length Dvl2 also inhibited Rac1 activation by Wnt3a (Figure 2B). This result is unexpected because previous studies have shown that overexpression of Dvl proteins activate canonical signaling in the absence of exogenous Wnt ligands. We suspected that Dvl2 overexpression might have a different effect on β-catenin signaling when Wnt ligands were present. To test this possibility, we examined β-catenin signaling in ST2 cells overexpressing Dvl2 with or without Wnt3a stimulation. Consistent with previous reports, at basal conditions Dvl2 overexpression increased both cytoplasmic and nuclear β-catenin levels and activated *Lef1-luciferase* expression, but the activation level as judged by all three parameters was significantly lower than that induced by Wnt3a (Figures 2C–2E). Importantly, in the presence of Wnt3a, both nuclear β-catenin levels and *Lef1-luciferase* expression were reduced by 50% in the Dvl2-overexpressing cells when compared to the control cells expressing IE virus (Figures 2D and 2E), although the level of β-catenin in the cytoplasm was not decreased (Figure 2C). The mechanism through which Dvl2 overexpression activates β-catenin signaling under basal conditions may be different from that employed by Wnt ligands, as Dvl2 overexpression did not activate Rac1 (Figure 2B). Taken together, the data so far indicate that Dvl2 overexpression inhibits both β-catenin signaling and Rac1 activation in response to Wnt, and that the DEP domain appears to mediate the inhibition. To test the role of endogenous Dvl2, we knocked down Dvl2 using siRNA. Reduction of Dvl2 protein level by 60%–70% (Figure 2F) correlated with a significant decrease in both Rac1 activation and *Lef1-luciferase* expression in response to Wnt3a (Figures 2G and 2H). The remaining activity is likely due to the residual Dvl2 and/or Dvl1 and Dvl3 that are also expressed in these cells (Tu et al., 2007). Overall, the data indicate that Dvl proteins play an important role in mediating Wnt-induced Rac1 activation.

Additional molecules were evaluated for their role in Wnt-induced Rac1 activation. To assess the potential involvement of LRP5/6 signaling, cells overexpressing Dkk1 were assayed; Rac1 activation by Wnt3a was completely abolished by Dkk1 (Figure 2A). Because overexpression of G_qI, a dominant-negative reagent for $G\alpha_{q/11}\beta\gamma$ signaling, impaired PKCδ activation but not β-catenin stabilization in response to Wnt (Tu et al., 2007), we examined whether it would affect Wnt-induced Rac1 activation. G_qI not only negated Rac1 activation by Wnt3a but also reduced the basal level of Rac1-GTP (Figures 2I and 2J). Because Dkk1 also similarly inhibited the basal level of Rac1-GTP (Figure 2J), it appears that the basal level Rac1 activation is in part due to endogenous Wnt signaling. G_qI also significantly reduced *Lef1-luciferase* expression in response to Wnt3a (Figure 2E), consistent with a decrease in nuclear β-catenin levels even though the cytoplasmic amount was not significantly affected (Figures 2C and 2D). Since PI3K is known to mediate Rac1 activation by heterotrimeric G protein signaling, we next examined whether it is required for Rac1 activation by Wnt. Wortmannin, a potent inhibitor for PI3K, dose-dependently inhibited Rac1 activation by Wnt3a (Figure 2K) and markedly reduced Wnt-induced *Lef1-luciferase* expression (Figure 2L). On the other hand, wortmannin under these conditions had no significant effect on cell survival or general gene expression (Figure S3 available online). Finally, siRNA knockdown of the PI3K p110 catalytic subunit alpha (PIK3CA) (Figure 2M) markedly reduced *Lef1-luciferase* expression in response to Wnt3a (Figure 2H). Taken together, these data indicate that Wnt3a activates Rac1 through a signaling cascade involving LRP5/6, Dvl, $G\alpha_{q/11}\beta\gamma$, and PI3K.

Constitutively Active Rac1 Enables Canonical Signaling by an Otherwise Inactive Wnt

To further substantiate the role of Rac1 in the canonical Wnt pathway, we studied Wnt7b signaling in ST2 cells, wherein it did not activate *Lef1-luciferase* expression (Tu et al., 2007). Interestingly, cells overexpressing Wnt7b accumulated >5-fold (versus ~4-fold induced by Wnt3a, see Figure 1I) more β-catenin over the control in the cytoplasm (Figure 3A) but showed little increase in the nucleus (Figure 3B). Similarly, immunofluorescence confocal microscopy confirmed that no significant levels of endogenous β-catenin were detected in the nucleus following Wnt7b expression (Figure 3F, compare with Figure 3E). Importantly, Wnt7b did not activate Rac1 in these cells (Figure 3C). Thus, the inability for Wnt7b to activate canonical signaling in ST2 cells is not due to deficiency in stabilizing β-catenin but instead correlates with the lack of Rac1 activation and β-catenin nuclear localization.

To test whether Rac1 activation is sufficient to enable Wnt7b in activating the canonical pathway, we overexpressed a constitutively active form of Rac1 (V12Rac1, termed caRac1 hereafter) that lacks GTPase activity. Cells expressing caRac1 activated *Lef1-luciferase* expression in response to Wnt7b, whereas caRac1 alone did not have any effect (Figure 3D). Consistent with this result, caRac1 increased the nuclear amount of β-catenin by more than 2-fold (Figure 3B), even though it had little effect on Wnt7b-induced β-catenin accumulation in the cytoplasm (Figure 3A). When examined by immunofluorescence confocal microscopy, cells expressing caRac1 alone exhibited the typical "fried egg" morphology due to exaggerated lamellipodia, as previously described (Welch et al., 2002), but did not show an increase in nuclear β-catenin (data not shown). However, when Wnt7b and caRac1 were coexpressed in the culture, the cells exhibited both the "fried egg" morphology and prominent nuclear accumulation of β-catenin (Figure 3G). Thus, constitutively active Rac1 is sufficient to localize Wnt7b-stabilized cytoplasmic β-catenin to the nucleus and enable canonical signaling in ST2 cells.

Rac1-Mediated JNK2 Activation by Wnt3a Controls β-catenin Signaling

To explore downstream events mediating the role of Rac1 in β-catenin nuclear localization, we investigated the potential relevance of JNK. Western analyses using an antibody specific for dual phosphorylation of JNK1 or JNK2 at Thr183 and Tyr185, a prerequisite for JNK activation, revealed that JNK2 was specifically activated by approximately 4-fold at 30 or 60 min after Wnt3a stimulation (Figure 4A) and that the activation was essentially abolished by expression of dnRac1 (Figure 4B). Inhibition of JNK activity by SP600125 dose-dependently diminished activation of *Lef1-luciferase* expression by Wnt3a (Figure 4C). Likewise, SP600125 decreased Wnt3a-activated AP expression by as much as 70% (Figure 4D), a reduction equivalent to that caused by disruption of Rac1 activity (Figures 1G and 1H). Also similar to Rac1 disruption, SP600125 dose-dependently

