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Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3–independent β-catenin degradation

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Wnts are secreted signaling molecules that can transduce their signals through several different pathways. Wnt-5a is considered a noncanonical Wnt as it does not signal by stabilizing β-catenin in many biological systems. We have uncovered a new noncanonical pathway through which Wnt-5a antagonizes the canonical Wnt pathway by promoting the degradation of β-catenin. This pathway is Siah2 and APC dependent, but GSK-3 and β-TrCP independent. Furthermore, we provide evidence that Wnt-5a also acts in vivo to promote β-catenin degradation in regulating mammalian limb development and possibly in suppressing tumor formation.

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Introduction

Wnts are secreted proteins that control diverse developmental processes such as gastrulation, limb, and CNS development (Wodarz and Nusse, 1998; Peifer and Polakis, 2000; Thorpe et al., 2000; Huelsken and Birchmeier, 2001). Recent studies have also shown that Wnt signaling may regulate the maintenance and differentiation of stem cells (Taipale and Beachy, 2001; Moon et al., 2002). In humans, inappropriate activation of the canonical Wnt signaling pathway is associated with a high frequency of tumors in specific tissues such as the intestine, liver, skin, and mammary gland (Taipale and Beachy, 2001; van Noort and Clevers, 2002).

19 different Wnt genes have been identified in the mouse and human genomes (http://www.stanford.edu/~rnusse/wntwindow.html) and they signal through either the canonical pathway or at least two different noncanonical pathways (Kuhl et al., 2000b; Niehrs, 2001; Winklbauer et al., 2001). The canonical Wnt pathway, through which *Drosophila* Wingless and vertebrate Wnt-1, 3a, and 8 transduce their signals, contains many evolutionarily conserved cellular components and plays a pivotal role in controlling cell proliferation and differentiation. Central to this pathway is the regulation of β-catenin activity, which depends on its protein abundance and nuclear localization. In the absence of Wnt, glycogen synthase kinase 3 (GSK-3) phosphorylates β-catenin, which allows β-TrCP, an F-box protein in the E3 ubiquitin ligase complex, to bind and tag β-catenin for proteasome-mediated degradation. When the Wnt ligand binds to its coreceptors Frizzled and LRP5/6, Dishevelled is activated and suppresses GSK-3 activity. As a result, β-catenin is not phosphorylated and remains free from β-TrCP–mediated degradation. The accumulated β-catenin binds the transcription factor of the LEF/TCF family and converts them from repressors to activators, which triggers downstream gene transcription (Peifer and Polakis, 2000; Chan and Struhl, 2002; Bienz and Clevers, 2003). β-Catenin activity can also be regulated in the canonical Wnt pathway in a GSK-3–independent manner as has been shown recently in *Drosophila* (Tolwinski et al., 2003). In this pathway, Arrow (LRP5/6) recruits Axin to the membrane and this interaction leads to Axin degradation upon canonical Wnt signaling. As a consequence, β-catenin is no longer bound by Axin, resulting in reduced β-catenin degradation and cytoplasmic sequestration. Thus, canonical Wnt signaling controls gene expression by allowing β-catenin to accumulate when Wnts are present and by reducing nuclear β-catenin levels when Wnts are absent.

The canonical Wnt pathway is highly susceptible to alterations of β-catenin protein stability. Mutations that increase β-catenin protein levels result in constitutive activation of this pathway, which, in turn, leads to cell fate changes during development and tumor formation in adult animals (Wodarz and Nusse, 1998; Peifer and Polakis, 2000). One of the strategies that a cell uses to combat this deleterious condition is to utilize additional regu-

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Abbreviations used in this paper: AER, apical ectodermal ridge; CEF, chick embryonic fibroblast; CsA, Cyclosporin A.

latory pathways that can modulate the level of β-catenin. For instance, β-catenin induces its own negative regulators *Naked* and *Axin2* (Zeng et al., 2000; Jho et al., 2002). It has also been shown that both phosphorylated and unphosphorylated β-catenin can be targeted for proteasome-mediated degradation through an alternative E3 ubiquitin ligase complex that contains APC, Ebi, and Siah1 or 2 (Liu et al., 2001; Matsuzawa and Reed, 2001). This pathway can be regulated by controlling the transcription of *Siah*. However, it is unknown whether *Siah* expression is regulated by Wnt signaling.

Wnt-5a signals through noncanonical pathways in a variety of assays, which may involve stimulation of intracellular Ca^{2+} release and activation of PKC and CaMKII (Sheldahl et al., 1999; Kuhl et al., 2000b). Wnt-5a has also been suggested to antagonize canonical Wnt activity both in *Xenopus* embryos and mammalian cells (Torres et al., 1996; Olson and Gibo, 1998; Ishitani et al., 2003). However, the underlying mechanism is not clear and direct genetic evidence for this antagonistic interaction is lacking.

Here, we demonstrate that Wnt-5a signaling can antagonize the canonical Wnt signaling pathway by promoting β-catenin degradation. We further show that the β-catenin degradation induced by Wnt-5a is independent of phosphorylation by GSK-3 and involves the induction of *Siah2* expression. We also find that Wnt-5a–induced β-catenin degradation does not require activation of CaMKII or NF-AT. Finally, we demonstrate through studies of *Wnt-5a*$^{-/-}$ embryos that in vivo, *Wnt-5a* functions at least in part through promoting β-catenin degradation. We also provide evidence that *Wnt-5a* may act as an oncosuppressive gene in the adult gut.

Results

Wnt-5a antagonizes the canonical Wnt signaling pathway by promoting GSK-3–independent degradation of β-catenin

To understand the mechanism by which Wnt-5a inhibits the canonical Wnt signaling, we used a TCF reporter (TOPFLASH; Korinek et al., 1997) as a readout for the canonical Wnt pathway activity in a variety of cell lines. In immortalized human embryonic kidney cells (HEK 293 cells), *Wnt-3a* expression led to up-regulation of the canonical Wnt activity as indicated by the increased luciferase activity (Fig. 1 A). When *Wnt-5a* was cotransfected with *Wnt-3a*, we found that the luciferase activity was largely reduced. Interestingly, although lower levels of *Wnt-3a* expression (10 and 33 ng) activated TOPFLASH in a dose-dependent manner, higher levels of *Wnt-3a* (167 ng–1 μg) resulted in gradually reduced stimulation of TOPFLASH activity (Fig. 1 A). However, inhibition of the canonical Wnt signaling by Wnt-5a was not simply a result of higher *Wnt* expression. This is because *Wnt-5a* did not stimulate TOPFLASH when expressed at both low and high levels (not depicted), and a very low level of *Wnt-5a* expression (10 ng) inhibited TOPFLASH activity stimulated by a low level of *Wnt-3a* expression (33 ng; Fig. 1 A).

Next, we considered the possibility that Wnt-5a might suppress canonical Wnt pathway activity through inhibition of the binding of canonical Wnts with their receptors. If this were true, Wnt-5a would be unable to suppress the canonical Wnt pathway activity stimulated by β-catenin. However, we found that Wnt-5a strongly suppressed the luciferase ac-

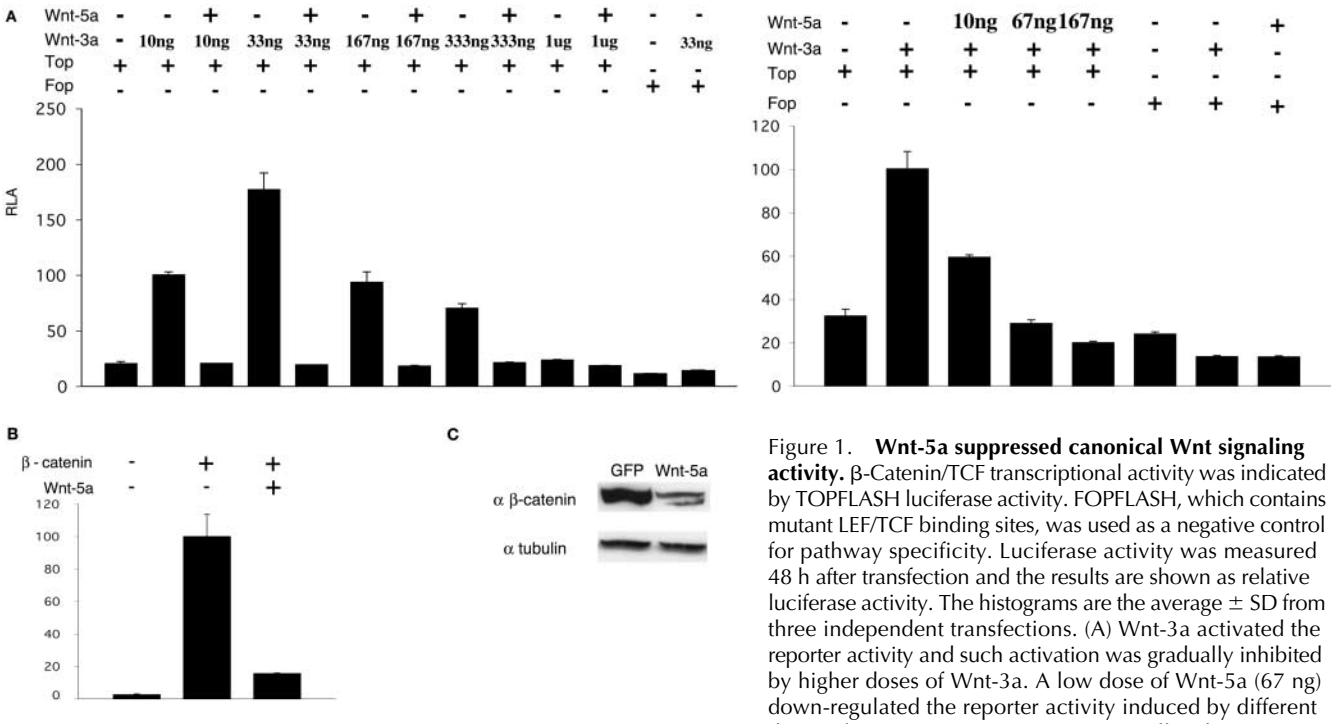

Figure 1. **Wnt-5a suppressed canonical Wnt signaling activity.** β-Catenin/TCF transcriptional activity was indicated by TOPFLASH luciferase activity. FOPFLASH, which contains mutant LEF/TCF binding sites, was used as a negative control for pathway specificity. Luciferase activity was measured 48 h after transfection and the results are shown as relative luciferase activity. The histograms are the average ± SD from three independent transfections. (A) Wnt-3a activated the reporter activity and such activation was gradually inhibited by higher doses of Wnt-3a. A low dose of Wnt-5a (67 ng) down-regulated the reporter activity induced by different doses of Wnt-3a (10 ng–1 μg) in 293 cells. The reporter activity activated by a low dose of Wnt-3a (33 ng) was inhibited by Wnt-5a in a dose dependent manner (10–167 ng). The mutant reporter FOPFLASH did not respond to Wnt-3a or Wnt-5a. (B) Wnt-5a down-regulated the reporter activity induced by β-catenin in 293 cells. (C) 293 cells were transfected with *β-catenin* and *GFP* or *Wnt-5a* where indicated. Wnt-5a expression led to a decrease in β-catenin protein levels.

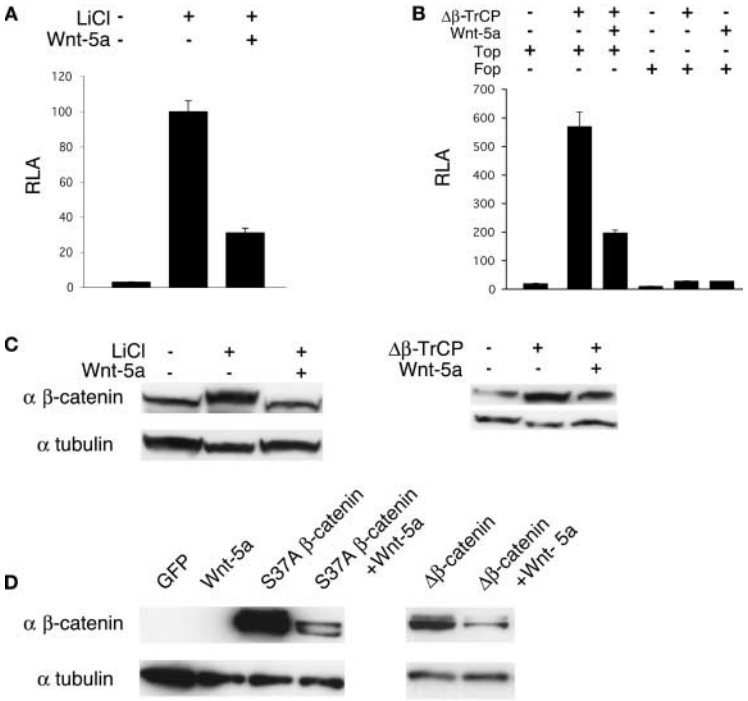

Figure 2. **Wnt-5a promoted β-catenin degradation through a mechanism independent of GSK-3 and β-TrCP.** (A) 24 h after transfection, 293 cells were treated with 40 mM LiCl for 16 h before cells were harvested. LiCl treatment up-regulated TOPFLASH reporter activity. Wnt-5a suppressed the effect of LiCl. (B) Cells were transfected with the indicated plasmids and harvested 48 h later for luciferase assay. Δβ-TrCP up-regulated the TOPFLASH reporter activity and this effect was suppressed by Wnt-5a. (C) 293 cells were transfected with the indicated plasmids and treated with LiCl as in A. LiCl treatment or Δβ-TrCP expression stabilized the endogenous β-catenin and Wnt-5a inhibited this effect. (D) Wnt-5a promoted the degradation of two mutant stabilized forms of β-catenin in 293 cells. Cells were transfected with the indicated plasmids and 48 h after transfection, cells were harvested for western analysis.