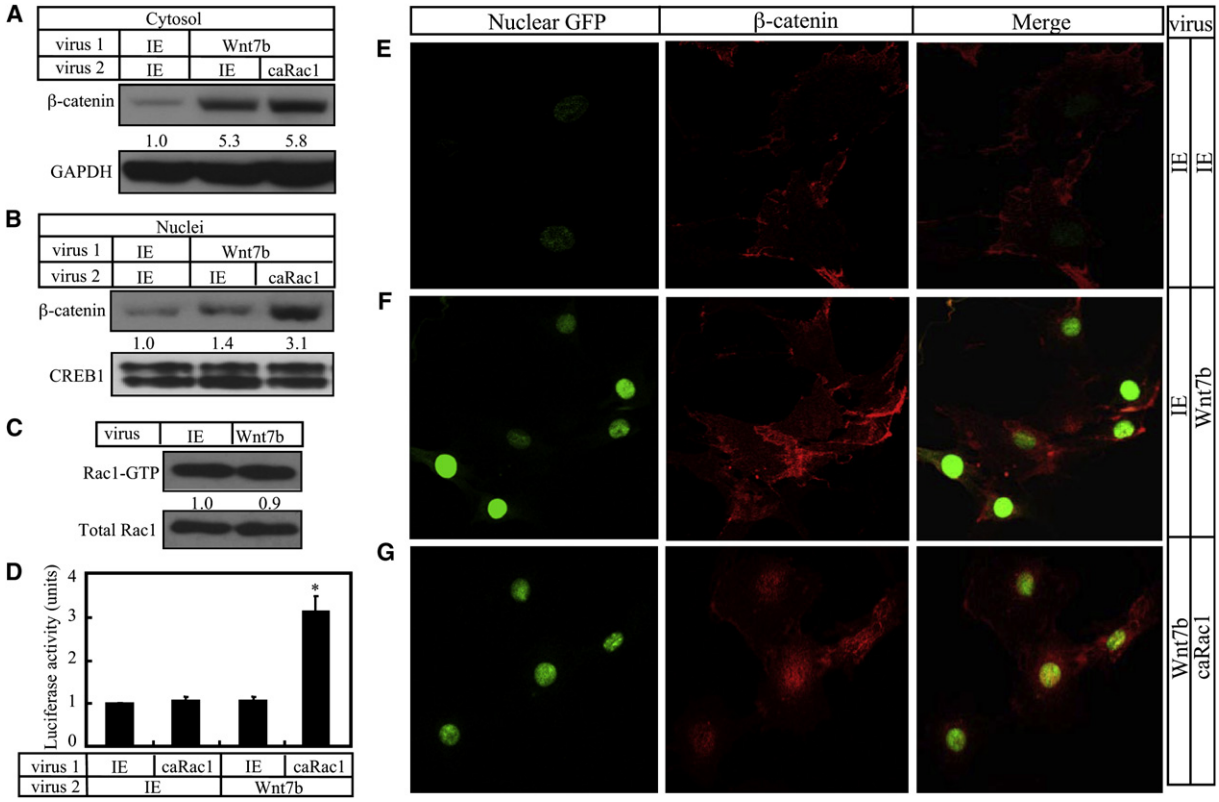

Figure 3. Rac1 Activation Enables Wnt7b to Activate Canonical Wnt Signaling in ST2 Cells

(A and B) Western analyses of β-catenin in cytosolic (A) or nuclear (B) fractions of ST2 cells following coinfection of viruses.

(C) Rac1 activation assay in cells infected with control IE or Wnt7b virus.

(D) Expression of *Lef1-luciferase* following coinfection.

(E–G) Immunofluorescence confocal microscopy of cells following viral infections. Nuclei were labeled green due to expression of nuclear GFP via IRES by all viruses. β-catenin signal is in red. Error bars: SD, *$p < 0.05$, $n = 3$.

inhibited nuclear accumulation of β-catenin (Figure 4F), without significantly affecting its stabilization in the cytoplasm (Figure 4E). We confirmed the importance of JNK2 by siRNA experiments, which showed that JNK2 siRNA reduced the protein level by 60% and diminished Wnt3a-induced *Lef1-luciferase* induction by ~50%, whereas the nontargeting control siRNA did not have any effect (Figure 4G). Knockdown of JNK1 by 80% modestly reduced Wnt3a-induced *Lef1-luciferase* expression by ~25%, whereas double knockdown of JNK1 and JNK2 had an effect similar to the JNK2 single knockdown (Figure 4G). Further supporting the predominant role of JNK2, immunofluorescence confocal microscopy showed that JNK2 siRNA significantly reduced the nucleus β-catenin level in the presence of Wnt3a, whereas JNK1 siRNA had only a modest effect (Figure 4H). Similarly, SP600125 abolished Wnt-induced nuclear localization of β-catenin but appeared to enrich β-catenin in certain areas associated with the cell membrane (Figure 5, compare E′ with B′). Thus, Wnt3a activates JNK2 via Rac1, and JNK activity appears to be required for nuclear localization of β-catenin.

To assess potential physical interactions among endogenous β-catenin, Rac1, and JNK, coimmunoprecipitation experiments were performed using cytosolic versus nuclear fractions from ST2 cells incubated with either L or Wnt3a conditioned medium. In the cytosolic fraction, with or without Wnt3a treatment, protein complexes precipitated with a β-catenin antibody contained total Rac1 and total JNK1 and JNK2 in addition to β-catenin as expected (Figure 4I). Importantly, phospho-JNK2, but not JNK1, was detected in the β-catenin complex only under Wnt3a stimulation. In addition, although the amount of β-catenin detected in the precipitate was markedly increased in response to Wnt3a, the amount of Rac1 was not significantly changed, indicating that Rac1 may be the rate-limiting partner in the complex, and that β-catenin stabilization does not significantly enhance the interaction between the two proteins. Consistent with this notion, the converse experiment using a Rac1 antibody for immunoprecipitation showed that a similar level of β-catenin was detected in the precipitates with or without Wnt3a (Figure 4I). The Rac1 immunoprecipitates also contained total JNK1 and JNK2 regardless of Wnt3a but contained a significantly higher level of phospho-JNK2 in the presence of Wnt3a. On the other hand, the same procedures did not detect any coprecipitation among β-catenin, JNK, and Rac1 in the nuclear fractions, regardless of Wnt3a (Figure 4J), even though TCF-4, a member of the LEF/TCF family known to interact with β-catenin, coprecipitated with nuclear β-catenin as expected (data not shown). In all cases, the control experiments using purified IgG1 did not precipitate

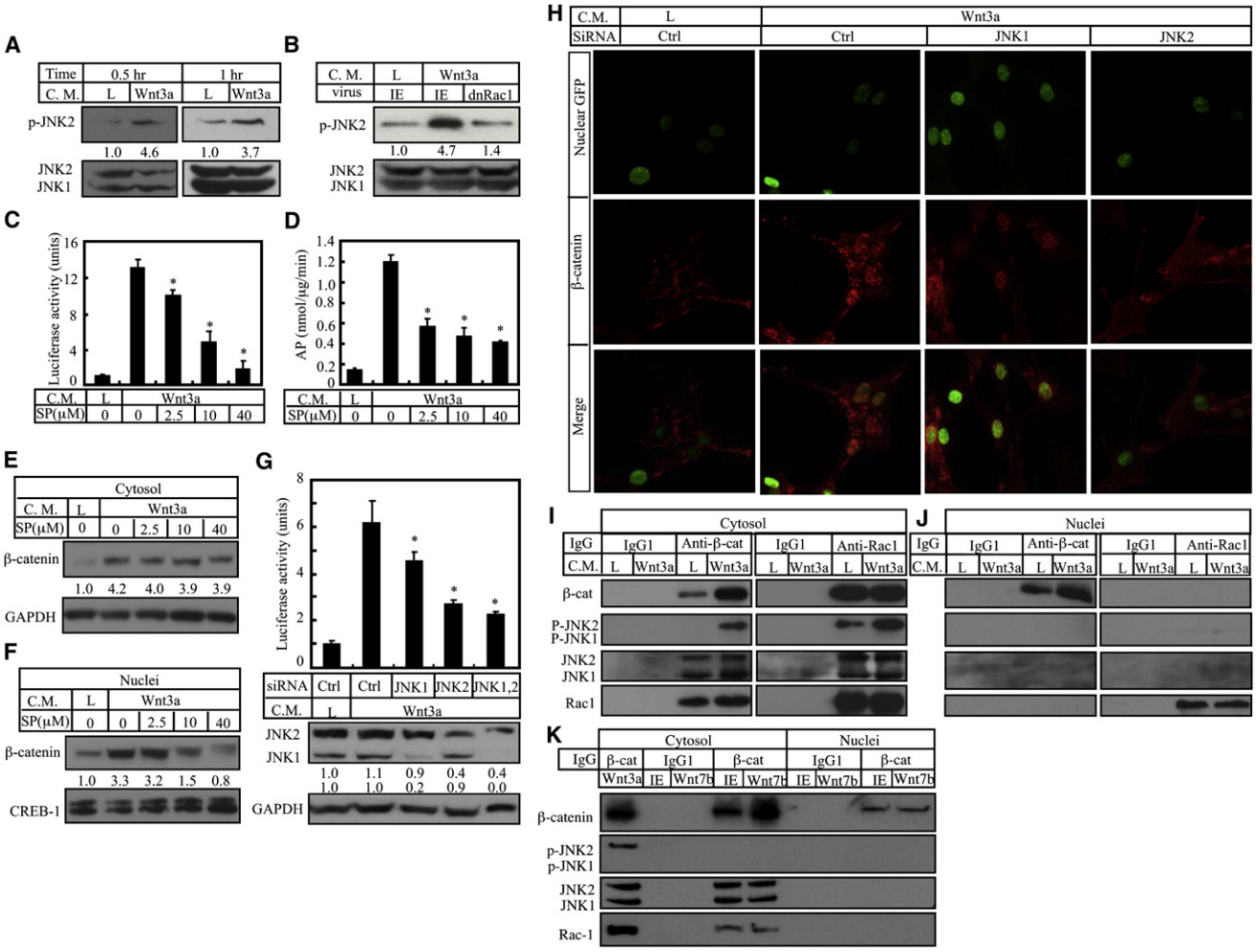

Figure 4. JNK2 Activation Is Required for Canonical Wnt Signaling

(A) Western analyses of phospho-JNK and total JNK in ST2 cells cultured in L versus Wnt3a medium. Phospho-JNK2 level was normalized to total JNK2.

(B) Western analyses of phospho-JNK and total JNK in ST2 cells cultured in L versus Wnt3a medium for 1 hr following viral infections.

(C) Effect of SP600125 on expression of *Lef1-luciferase* in ST2 cells.

(D) Effect of SP600125 on AP activity in ST2 cells.

(E and F) Western analyses of β-catenin in cytolosic (E) or nuclear (F) fractions of ST2 cells cultured in L versus Wnt3a medium for 1 hr with varying concentrations of SP600125.

(G) Effects of JNK1/2 knockdown on *Lef1-luciferase* expression. Western analyses of JNK performed at ~96 hr after siRNA transfection.

(H) Effects of JNK1/2 knockdown on nuclear localization of endogenous β-catenin.

(I and K) Coimmunoprecipitation of endogenous β-catenin, JNK1/2, and Rac1 in cytosolic versus nuclear fractions of cells cultured in L versus Wnt3a medium for 1 hr (I and J) or infected with control (IE) or Wnt7b-expressing virus (K). IgG1: control antibody for immunoprecipitation. Error bars: SD, *$p < 0.05$, $n = 3$.

any of the proteins. Thus, the endogenous β-catenin, Rac1, JNK1, and JNK2 constitutively interact with each other in the cytoplasm of the ST2 cell, and Wnt3a signaling specifically activates JNK2 in this context.

To determine whether JNK2 activation within the Rac1-JNK1/2-β-catenin complexes is specific to canonical Wnt signaling, we examined the effect of Wnt7b, which does not activate canonical Wnt signaling in ST2 cells. These experiments detected no phospho-JNK2 in the cytosolic β-catenin immunoprecipitates with or without Wnt7b stimulation (Figure 4K). This result is consistent with the lack of Rac1 activation by Wnt7b and indicates that Rac1-mediated JNK2 activation within Rac1-JNK1/2-β-catenin complexes is likely specific to canonical Wnt signaling.

To test further the functional relationship between JNK and Rac1, we examined the effect of SP600125 on caRac1-enhanced β-catenin nuclear localization in response to Wnt3a (Figure 5). Overexpression of caRac1 accentuated β-catenin in the nucleus in response to Wnt3a (Figure 5D′). However, SP600125 markedly reduced nuclear β-catenin in caRac1-expressing cells stimulated with Wnt3a and instead caused prominent accumulation of β-catenin in the perinuclear region (Figure 5F′). Interestingly, the cells maintained the characteristic "fried egg" morphology caused by caRac1 but now also exhibited β-catenin enrichment

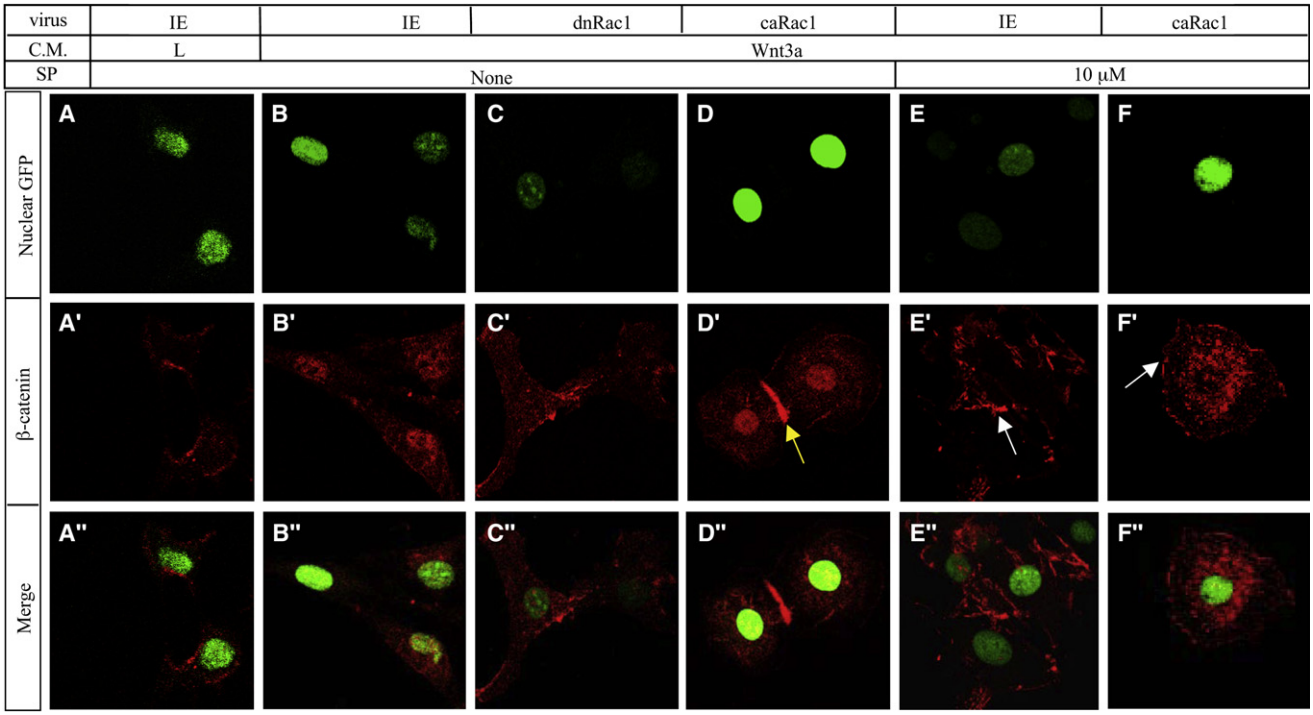

Figure 5. Rac1 and JNK Activity Are Required for β-catenin Nuclear Localization in Response to Wnt

ST2 cells were virally infected and cultured in L or Wnt3a medium for 1 hr, with or without SP600125 (10 μM), before being subjected to immunofluorescence confocal microscopy. (A–F) Nuclei as revealed by NLS-EGFP expressed by each virus via IRES. (A′–F′) Staining of endogenous β-catenin. Yellow arrow in (D′), enrichment of β-catenin at a cell-cell junction. White arrows in (E′) and (F′), enrichment of β-catenin at cell periphery. (A″–F″) Merged pictures.

in certain areas of the plasma membrane, a feature common to cells treated with SP600125 (Figures 5E′and 5F′, white arrows). Overall, JNK appears to function downstream of Rac1 in regulating nuclear localization of β-catenin during canonical Wnt signaling.