tivity stimulated by β-catenin in various cells including 293 cells (Fig. 1 B and not depicted). Thus, the interaction of Wnt5a signaling and the canonical Wnt pathway lies downstream of the receptor–ligand interaction.

Then, we tested whether Wnt-5a inhibited the canonical Wnt pathway by regulating β-catenin degradation. In contrast to what has been reported in a recent publication (Ishitani et al., 2003), we found that *Wnt-5a* expression led to a significant decrease in β-catenin protein levels in 293 cells (Fig. 1 C), suggesting that Wnt-5a antagonizes the canonical Wnt pathway by promoting the degradation of β-catenin protein.

Because phosphorylation of β-catenin by GSK-3 followed by β-TrCP binding results in β-catenin degradation, we then tested whether Wnt-5a promoted β-catenin degradation requires GSK-3 or β-TrCP. We used LiCl to specifically inhibit GSK-3 (Klein and Melton, 1996). LiCl treatment resulted in the enhancement of TOPFLASH reporter activity and accumulation of endogenous β-catenin in 293 cells (Fig. 2, A and C). However, *Wnt-5a* was still able to inhibit β-catenin activity substantially in the presence of LiCl (Fig. 2 A). In addition, when the activity of β-TrCP was blocked by a dominant negative β-TrCP (Δβ-TrCP), Wnt-5a was still able to suppress β-catenin activity (Fig. 2 B). Consistent with the decrease in TOPFLASH activity, Wnt-5a signaling reduced endogenous β-catenin protein levels in the presence of LiCl and Δβ-TrCP (Fig. 2 C). These results indicate that the β-catenin protein degradation promoted by *Wnt-5a* was independent of GSK-3 and β-TrCP. Therefore, Wnt-5a might potentially be able to promote the degradation of two different forms of mutant β-catenin, which cannot be phosphorylated by GSK-3 and are constitutively active. One mutant, β-catenin S37A, is a Ser to Ala mutation that abolishes the GSK-3 dependent phosphorylation of β-catenin at Ser 37, which is required for β-TrCP binding. A second mutant, Δβ-catenin, lacks amino acids 29–48 and contains none of the GSK-3 phosphorylation sites that are required for β-catenin degradation (Tetsu and McCormick, 1999). Both mutants stabilized β-catenin. However, when they were coexpressed with *Wnt-5a* in 293 cells, we found that they were destabilized by Wnt-5a (Fig. 2 D), demonstrating that Wnt-5a functions to inhibit canonical Wnt/β-catenin pathway independently of GSK-3 and β-TrCP.

Wnt-5a promoted β-catenin degradation does not require CaMKII or NF-AT activation

Wnt-5a signaling may activate PKC and intracellular Ca^{2+} mobilization to trigger a series of downstream effects including activation of NF-AT and CaMKII (Kuhl et al., 2000a; Saneyoshi et al., 2002). During early *Xenopus* development, *Wnt-5a* activates calcineurin, which leads to NF-AT nuclear localization and increased β-catenin degradation (Saneyoshi et al., 2002). To address how Wnt-5a transduces its signal in mammalian cells, we checked whether Wnt-5a activates NF-AT transcriptional activity and whether such activation is required for Wnt-5a–induced β-catenin degradation. We found that NF-AT transcriptional activity was only weakly activated by Wnt-5a (~50%), whereas it was strongly up-regulated by activated calcineurin (~300%; Fig. 3 A). In addition, we found that although activated calcineurin was able to inhibit the TOPFLASH activity up-regulated by β-catenin S37A, Wnt-5a had an additive effect in its presence (Fig. 3 B). Moreover, specific inhibition of calcineurin by Cyclosporin A (CsA; Crabtree and Olson, 2002) did not block the inhibitory effect of Wnt-5a on β-catenin activity and protein stability (Fig. 3, B and D), although it strongly inhibited NF-AT transcriptional activity (Fig. 3 A) and up-regulated β-catenin protein level in the absence of Wnt-5a (Fig. 3 D). These data indicate that the calcineurin–NF-AT pathway is not the major one mediating the effect of Wnt-5a on β-catenin degradation in 293 cells. Next, we also exam-

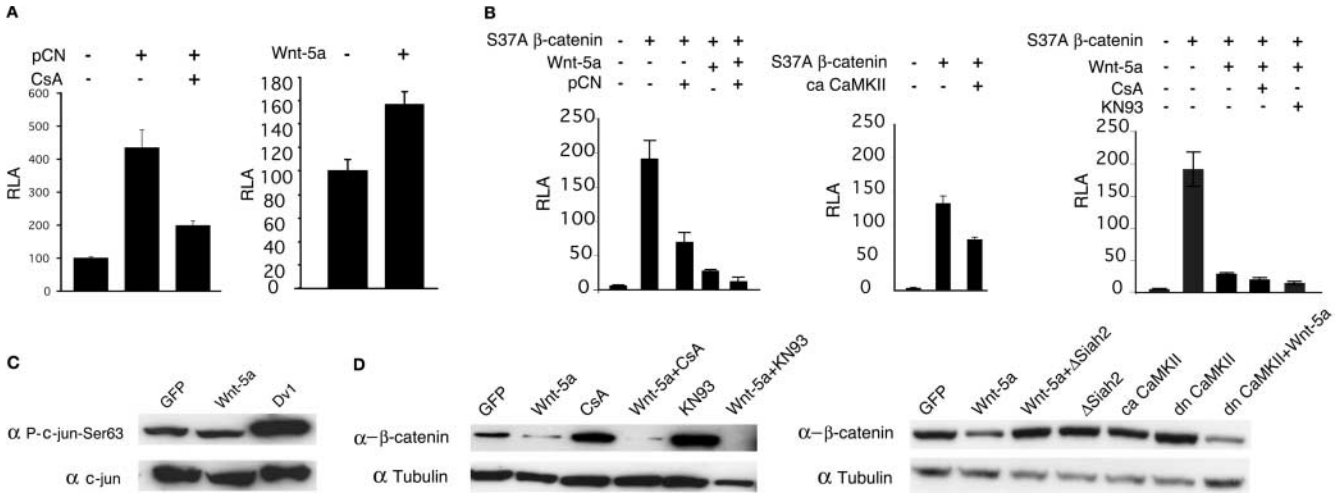

Figure 3. **Activation of NF-AT and CaMKII is not required for Wnt-5a–induced β-catenin degradation.** Cyclosporin A (CsA) inhibited NF-AT reporter activity. Luciferase activity was measured 40 h after transfection. (B) Activated calcineurin (pCN) or CaMKII (ca CaMKII) inhibited the TOPFLASH activity stimulated by the mutant β-catenin (S37A). Inhibiting calcineurin–NF-AT pathway by CsA or inhibiting CaMKII by KN93 did not block Wnt-5a–induced inhibition of TOPFLASH activity in 293 cells. 24 h after transfection with indicated plasmids, CsA or KN93 were added. After 16 h of incubation with the inhibitors, cells were lysed for luciferase assay. (C) 293 cells were transfected with c-Jun and indicated plasmids. JNK activation was detected by anti–phospho-c-Jun (Ser 63) antibodies. Dishevelled, but not Wnt-5a, activated JNK. (D) CHO cells were transfected with mutant *β-catenin* (S37A) and *Wnt-5a* or *GFP* where indicated, and then treated with CsA or KN93 to inhibit calcineurin or CaMKII, respectively. CsA or KN93 treatment or expression of a dominant negative CaMKII (dn CaMKII) appeared to stabilize the mutant β-catenin, but neither treatment inhibited Wnt-5a–induced β-catenin degradation.

ined in 293 cells whether Wnt-5a activated PKC, CaMKII or JNK, all of which have been implicated in transducing the noncanonical Wnt-5a signal (Kuhl et al., 2000a; Yamanaka et al., 2002). Consistent with what has been shown before, we found that Wnt-5a signaling led to the activation of PKC and CaMKII in 293 cells (unpublished data). However, no significant JNK activation was detected (Fig. 3 C). In addition, we found that, in contrast to Wnt-5a signaling, PKC activation led to stabilization of β-catenin as shown previously (Chen et al., 2000; unpublished data). As an activated form of CaMKII was able to inhibit TOPFLASH activity (Fig. 3 B), we tested whether CaMKII activation is required for Wnt-5a–induced β-catenin degradation. We blocked CaMKII activity with a dominant negative CaMKII (Kuhl et al., 2000a) or KN93 (Rich and Schulman, 1998). We found that blocking CaMKII activity did not rescue the TOPFLASH activity or β-catenin protein stability inhibited by Wnt-5a (Fig. 3, B and D), indicating that CaMKII is not required in the Wnt-5a signaling pathway for β-catenin degradation. Interestingly, in the absence of Wnt-5a, inhibition of CaMKII by a dominant negative CaMKII or KN93 also resulted in the up-regulation of β-catenin protein level (Fig. 3 D). Together, our results indicate that activation of NF-AT or CaMKII may play a role in promoting β-catenin degradation. However, inhibition of canonical Wnt activity by Wnt-5a can be mediated by pathways other than the activation of PKC, NF-AT, and CaMKII in mammalian cells.

Siah2 is induced by *Wnt-5a* and is involved in Wnt-5a–induced β-catenin degradation

To test whether Wnt-5a–induced degradation of β-catenin S37A is also mediated by proteasome, we used epoxomicin to block proteasome-mediated protein degradation (Meng et al., 1999). We found that β-catenin S37A degradation induced by Wnt-5a was substantially inhibited when the cells were treated with Epoxomicin (Fig. 4 A). As the Siah–APC–Ebi E3 ubiquitin ligase complex has been implicated in GSK-3–independent β-catenin degradation (Liu et al., 2001; Matsuzawa and Reed, 2001), we then examined whether Siah activity is required for Wnt-5a–induced β-catenin degradation. We found that both Siah1 and 2 exhibit activities similar to Wnt-5a in inhibiting canonical Wnt activities in 293 cells (unpublished data). Moreover, a dominant negative Siah2 (ΔSiah2) was able to rescue the β-catenin activity that had been suppressed by *Wnt-5a* (Fig. 4 B). In addition, degradation of wild-type and an activated form of β-catenin by Wnt-5a signaling were also suppressed by ΔSiah1 and ΔSiah2 (Fig. 4 C and not depicted). Furthermore, we found that, as a result of deceased degradation of the endogenous β-catenin, both ΔSiah1 and ΔSiah2 up-regulated the TOPFLASH activity and this was not inhibited by Wnt-5a (Fig. 4 B). As it has been shown that Siah-dependent β-catenin degradation requires APC activity (Liu et al., 2001; Matsuzawa and Reed, 2001), we next examined whether a dominant negative form of APC (ΔAPC), which has been shown to sequester Siah proteins, could also block β-catenin degradation induced by Wnt-5a signaling. We found that indeed both the activity and protein levels of β-catenin were rescued by ΔAPC (Fig. 4 D). In addition, Wnt-5a hardly inhibited the TOPFLASH activity up-regulated by ΔAPC when there was no exogenous β-catenin (Fig. 4 D). All these results demonstrate that Wnt-5a–induced β-catenin degradation requires functional Siah and APC.

Next, we investigated whether Wnt-5a might promote β-catenin degradation by regulating *Siah* gene expression. We found that *Siah2* was normally expressed at very low levels as

compared with *Siah1*, but that only *Siah2* expression was induced by *Wnt-5a* in 293 cells (Fig. 4 E and not depicted). Although *p53* activates the expression of *Siah1* and *2* (Liu et al., 2001 and Fig. 4 E), we found that *Wnt-5a* did not activate the transcriptional activity of p53 (Fig. 4 F). These data suggest that Wnt-5a may promote the degradation of β-catenin by activating the expression of *Siah2*, possibly through a pathway parallel to p53 in 293 cells.