To investigate the specificity of JNK in mediating canonical Wnt signaling, we examined the other members of the mitogen-activated protein kinase (MAPK) family. Although Wnt3a failed to activate p38 (Figure S1A), it activated extracellular signal-related kinase 2 (ERK2) by 2- to 3-fold at 30 or 60 min post-stimulation (Figure S1B). However, inhibition of ERK activation by PD98059 or U0126 did not affect Wnt3a-induced *Lef1-luciferase* expression (Figure S1D), even though the drugs potently inhibited ERK2 activation (Figure S1C). Thus, within the MAPK family, JNK appears to be specifically required for canonical Wnt signaling in ST2 cells.

To determine whether the PI3K-Rac1-JNK mechanism is unique to ST2 cells, we examined several other cell lines. In HEK293 cells, Wnt3a treatment for 1 hr activated Rac1 by approximately 2-fold, and the induction was abolished by wortmaninn (Figure S2A). Importantly, wortmannin or SP600125 completely inhibited Wnt3a-induced *Lef1-luciferase* expression (Figures S2B and S2C). Similarly, in NIH 3T3 cells, Wnt3a induced Rac1 activation by ~4-fold and *Lef1-luciferase* expression by ~3-fold; wortmannin again significantly inhibited both responses, although to a lesser degree than in the ST2 and HEK293 cells (Figures S2D and S2E). SP600125 also abolished the induction of *Lef1-luciferase* expression by Wnt3a in NIH 3T3 cells (Figure S2F). Thus, the role of PI3K/Rac1/JNK is likely a common feature of canonical Wnt signaling.

Ser191 and Ser605 Are Critical for β-catenin Nuclear Localization

Both the role of JNK2 in β-catenin nuclear localization and the physical association between the two proteins prompted us to examine whether JNK directly phosphorylates β-catenin. A search of the β-catenin protein sequence revealed three potential JNK phosphorylation sites, i.e., Ser191, Ser246, and Ser605, with the first two conserved among *Drosophila*, *Xenopus*, mouse, and human and the third one common between mouse and *Xenopus*. To assess their potential importance, we expressed β-catenin variants that harbor mutations at the consensus serine residues (Ser to Ala) individually (S191A, S246A, S605A) or in combination (triple) and evaluated their capacity to mediate *Lef1-luciferase* expression in ST2 cells in response to Wnt3a. Overexpression of wild-type β-catenin increased the expression level by approximately 6-fold over the control cells in response to Wnt3a. Whereas the S246A variant behaved essentially the same as wild-type β-catenin, the S191A and S605A variants each caused only a 2-fold increase over the control, and importantly, the triple mutant resulted in no significant increase (Figure 6A, left top). The differential effects of the variants did not reflect differences in their expression levels, as S605A and the triple mutant were expressed at comparable levels as the wild-type, and S191A and S246A were at 85% and 47% of the wild-type, respectively (Figure 6A, left bottom).

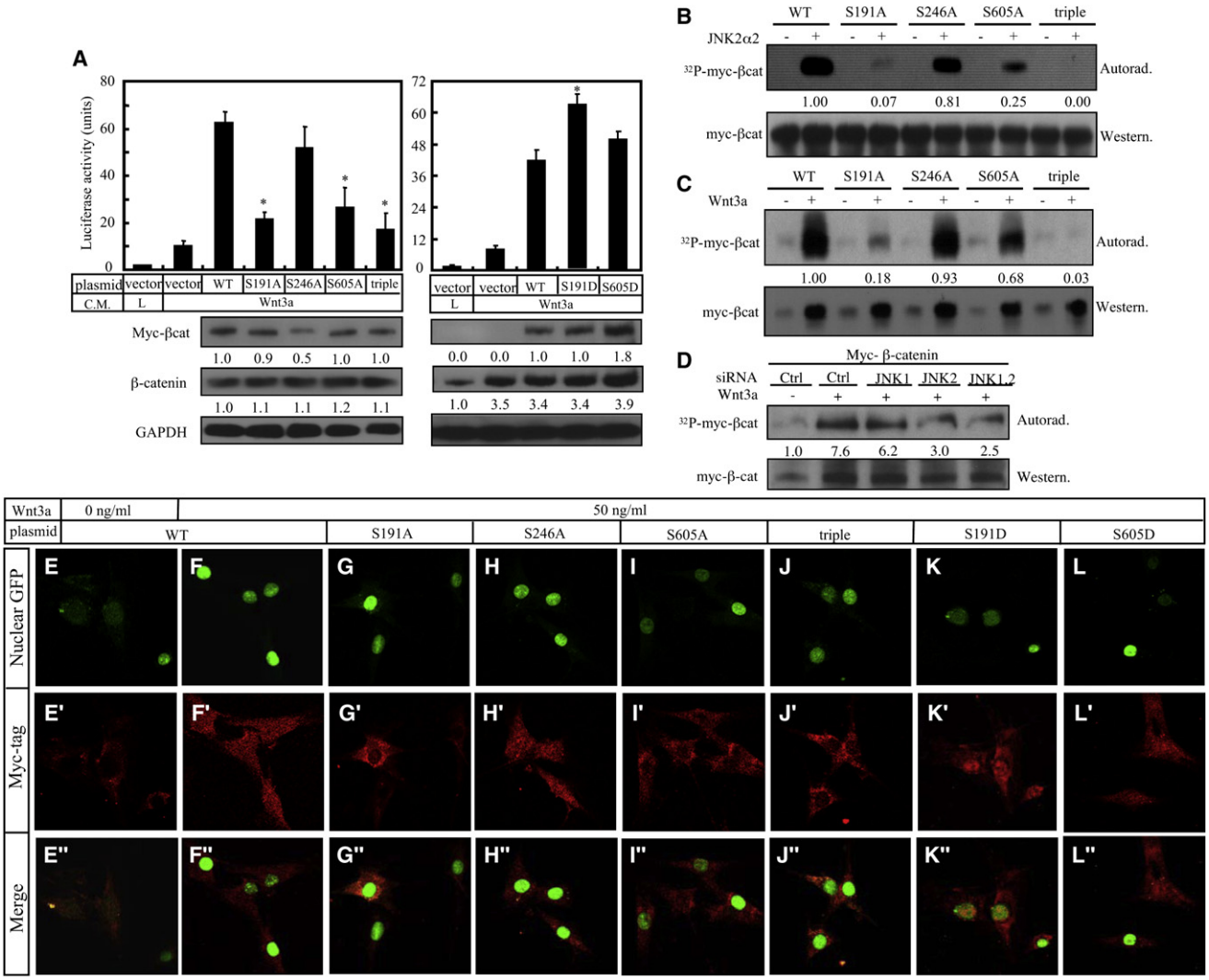

Figure 6. Phosphorylation of Ser191 and Ser605 Is Critical for Wnt-Induced Nuclear Localization of β-catenin

(A) Expression of *Lef1-luciferase* following transient transfection of β-catenin variants and western analyses of Myc-tagged β-catenin variants and endogenous β-catenin in the same lysates used for luciferase assays.