β-Catenin protein is accumulated in the distal limb bud of *Wnt-5a*$^{-/-}$ embryos

To investigate whether Wnt-5a signaling also antagonizes the canonical Wnt pathway in vivo, we analyzed the limb development defects in the *Wnt-5a*$^{-/-}$ embryos. *Wnt-5a* is strongly expressed in the distal limb bud and its expression gradually fades proximally (Yamaguchi et al., 1999). In the *Wnt-5a*$^{-/-}$ limb, the distal digits are missing. It has been shown that this is not due to earlier regression of the apical ectodermal ridge (AER) or absence of the distal limb mesenchyme (Yamaguchi et al., 1999). Consistent with previous observations, we found that the expression of *Sox9*, the earliest marker for mesenchymal condensation and chondrocyte differentiation (Zhao et al., 1997), was not detected in the distal-most cells in the *Wnt-5a*$^{-/-}$ limb at 12.5 dpc (Fig. 5 A). These data demonstrate that chondrocyte differentiation, which is required for digit formation, was inhibited before mesenchymal condensation in the distal limb of the *Wnt-5a*$^{-/-}$ limb. This phenotype is different from that in the *LRP6*$^{-/-}$ limb, in which skeletal elements are missing along the anterior–posterior axis due to a disrupted AER (Pinson et al., 2000). As LRP6 is a Wnt coreceptor which acts specifically in the canonical Wnt pathway (Wehrli et al., 2000), it appears that Wnt-5a signals predominantly through a noncanonical Wnt pathway during mouse limb development. Because canonical Wnt signaling has been shown to inhibit chondrogenesis (Rudnicki and Brown, 1997), we directly assessed canonical Wnt pathway activity in the *Wnt-5a*$^{-/-}$ mutant limb using *TOPGAL* transgenic mice in which *LacZ* expression is under the control of LEF/TCF binding sites (DasGupta and Fuchs, 1999). Ectopic LacZ staining was detected in the distal limb of *Wnt-5a*$^{-/-}$/*TOPGAL* mice at 12.5 dpc (Fig. 5 B), indicating that canonical Wnt activity has been elevated to a higher level in the distal part of the limb in the absence of Wnt-5a activity. Consistent with this, we found that β-catenin protein level was higher in *Wnt-5a*$^{-/-}$ limbs as compared with wild-type at 11.5 dpc (Fig. 5 C), suggesting that Wnt-5a signaling also promoted β-catenin degradation in the developing limb.

Next, we tested whether decreasing the canonical Wnt activity by applying a secreted Wnt antagonist could rescue distal chondrogenesis in the *Wnt-5a*$^{-/-}$ limb. We generated chick embryonic fibroblast (CEF) cells that express a se-

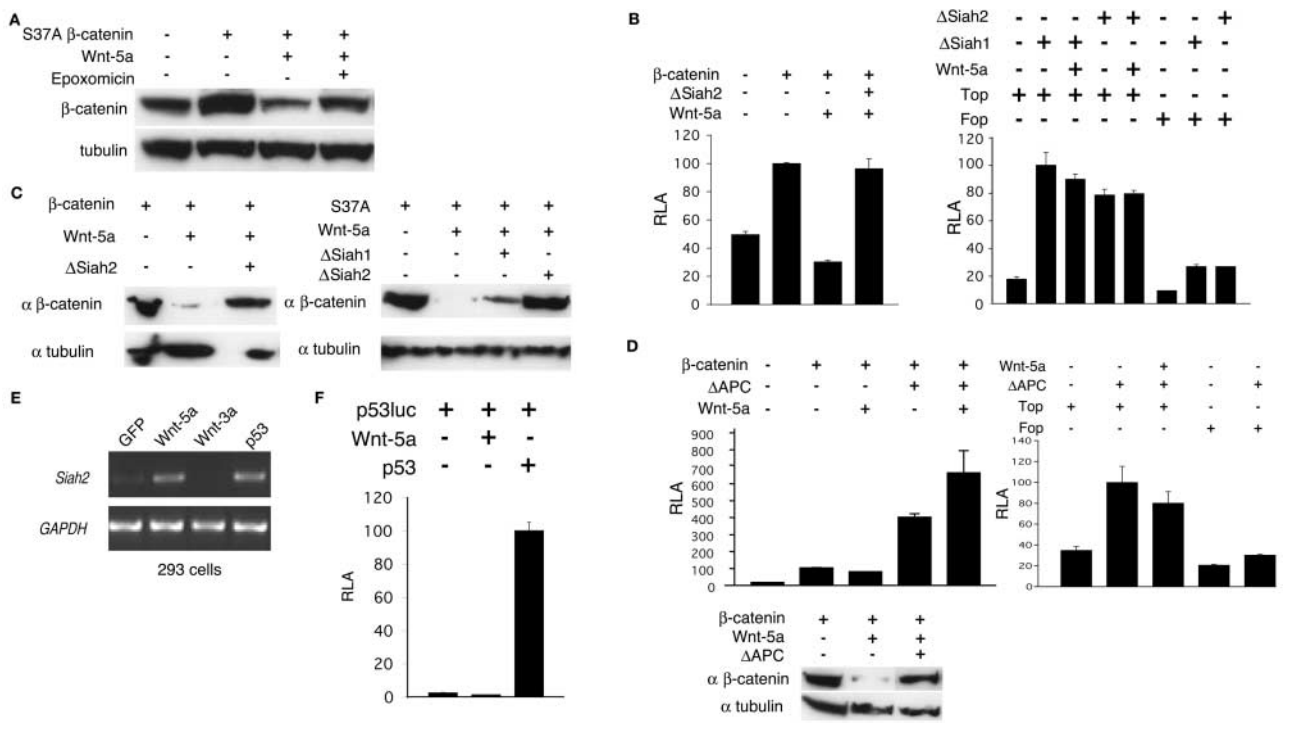

Figure 4. **Wnt-5a promoted β-catenin degradation through a mechanism that requires Siah2 and APC.** (A) β-catenin degradation in response to Wnt-5a was inhibited by the proteasome inhibitor epoxomicin. 293 cells were transfected with indicated plasmids. 38 h after transfection, cells were treated with 100 nM epoxomicin for 8 h before they were harvested. (B) A dominant negative Siah2 (ΔSiah2) blocked Wnt-5a mediated inhibition of TOPFLASH activity activated by exogenous β-catenin. ΔSiah1 or ΔSiah2 alone also up-regulated TOPFLASH activity and this was not inhibited by Wnt-5a. FOPFLASH responded to ΔSiah1 or ΔSiah2 very weakly. (C) ΔSiah2 blocked the degradation of both wild-type and mutant β-catenin promoted by Wnt-5a. (D) ΔAPC blocked Wnt-5a–induced inhibition of β-catenin activity and degradation. ΔAPC alone also up-regulated TOPFLASH, which was hardly inhibited by Wnt-5a. FOPFLASH did not respond to ΔAPC. (E) RT-PCR was performed to detect *Siah2* transcript. Expression of *Siah2* was induced by Wnt-5a and p53 in 293 cells. (F) Wnt-5a did not activate p53 transcriptional activity.