(B) In vitro phosphorylation of Myc-tagged β-catenin variants by JNK2α2. Autoradiography signals were normalized to levels of Myc-β-catenin variants (same is true in C and D).

(C) In vivo phosphorylation of Myc-β-catenin variants in intact cells.

(D) In vivo phosphorylation of Myc-β-catenin (wild-type) in intact cells transfected with siRNA.

(E–L, E′–L′, and E″–L″) Confocal images for nuclear GFP (E–L), exogenous Myc-β-catenin variants (E′–L′), and the merge (E″–L″) in cells cultured with or without recombinant Wnt3a at 50 ng/ml for 1 hr, following infection with IE virus (expressing nuclear GFP) and transient transfection with plasmids expressing Myc-β-catenin variants. Error bars: SD, *$p < 0.05$, $n = 3$.

Moreover, endogenous β-catenin levels were similar among the cells expressing the different β-catenin variants. To evaluate the potential importance of phosphorylation at Ser191 and Ser605, we mutated the serine residues to the phosphomimetic aspartate (D). Although either S191D or S605D only minimally affected *Lef1-luciferase* expression under basal conditions (~10% increase over the wild-type construct), S191D showed significant synergy with Wnt3a in further upregulating *Lef1-luciferase* expression, whereas S605D had a similar but more modest effect (Figure 6A, right top). Western blotting showed that S191D and the wild-type β-catenin construct were expressed at the same level but S605D was ~80% higher, and that the endogenous β-catenin levels were similar among the cells expressing different constructs (Figure 6A, right bottom). Thus Ser191, and to a lesser degree Ser605, play an important role in canonical Wnt signaling.

We next determined whether Ser191, Ser246, or Ser605 could indeed be phosphorylated by JNK. Here, overexpressed Myc-tagged β-catenin variants were immunoprecipitated from ST2 cells with a Myc antibody and subsequently subjected to

phosphorylation assays in vitro using purified active JNK2α2, followed by SDS-PAGE and autoradiography. The wild-type β-catenin incorporated robust levels of ^{32}P, and S246A showed a 20% reduction, but S191A virtually eliminated phosphorylation and S605A reduced it by 75% (Figure 6B, top). As expected, the triple mutant β-catenin incorporated no ^{32}P. Subsequent western blotting using the Myc antibody confirmed that the ^{32}P-labeled bands corresponded to the β-catenin variants, and that similar levels of Myc-tagged β-catenin variants were present among the various immunoprecipitates (Figure 6B, bottom). Thus, consistent with their importance for canonical Wnt signaling, S191 and, to a lesser extent, S605 appear to be the predominant phosphorylation sites for JNK2 in vitro.

To confirm that S191 and S605 are phosphorylated in intact cells responding to Wnt, we performed in vivo phosphorylation assays. Cells expressing the Myc-tagged β-catenin variants were incubated with ^{32}P orthophosphate with or without Wnt3a stimulation, and their lysates were immunoprecipitated with the Myc antibody before being resolved on SDS-PAGE and subjected to autoradiography. Under basal conditions, no significant phosphorylation was detected for any of the β-catenin variants. However, upon Wnt3a stimulation, the wild-type β-catenin became highly phosphorylated (Figure 6C, top). Whereas S246A had little effect (7% reduction), S605A and S191A reduced phosphorylation by 32% and 82%, respectively; the triple mutation completely abolished phosphorylation in response to Wnt3a. Western blotting using the Myc antibody revealed similar low levels of Myc-β-catenin variants among the immunoprecipitates under the basal conditions and similar high levels upon Wnt3a stimulation (Figure 6C, bottom), indicating that the different variants were expressed at similar levels and that the mutations had no obvious effect on the stabilization of Myc-β-catenin by Wnt3a. Normalization of the ^{32}P signal to the Myc-β-catenin protein level revealed that Wnt3a induced a ~3-fold increase in phosphorylation on per molecule basis. Thus, β-catenin is phosphorylated in intact cells upon Wnt signaling, and Ser191 and Ser605 are the predominant phosphorylation sites.

To examine directly the role of JNK1/2 in Myc-β-catenin phosphorylation in intact cells in response to Wnt3a, we performed in vivo phosphorylation assays in ST2 cells transfected with JNK1/2 siRNA. JNK2 knockdown notably reduced ^{32}P levels of Myc-β-catenin, whereas JNK1 knockdown had a smaller effect, and the double knockdown exhibited an additive effect (Figure 6D). Thus, JNK2 appears to be mainly responsible for Wnt3a-induced β-catenin phosphorylation in ST2 cells.

To confirm the effect of specific phosphorylation on nuclear localization of β-catenin, we examined the subcellular localization of the Myc-tagged β-catenin variants in response to Wnt3a by immunofluorescence confocal microscopy. Detection of the Myc tag revealed that the exogenous wild-type β-catenin was present in the cytoplasm at a relatively low level, and with clear exclusion from the nucleus under basal conditions (Figures 6E–6E″), but was detected at high levels in both compartments in response to Wnt3a (Figures 6F–6F″). Note that the overexpressed myc-β-catenin did not exhibit the same prominent nuclear localization as the endogenous β-catenin in response to Wnt3a (see Figures 4H and 5B′). Importantly, the variants harboring either S191A (Figures 6G–6G″), S605A (Figures 6I–6I″), or the triple mutation (Figures 6J–6J″) failed to accumulate in the nucleus when the cells were stimulated with Wnt3a, whereas the S246A form, like the wild-type form, was detected in the nucleus in response to Wnt3a (Figures 6H–6H,″ compare with Figures 6F–6F″). Conversely, the S191D form exhibited more prominent nuclear localization than the wild-type construct (Figures 6K–6K,″ compare with Figures 6F–6F″), whereas S605D also slightly increased the nuclear signal (Figures 6L–6L″). These results therefore support the notion that both Ser191 and Ser605 participate in the nuclear localization of β-catenin in response to Wnt signaling.

Genetic Removal of Rac1 in the Mouse Limb Bud Ectoderm Phenocopies β-catenin Deletion

To determine the physiological relevance of Rac1 in canonical Wnt signaling, we genetically ablated Rac1 from the apical ectodermal ridge (AER) of the mouse embryonic limb bud, where Wnt signaling through β-catenin is critical for limb outgrowth (Barrow et al., 2003). Specifically, we generated embryos of *Msx2-Cre;Rac1$^{n/c}$* (*Rac1-CKO*) by crossing males of *Rac1$^{c/c}$* with females of *Msx2-Cre;Rac1$^{c/+}$*, taking advantage of the fact that *Msx2-Cre* is expressed both in the female germline and in the limb bud ectoderm that gave rise to the AER (Sun et al., 2000). *Rac1-CKO* embryos at E16.5 lacked all hindlimb structures and exhibited truncations at various levels in the forelimb (Figures 7A–7D and 7A′–7D′). These defects are identical to those previously characterized by others in embryos of *Msx2-Cre;β-catenin$^{n/c}$* (Barrow et al., 2003). The discrepancy between the fore- and hindlimb, as well as the phenotypic variation among forelimbs, were also noted in the β-catenin mutants and most likely reflect the earlier onset of *Msx2-Cre* in the hindlimb and the potential temporal variation in Cre expression among the forelimbs. Overall, genetic removal of either *Rac1* or *β-catenin* from the limb bud ectoderm results in identical limb truncation phenotypes in the mouse.