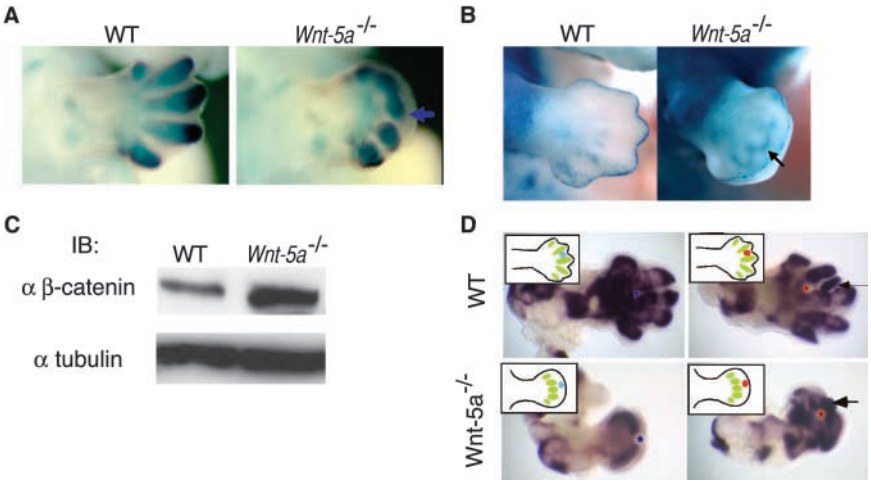

Figure 5. **Canonical Wnt signaling was increased in the *Wnt-5a*$^{-/-}$ limb buds.** Gene expression in embryos was examined by whole mount in situ hybridization. (A) At 12.5 dpc, the expression of *Sox9*, which marks early chondrogenesis, was not detected in the distal-most limb bud of the *Wnt-5a*$^{-/-}$ limb (arrow). (B) Ectopic LacZ staining (arrow) was detected in the *Wnt-5a*$^{-/-}$*/TOPGAL* distal limb at 12.5 dpc. (C) The β-catenin protein level was increased in the distal *Wnt-5a*$^{-/-}$ limb at 11.5 dpc. (D) *Sfrp-2* expressing cells (red), but not control cells (blue), induced ectopic expression of *ColII* (arrows), a marker for chondrocyte differentiation in both wild-type and *Wnt-5a*$^{-/-}$ limbs.

creted Wnt antagonist *Sfrp-2*. *Sfrp-2* is normally expressed in the surrounding mesenchyme of newly formed cartilage in the distal limb (Lescher et al., 1998). It was shown that SFRP-2 is able to antagonize the activity of some canonical Wnts during embryonic development (Lee et al., 2000). The *Sfrp-2* expressing CEF cells were grafted into the interdigit area of wild-type limb bud or the distal mesenchyme of *Wnt-5a*$^{-/-}$ limb bud at 11.5 dpc and the limb buds were culture for 3 d in vitro. We found that *Sfrp-2* expressing CEF cells induced ectopic expression of *ColII*, a marker for differentiated chondrocytes, in both wild-type and *Wnt-5a*$^{-/-}$ limb buds (Fig. 5 D). These data demonstrate that suppression of canonical Wnt signaling allows distal chondrogenesis to occur and rescues the limb phenotype of *Wnt-5a*$^{-/-}$ embryos to a certain extent.

To test whether *Wnt-5a* also regulates β-catenin protein stability by controlling *Siah2* expression during mouse limb development, we compared the expression of *Siah2* in wild-type and *Wnt-5a* mutant embryos by whole mount in situ hybridization. We found that *Siah2* was expressed at very low levels in the wild-type and *Wnt-5a*$^{-/-}$ limb (unpublished data), precluding accurate comparison.

Wnt-5a promotes β-catenin degradation in a colon cancer cell line

Because mutations that cause β-catenin stabilization are associated with the development of colon cancers and *Wnt-5a* is expressed in the gut mesoderm (Lickert et al., 2001), we first tested whether *Wnt-5a* can inhibit the accumulation of β-catenin protein in the colon cancer cell line SW48, which contains intact APC and a Ser 33 to Tyr missense mutation in β-catenin that results in its stabilization. *Wnt-5a* expression led to reduced TOPFLASH reporter activity and decreased β-catenin protein levels (Fig. 6, A and B). Next, we found that *Siah2* was activated by *Wnt-5a* in the SW48 cells (Fig. 6 C). Finally, we found that dominant negative forms of Siah1 and 2 were able to block the activity of Wnt-5a in degrading β-catenin (Fig. 6 B). In contrast, in the colon

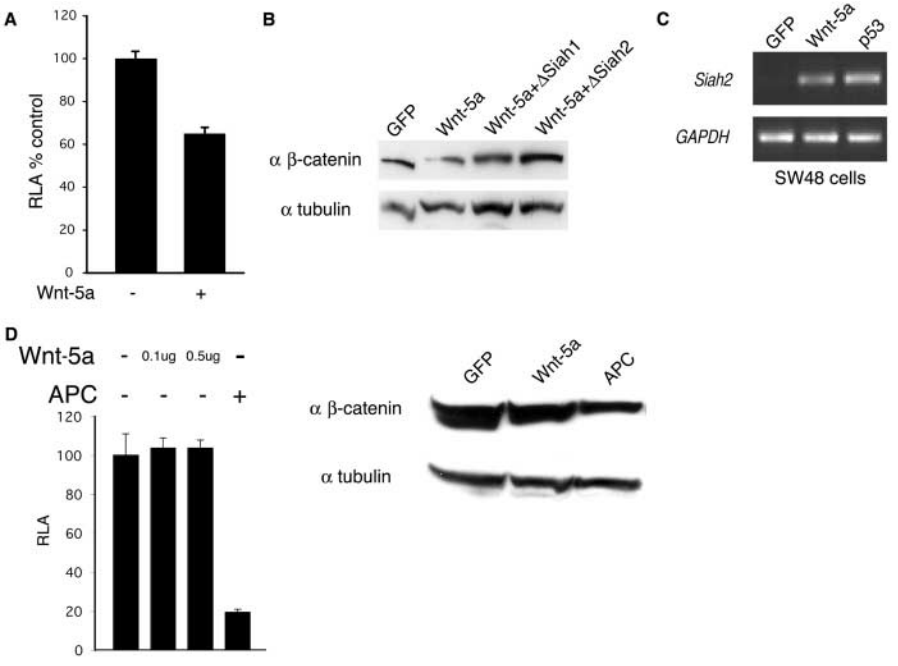

Figure 6. **Wnt-5a decreased β-catenin activity and protein stability in the SW48 cells but not in SW480 cells.** (A) *Wnt-5a* expression inhibits TOPFLASH activity in SW48 cells. (B) *Wnt-5a* expression reduced the protein level of β-catenin in SW48 cells. Both ΔSiah1 and ΔSiah2 blocked the activity of Wnt-5a in reducing β-catenin protein. (C) *Wnt-5a* also induces *Siah2* expression in SW48 cells. (D) *Wnt-5a* expression in SW480 cells did not lead to the inhibition of β-catenin activity and protein degradation. Expression of APC inhibited β-catenin activity and promoted β-catenin degradation.