To directly monitor the effect of *Rac1* removal on canonical Wnt signaling in the limb bud ectoderm, we took advantage of the *TOPGAL* reporter mouse strain engineered to reflect β-catenin signaling in vivo (DasGupta and Fuchs, 1999). Specifically, we generated E10 embryos with the genotype of *Msx2-Cre;Rac1$^{n/c}$;TOPGAL* (*Rac1-CKO-TOPGAL*) and control littermates also carrying *TOPGAL*. The control *TOPGAL* embryos (e.g., *Rac1$^{c/c}$;TOPGAL*) exhibited robust activities in multiple tissues including the limb buds (Figure 7E). Within the forelimb bud, both the distal ectoderm (DE) and the proximal mesenchyme (DM and VM) showed strong signals (Figures 7E′–7E″). In contrast, the forelimb of the *Rac1-CKO-TOPGAL* embryo had little if any LacZ activity, even though the activities in other parts of the embryo appeared normal (Figures 7F–7F″). Moreover, consistent with the limb truncation phenotype, the sizes of both fore- and hindlimb buds were significantly smaller in the *Rac1-CKO-TOPGAL* embryo (Figure 7E′ versus Figure 7F′ and data not shown). Importantly, the limb bud of *Rac1-CKO* embryos maintained an intact epithelium and a morphologically identifiable AER at E10.5 (Figures 7G1 and 7G2, red arrows). Immunostaining of E-cadherin revealed normal adherens junctions among epithelial cells and confirmed a thinner AER in the mutant embryo (Figures 7H1 and 7H2). These results argue against a nonspecific effect of Rac1 removal on limb bud ectoderm but instead support a specific role for *Rac1* in regulating canonical Wnt signaling.

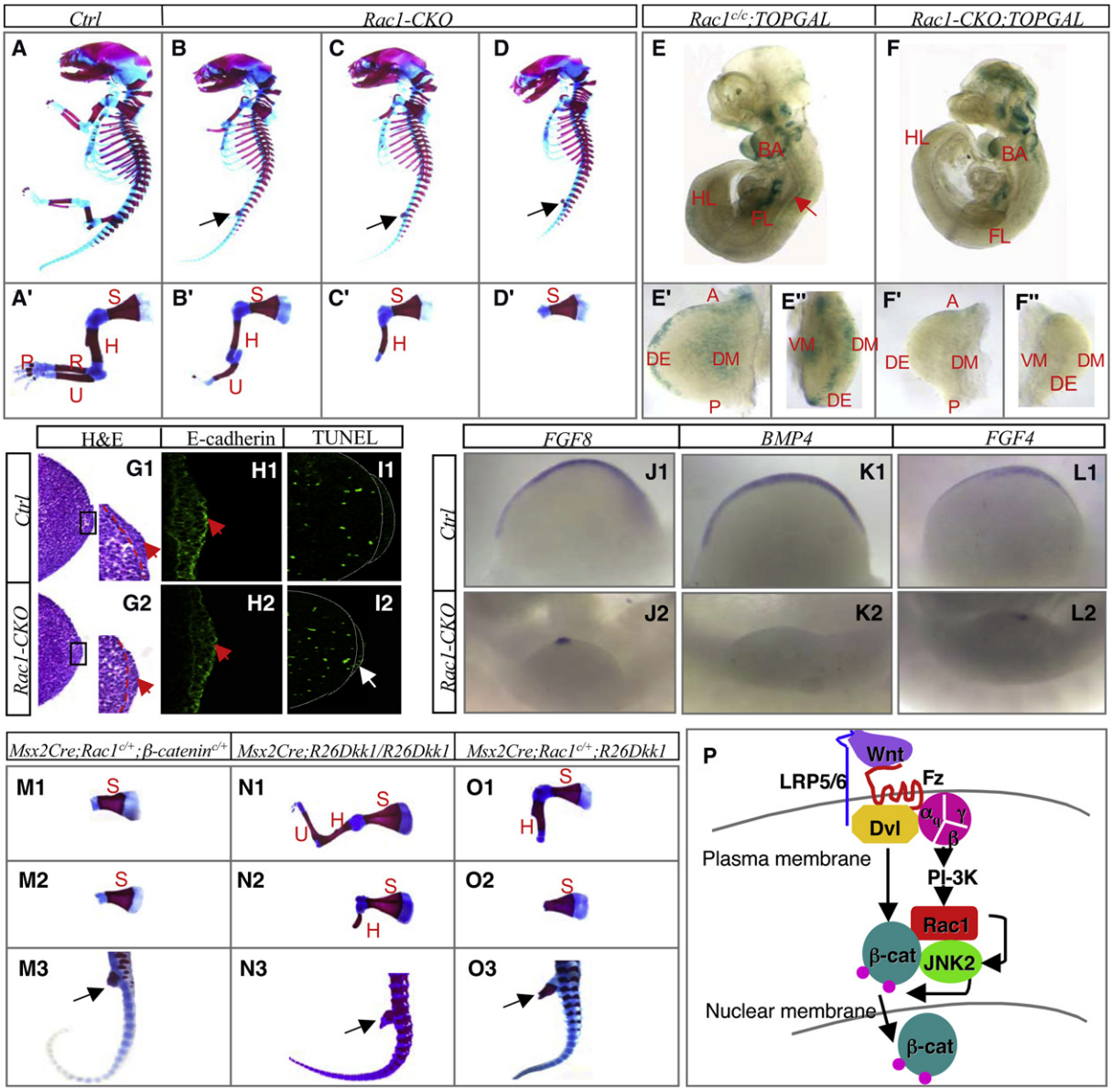

Figure 7. Rac1 Interacts Genetically with β-catenin and Dkk1 in the Limb Bud Ectoderm of Mouse Embryos

(A–D) E16.5 skeletons. Black arrows denote absence of hindlimbs in all Rac1-CKO embryos. The control genotype is Msx2-Cre;Rac1$^{c/+}$. (A′–D′) Forelimbs from embryos above at a higher magnification. S: scapula; H: humerus; R: radius; U: ulna; P: phalanges.

(E–F) LacZ staining of E10 littermate embryos. Note that the control embryo (E) was partially squashed during staining so that the dorsal neural tube (red arrow) is now facing the reader instead of to the right. BA: 1st branchial arch; FL: forelimb; HL: hindlimb. (E′–F″) Forelimb buds from the embryos above at a higher magnification with either the dorsal (E′ and F′) or the distal view (E″and F″). A: anterior; P: posterior; DE: distal ectoderm; DM: dorsal mesenchyme; VM: ventral mesenchyme.

(G1–G2) H&E staining of forelimb sections through the AER region at E10.5. Boxed areas shown at a higher magnification. Red dotted line separates mesenchyme from ectoderm. Red arrow: AER.

(H1–H2) Immunostaining of E-cadherin in the AER region of the forelimb at E10.5.

(I1–I2) TUNEL staining of forelimb sections at E10.5. Dotted white line demarcates ectoderm and AER. White arrow: TUNEL signal in the mutant AER.

(J1–L2) Whole-mount in situ hybridization of the forelimb at E10.5. Ventral view for all limb buds, anterior to the left and posterior to the right.

(M1–M3) Representative forelimbs (M1–M2) and hindlimb (M3, black arrow) of Msx1-Cre;Rac1$^{c/+}$;β-catenin$^{c/+}$ embryos at E16.

(N1–N3) Representative forelimbs (N1–N2) and hindlimb (N3, black arrow) of Msx1-Cre;R26-Dkk1/R26-Dkk1 mice at birth.

(O1–O3) Representative forelimbs (O1–O2) and hindlimb (O3, black arrow) of Msx1-Cre;Rac1$^{c/+}$;R26-Dkk1 embryos at E16.5.

(P) An integrated model for canonical Wnt signaling.