cancer cell line SW480, which contains wild-type β-catenin but no APC activity, expression of *Wnt-5a* did not lead to inhibition of TOPFLAH activity or β-catenin degradation (Fig. 6 D). These results are consistent with the conclusions drawn from our experiments in 293 cells: the activity of Wnt-5a in promoting β-catenin degradation requires Siah and APC. Together, our findings show that Wnt-5a, in contrast to many other Wnts, signals through a novel pathway that may involve the regulation of *Siah2* expression to antagonize the canonical Wnt pathway in regulating embryonic development and, possibly, in suppressing the formation of a subset of tumors.

Discussion

Our results argue for a novel Wnt-5a signaling pathway that antagonizes the canonical Wnt pathway by promoting β-catenin degradation through the Siah–APC pathway, rather than regulating the GSK-3–βTrCP pathway. β-Catenin is the key component that transduces the canonical Wnt signal through its reduced degradation, which permits association with the transcription factors of the TCF family to activate Wnt downstream target genes (van de Wetering et al., 1997). Therefore, modulating β-catenin protein stability has a profound impact on embryonic development and tumor formation in which precise regulation of Wnt signaling strength is required. Here, we show that in addition to secreted Wnt inhibitors such as Dickkopf and SFRP, Wnt-5a signaling also plays an important role in modulating the signaling strength of the canonical Wnt pathway in embryonic development.

Wnt-5a promotes GSK-3–independent β-catenin degradation

One of our important findings is that Wnt-5a signaling can promote β-catenin degradation by a GSK-3–independent pathway. As the canonical Wnt pathway stabilizes β-catenin degradation through inhibiting its phosphorylation by GSK-3, our results are consistent with the previous observation that Wnt-5a signals through a noncanonical pathway to inhibit the canonical Wnt signaling activity in early *Xenopus* embryos (Torres et al., 1996). However, it was not clear whether Wnt-5a signaling directly inhibited components in the canonical Wnt pathway or indirectly suppressed cellular response to canonical Wnts by altering cell adhesion. Furthermore, the functional mechanism of Wnt-5a signaling in vivo was never properly addressed in previous studies. Our data, together with those obtained in Slusarski's Lab (Westfall et al., 2003, in this issue), demonstrated for the first time that at least part of activities of Wnt-5a in vivo is to inhibit the canonical Wnt signaling activity by promoting β-catenin degradation.

The canonical Wnt signaling can also up-regulate β-catenin activity through a GSK-3–independent pathway in a recently published work (Tolwinski et al., 2003). This pathway depends on the degradation of Axin, which can sequester β-catenin in the cytoplasm. Because this pathway appears to enhance β-catenin activity without affecting β-catenin protein stability, it is unlikely that Wnt-5a signaling inhibits the canonical Wnt signaling pathway through interfering the interaction of LRP5/6, Axin, and β-catenin.

We propose that Wnt-5a signaling acts through a novel pathway that requires Siah2/APC activity to modulate β-catenin stability. Consistent with this, we found that *Siah2* expression can be up-regulated by Wnt-5a in 293 cells (Fig. 4 E). However, Siah1 and 2 appear to have similar activities in promoting β-catenin degradation and the dominant negative Siah2 we used in this work blocks the protein degradation pathway mediated by both Siah1 and 2. As the Siah proteins exhibit functional redundancy and they promote the degradation of themselves, it is likely Siah1 is more stable in the absence of Siah2 so that *Siah2* function can be largely compensated by *Siah1* in *Siah2*$^{-/-}$ mice (Hu and Fearon, 1999). This may explain the lack of developmental defects in *Siah2*$^{-/-}$ mice (Frew et al., 2002). In that sense, Wnt-5a might also regulate another factor that acts in the Siah-mediated protein degradation pathway so that β-catenin protein degradation defect is more severe in *Wnt-5a*$^{-/-}$ mutant embryos. Further investigation of Wnt-5a downstream targets will provide more insight into the molecular mechanism of Wnt-5a in regulating β-catenin protein stability.

It is interesting to note that Δβ-TrCP has much stronger activity in up-regulating TOPFLASH activity and stabilizing β-catenin (Fig. 2, B and C) as compared with ΔSiah1, ΔSiah2, and ΔAPC (Fig. 2 B; Fig. 4, B and D) in nonstimulated cells. These results indicate that β-catenin stability is predominantly regulated by GSK-3–β-TrCP pathway. Wnt-5a may act through the Siah/APC pathway to modulate canonical Wnt pathway activation so that inappropriate high activation of the canonical Wnt pathway is prevented.

Wnt-5a–induced β-catenin degradation and Ca^{2+} signaling

A revealing observation we made is that blocking CaMKII or calcineurin activities with either a dominant negative construct or specific pharmacological inhibitors did not block the activity of Wnt-5a in β-catenin degradation, indicating that activation of NF-AT or CaMKII is not required for the activity of Wnt-5a in antagonizing the canonical Wnt pathway. The previously identified downstream signaling events of Wnt-5a such as activation of PKC and CaMKII appears to have different effects in β-catenin degradation. PKC increases β-catenin activity and protein stability, whereas CaMKII decreases β-catenin activity. It is also interesting to note that we did not detect significant activation of NF-AT in response to Wnt-5a signaling. In addition, an activated form of calcineurin had much stronger activity than Wnt-5a in activating NF-AT transcriptional activity and yet its activity in inhibiting the TOPFLASH reporter was weaker than Wnt-5a in 293 cells (Fig. 3, A and B). Although it was never shown that a dominant negative NF-AT could rescue the phenotype of *Wnt-5a* overexpression in the *Xenopus* embryo, a constitutively active NF-AT was shown to enhance the *Wnt-5a* overexpression phenotype (Saneyoshi et al., 2002). These results can certainly be explained by Wnt-5a and NF-AT acting in parallel pathways so they exhibit an additive effect. This is consistent with our finding in 293 and CHO cells that NF-AT or CaMKII activation is sufficient but not necessary in mediating Wnt-5a–induced β-catenin degradation, which may imply that Wnt-5a signals through the

combined effects of NF-AT and CaMKII activation in promoting β-catenin degradation. In this case, one would expect that blocking either NF-AT or CaMKII activation would result in partial rescue of the β-catenin activity and protein stability inhibited by Wnt-5a. However, our observation that Wnt-5a–induced β-catenin inhibition was not rescued by blocking NF-AT or CaMKII activation argues that there may be a NF-AT– and CaMKII-independent pathway that is activated by Wnt-5a signaling to promote β-catenin degradation in 293 cells. Although this is our favored model, we could not rule the possibility that KN93 treatment or expression of a dominant negative CaMKII only partially inhibited CaMKII activity. Therefore, CaMKII activated by Wnt-5a was not sufficiently inhibited to allow us to see the blockage of Wnt-5a activity. It is also possible that the abundance of some signaling components in these parallel pathways may rely on specific cellular context, which will determine that the dominant pathways in mediating Wnt-5a–induced β-catenin degradation vary in different cells, tissues, and organisms.