The striking morphological similarity between the Rac1 and the β-catenin conditional knockout embryos prompted us to explore their potential resemblance at the molecular level. Barrow et al. demonstrated that the forelimbs of *Msx2-Cre;β-catenin*$^{n/c}$ embryos failed to maintain FGF8 expression in the AER and failed to express BMP4 in the distal ventral ectoderm at 34-somite stage (Barrow et al., 2003). Similarly, with the *Rac1-CKO* embryos we detected only residual FGF8 in the forelimb AER and no BMP4 expression in the ventral ectoderm at E10.5 (34–35 somites) (Figures 7J1 and 7J2 and Figures 7K1 and 7K2, respectively). Moreover, AER expression of FGF4 was markedly reduced in *Rac1-CKO* embryos (Figures 7L1 and 7L2). Also similar to the *β-catenin* mutant embryos, none of the molecules were detected in the hindlimb of the *Rac1* mutant at E10.5 (data not shown). Finally, removal of *β-catenin* caused apoptosis in both mesenchyme and the ectoderm (Barrow et al., 2003); we observed an increase in TUNEL staining in both compartments in the forelimb of *Rac1-CKO* embryos at E10.5 (Figures 7I1 and 7I2). Thus, both morphologically and molecularly, the *Rac1* conditional mutant embryos resemble the *β-catenin* conditional mutants.

Rac1 Interacts Genetically with β-catenin and Dkk1 in the AER

To address further the specificity of the role of Rac1 in β-catenin signaling, we examined whether the two molecules genetically interact in the AER. Specifically, we generated mouse embryos lacking one copy each of Rac1 and β-catenin in the limb bud ectoderm (*Msx2-Cre;Rac1*$^{c/+}$*;β-catenin*$^{c/+}$) by crossing *Msx2-Cre;Rac1*$^{c/+}$ males with *β-catenin*$^{c/c}$ females. Remarkably, all double-heterozygous embryos (5/5) developed no hindlimb, and nearly all forelimbs (9/10 from 5 embryos) lacked structures distal to the scapula (Figures 7M1–7M3), resembling the limb phenotypes of the *Msx2-Cre;Rac1*$^{n/c}$ or the *Msx2-Cre;β-catenin*$^{n/c}$ embryos. The one exception forelimb lacked digits 4 and 5 and the deltoid tuberosity of the humerus (data not shown). The single heterozygous mice for either *Rac1* or *β-catenin* showed no phenotype (data not shown). Thus, Rac1 in AER controls limb outgrowth likely through regulation of β-catenin.

To further substantiate that the limb phenotype caused by the removal of Rac1 or β-catenin is indeed due to disruption of canonical Wnt signaling, we genetically manipulated the expression levels of Dkk1. Specifically, we generated a mouse strain (*R26-Dkk1*) with the full-length *Dkk1* cDNA knocked into the *Rosa26* locus; the cDNA is proceeded by a transcriptional stop signal flanked by a pair of loxP sites and therefore can only be transcribed when the loxP sites are recombined by Cre (Soriano, 1999; Srinivas et al., 2001). We then crossed the *R26-Dkk1* and the *Msx2-Cre* mice to produce embryos carrying both alleles and hence expressing exogenous Dkk1 in the AER. Embryos with *Msx2-Cre* and one copy of the *R26-Dkk1* allele (*Msx2-Cre;R26-Dkk1*) did not have any obvious phenotype (data not shown). However, all embryos carrying *Msx2-Cre* and two copies of *R26-Dkk1* (*Msx2-Cre;R26-Dkk1/R26-Dkk1*) formed no hindlimb and exhibited varying degrees of truncations in the forelimb (Figures 7N1–7N3). Thus, overexpression of Dkk1 in the AER results in the same phenotype as removing either β-catenin or Rac1 in the same compartment. Moreover, the dosage effect of Dkk1 underscores both the robustness of the canonical Wnt signaling system and the sensitivity of the AER to a threshold level of Wnt signaling.

The findings above prompted us to hypothesize that a partial reduction of Rac1 on top of moderate Dkk1 overexpression may be sufficient to diminish Wnt signaling to below the critical threshold level in the AER. To test this notion, we generated embryos carrying *Msx2-Cre* and one copy each of *Rac1*^{c} and *R26-Dkk1* (*Msx2-Cre;Rac1*$^{c/+}$*;R26-Dkk1*). Remarkably, these embryos developed no hindlimbs and had severe truncations in the forelimbs (Figures 7O1–7O3). Thus, Rac1 interacts genetically with Dkk1 and β-catenin in the control of limb outgrowth. Overall, the genetic evidence, together with the in vitro data, argues for a critical role of Rac1 in canonical Wnt signaling.

DISCUSSION

By using both biochemical and genetic approaches, we have uncovered a signaling cascade that operates in conjunction with β-catenin stabilization to activate canonical Wnt signaling. Studies in ST2 cells support a model in which Wnt signals through LRP5/6, Dvl, and most likely Frizzled receptors to activate a signaling module composed of $G\alpha_{q/11}\beta\gamma$-PI3K-Rac1-JNK2 (Figure 7P). As a result, stabilized β-catenin is phosphorylated at Ser191 and Ser605 and thereby localized to the nucleus. Our results are not only consistent with previous findings that abnormally high levels of Rac1 activity promote β-catenin nuclear localization and TCF/LEF-mediated transcription in cancer cell lines (Esufali and Bapat, 2004), but they also identify Rac1 activation as an integral component of canonical Wnt signaling in a normal cellular context.

Both this and a previous study of ours have identified $G\alpha_{q/11}\beta\gamma$ as an important component of Wnt signaling. In the previous study, we provided evidence that $G\alpha_{q/11}\beta\gamma$ mediates a noncanonical Wnt pathway involving PLCβ-PKCδ (Tu et al., 2007). Here we show that $G\alpha_{q/11}\beta\gamma$ activates a PI3K-Rac1 cascade that participates in canonical Wnt signaling. Our data from both studies indicate that Wnt3a activates at least three distinct signaling cascades in ST2 cells: (1) stabilization of β-catenin, which does not require $G\alpha_{q/11}\beta\gamma$ but is inhibited by Dkk1; (2) activation of PLCβ-PKCδ, which requires $G\alpha_{q/11}\beta\gamma$ signaling but is not inhibited by Dkk1; (3) activation of PI3K-Rac1-JNK2, which both requires $G\alpha_{q/11}\beta\gamma$ signaling and is inhibited by Dkk1. It is not known whether Wnt3a induces formation of three distinct signaling complexes or a single complex with all three signaling properties. The specificity of signaling may be determined by the diverse Wnt receptors at the cell surface.

Rac1 has also been implicated in nuclear transport of other proteins. It was shown to regulate nuclear accumulation of an armadillo protein SmgGDS through a mechanism dependent upon both the C-terminal polybasic region (PBR) and the activation of Rac1 (Lanning et al., 2003). More recently, Rac1 was reported to control nuclear translocation of STAT transcription factors through interactions with a GTPase-activating protein MgcRacGAP and tyrosine-phosphorylated STAT3 or STAT5A (Kawashima et al., 2006). The present study has identified a mechanism in which Rac1 activation controls nuclear localization through JNK-dependent phosphorylation, but the molecular details for this mechanism remain to be elucidated.

In addition to the serine/threonine phosphorylation within the N terminus that is critical for β-catenin stability (Liu et al., 2002), several other phosphorylation events have been implicated in the turnover and/or subcellular localization of β-catenin. Ryo et al. reported that phosphorylation of Ser246 and the subsequent conformational changes of the p-Ser-Pro bond by the prolyl isomerase Pin1 increased both total and nuclear levels of β-catenin by inhibiting its interaction with APC (Ryo et al., 2001). However, in our studies mutation of Ser246 did not have any obvious effect on either stability or activity of β-catenin in response to Wnt. Moreover, Wnt stimulation did not cause significant phosphorylation at Ser246. The discrepancy could reflect differences in the experimental systems. More recently, phosphorylation of Ser552 by Akt has been implicated in β-catenin signaling in intestinal cells (He et al., 2007). Thus, multiple signal cascades may converge on β-catenin via phosphorylation to control canonical Wnt signaling.

Functional cooperation between JNK and β-catenin has been recently reported in the intestine (Nateri et al., 2005). There, JNK-mediated phosphorylation of c-Jun induced formation of a ternary complex among c-Jun, TCF4, and β-catenin, which in turn activated the c-Jun promoter. This result and our current finding, however, appear to be at odds with a recent report that sustained, high levels of nuclear JNK activity in early *Xenopus* embryos prevented nuclear accumulation of β-catenin (Liao et al., 2006). The discrepancy could indicate that the role of JNK in canonical Wnt signaling depends on the duration, degree, and/or subcellular location of its activation. In this regard, it is worth noting that in our study, the complexes among Rac1, β-catenin, and JNK1/2 were only detected in the cytoplasm but not in the nucleus, and that Wnt3a specifically activated JNK2 in this context. On the other hand, Wnt7b, which did not activate canonical Wnt signaling in ST2 cells, also failed to activate JNK2 in the β-catenin complexes. One may speculate that such an activation event of JNK2 could be "pathway specific" and may not be achieved by other mechanisms.

Future genetic studies are necessary to determine whether Rac1 participates in canonical Wnt signaling in other physiological settings besides the embryonic limb AER. Of note, *Rac1*$^{-/-}$ mouse embryos (Sugihara et al., 1998) share similar gastulation defects with *β-catenin*$^{-/-}$ (Huelsken et al., 2000), *Wnt3*$^{-/-}$ (Liu et al., 1999), and *LRP5*$^{-/-}$/*LRP6*$^{-/-}$ mutants (Kelly et al., 2004). It is therefore of interest to determine whether Rac1 genetically interacts with these other components of canonical Wnt signaling during gastulation of mouse embryos.

Genetic evidence for Rac in canonical Wnt signaling in *Drosophila* has not been reported to date. This could be due to functional redundancy among the three *Rac* homologs (*Rac1*, *2*, and *Mtl*) in the *Drosophila* genome (Hakeda-Suzuki et al., 2002). It is worth noting that triple mutant *Drosophila* embryos containing *Rac1* null alleles were embryonic lethal although the phenotype has not been reported (Hakeda-Suzuki et al., 2002). Moreover, loss-of-function mutations of RacGap50C, a Rac GTPase-activating protein (GAP, negative regulator of Rac activity), ectopically activate Wg signaling in *Drosophila* embryos (Jones and Bejsovec, 2005).

The role of JNK in canonical Wnt signaling in vivo remains to be further elucidated. Although the present work in ST2 cells indicates that JNK2 plays a principle role in mediating Wnt3a-induced phosphorylation of β-catenin, mouse embryos lacking both JNK1 and JNK2 do not exhibit the gastulation defect associated with loss of canonical Wnt signaling, even though they die around E11.5–E12.5 (Kuan et al., 1999). It is possible that other members of the MAPK family regulate β-catenin phosphorylation in other cell types. Of note, *ERK2*$^{-/-}$ mouse embryos are embryonic lethal at E6.5 with no mesoderm formation (Yao et al., 2003); this phenotype resembles that caused by loss of canonical Wnt signaling. Future studies are warranted to determine whether ERK2 participates in canonical Wnt signaling during gastulation of mouse embryos. In *Drosophila*, JNK and Wingless/Armadillo signaling cascades have been shown to genetically interact in promoting ventral patterning (McEwen et al., 2000). More recently, Rac, and a RacGAP of the chimaerin family, known as RhoGAP5A, were shown to interact genetically with ERK in regulating the distribution of Armadillo and cell number in the *Drosophila* eye (Bruinsma et al., 2007).

The finding that PI3K and Rac1 participate in canonical Wnt signaling has important implications in cancer. Activating mutations of PI3K have been frequently identified in ovarian, breast, hepatocellular, and colorectal carcinomas (Lee et al., 2005; Levine et al., 2005; Samuels et al., 2004). More recently, PI3K/Pten signaling was shown to cooperate with β-catenin in causing ovarian endometrioid adenocarcinoma in women and in mice (Wu et al., 2007). Similarly, high levels of Rac1 activation have been found in colon cancer cells containing elevated β-catenin levels and contribute to aberrant activation of canonical Wnt signaling (Esufali and Bapat, 2004). Overall, the Wnt-PI3K-Rac1 pathway may provide additional therapeutic targets for suppressing canonical Wnt signaling in cancer cells.

EXPERIMENTAL PROCEDURES

Mouse Strains and Embryo Analyses

Msx2-Cre (Sun et al., 2000), *Rac1*$^{c/c}$ (Gu et al., 2003), and *TOPGAL* (DasGupta and Fuchs, 1999) mouse strains were as reported. The *R26-Dkk1* mouse strain was generated as described in the Supplemental Data. LacZ staining and whole-mount skeletal preparations were based on methods as previously described (Long et al., 2001). Whole-mount in situ hybridization was based on a procedure as previously described (Wilkinson and Nieto, 1993). Immunostaining for E-cadherin and TUNEL assays (ApopTag Fluorescein In Situ Apoptosis Detection Kit, Chemicon International) were performed on paraffin sections.

Cell Cultures, Transfections, Infections, Viruses, Plasmids, and Oligonucleotides

ST-2 cells, Wnt3a-expressing and control L cells, transfection and infection procedures, retroviruses expressing G_qI, Dkk1, Dvl-2 derivatives, or Wnt7b were all as previously described (Tu et al., 2007). Additional viruses were produced in the same way to express N17Rac1, N17Cdc42 (cDNA Resource Center, University of Missouri, Rolla, MO), or V12Rac1. V12Rac1 was generated by site-directed mutagenesis (Stratagene). Full-length cDNA for β-catenin was cloned by PCR from a mouse 15 day embryo cDNA pool Marathon-Ready (BD Biosciences Clontech), and mutations were introduced by site-directed mutagenesis; all variants of β-catenin were cloned in Myc-tagged pCS2+MT vector. Gene-specific siRNA oligonucleotides (ON-TARGETplus SMARTpool) (see Supplemental Data) and the control siRNA (ON-TARGETplus siCONTROL Nontargeting siRNA #1) were from Dharmacon. MTT assay kit was form ATCC.

Antibodies, Proteins, and Chemicals

Antibodies for JNK, ERK, p38, phospho-JNK, phospho-ERK, and phospho-p38 were from Cell Signaling; antibody for CREB1 was from Upstate. GAPDH

antibody was from Chemicon; β-catenin and E-cadherin antibodies were from BD Biosciences Pharmigen. TCF4 antibody, Myc antibody, and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. Alexa555- and Alexa488-conjugated secondary antibodies were from Molecular Probes. Recombinant human active JNK2α2 and mouse Wnt3a were from Invitrogen and R&D Systems, respectively. Wortmannin, SP600125, U0126, and PD98059 were from Calbiochem.

Assays for Rac1, CDC42, RhoA Activation, and AP Activity

Activation of Rho family small GTPases was detected using an EZ-Detect Rac1, Cdc42, and RhoA Activation Kit (Pierce Biotechnology). AP activity was assayed by incubating cell lysates with p-nitrophenylphosphate as previously described (Tu et al., 2007).

Immunoprecipitation, Western Analyses, Immunohistochemistry, and Phosphorylation Assays

The cytosolic and nuclear fractions of cells were prepared as described in the Supplemental Data. Immunoprecipitation and western analyses were performed using standard protocols. The intensity of protein bands was quantified using NIH ImageJ. Immunocytochemistry was performed in cells cultured on chamber slides (Nalge Nunc International). In vitro and in vivo phosphorylation assays were performed as described in the Supplemental Data.

SUPPLEMENTAL DATA

Supplemental Data include Experimental Procedures and three figures and can be found with this article online at http://www.cell.com/cgi/content/full/133/2/340/DC1/.

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