It was reported recently that CaMKII mediates the activity of Wnt-5a in inhibiting canonical Wnt activity without affecting β-catenin protein stability in 293 cells (Ishitani et al., 2003), which disagrees with what we have observed in a large number of different cell lines. The difference may simply result from heterogeneity of 293 cells used in different research groups. Our in vivo observation that β-catenin stability was increased in the *Wnt-5a*$^{-/-}$ limb strongly supports the role of Wnt-5a in promoting β-catenin degradation. However, it is possible that Wnt-5a signals through both β-catenin degradation and CaMKII-activated phosphorylation of TCF transcription factors in inhibiting the canonical Wnt pathway. Genetic manipulation of CaMKII activity in a particular developmental process, for instance, limb development, will be necessary to directly access the role of CaMKII in Wnt-5a and the canonical Wnt signaling. Such studies will be important in understanding the mechanism of Wnt signaling not only in embryonic development but also in postembryonic physiological processes where Ca^{2+} signaling plays a critical role such as learning and memory and lymphocyte development and activation (Soderling et al., 2001; Crabtree and Olson, 2002).

The role of Wnt-5a in regulating limb development and tumor formation

Consistent with our findings in vitro, we found that loss of Wnt-5a function led to increased canonical Wnt signaling activity in the distal limb by directly assessing the canonical Wnt signaling activity in vivo in the *Wnt-5a*$^{-/-}$ limb using *TOPGAL* mice (DasGupta and Fuchs, 1999). Two lines of evidence support our conclusion that inhibited chondrogenesis in the *Wnt-5a*$^{-/-}$ limb bud results from up-regulated canonical Wnt activity. First, it has been shown both in vivo and in vitro that increased β-catenin activity leads to inhibition of chondrogenesis (Ryu et al., 2002). Second, SFRP-2, a secreted Wnt inhibitor was able to rescue chondrogenesis in the *Wnt-5a*$^{-/-}$ limb bud (Fig. 5 D). This implies that one important function of *Wnt-5a*, which is expressed at the highest level in the distal limb, is to inhibit the canonical Wnt activity to allow chondrogenesis to occur in the distal limb bud. The high canonical Wnt activity in the distal limb may come from the ectoderm and AER, which express many Wnt family members (Parr et al., 1993). Consistent with this, it has been shown that the limb ectoderm and AER exhibit strong activity in inhibiting chondrogenesis (Solursh et al., 1981). As SFRP-2 is also able to induce chondrogenesis in the interdigit area of the wild-type limb bud, the canonical Wnt activity is high in both the interdigit area and the distal limb. The canonical Wnt activity in the interdigit area may come from *Wnt-14*, which is expressed in the interdigit area and has activity similar to β-catenin in inhibiting chondrogenesis (Hartmann and Tabin, 2001).

It has been shown that cell proliferation is reduced in the distal limb bud of the *Wnt-5a*$^{-/-}$ embryos at the time of chondrogenesis (Yamaguchi et al., 1999). However, we do not think decreased cell proliferation per se in the *Wnt-5a*$^{-/-}$ limb bud results in failure of chondrogenesis because cell condensation before chondrocyte differentiation is not due to increased cell proliferation, rather, it is a process of cell aggregation, which involves alterations in cell adhesion and migration (Thorogood and Hinchliffe, 1975). In fact, cell proliferation in the condensing mesenchyme is drastically reduced and increasing cell proliferation does not affect chondrocyte differentiation (Rossi et al., 2002). Moreover, it has been shown that TGFβ inhibits cell proliferation and yet it strongly promotes chondrocyte differentiation (Shukunami et al., 1998). As noncanonical Wnt signaling has been implicated in the regulation of cytoskeleton rearrangement (Habas et al., 2001), which may cause changes in cell adhesion and migration during mesenchymal condensation, it will be interesting to further investigate whether *Wnt-5a* also controls chondrocyte differentiation through regulating cytoskeleton rearrangement.

Our finding that Wnt-5a signaling promotes GSK-3–independent β-catenin degradation suggests that Wnt-5a may play a role as an oncosuppressor. *Wnt-5a* is expressed in the mesenchymal layer of the gut (Lickert et al., 2001) that can signal to the epithelium where tumors arise. Because the activity of Wnt-5a requires APC, it is likely that mutations in Wnt-5a will have a synergistic effect with gain of function mutations in β-catenin or loss of function mutations in APC in colon cancer development. On the other hand, up-regulating Wnt-5a may prevent tumor formation in cells that has stabilized β-catenin and intact APC, but not in cells that contain loss of function mutations in APC as indicated by our experiments in SW48 and SW480 cells (Fig. 6). Further testing these hypotheses with genetic approaches will provide more insight into the processes of intestinal tumor formation.

Materials and methods

Generation of TOPGAL transgenic mice and in situ hybridization

The TOPGAL Transgenic mice were generated as described previously (DasGupta and Fuchs, 1999). Mouse embryos were dissected in PBS. For LacZ staining, embryos were fixed in 0.5% formaldehyde and 0.5% glutaraldehyde for 10 min at room temperature. For in situ hybridizations, embryos were fixed in 4% formaldehyde at 4°C overnight. Whole mount in situ hybridization was performed as described previously (Yang et al., 1998). RNA probes were described previously: *Sox9* (Zhao et al., 1997) and *ColII* (Lee et al., 1996).

Plasmids

The coding regions of Wnt-5a and Wnt-3a were inserted into pIRES-hrGFP-1a expression vector (Stratagene) and pCDNA3 (Invitrogen), respectively. The TCF reporter plasmid Kit including TOPFLASH and FOPFLASH plasmids was purchased from Upstate Biotechnology. The wild-type and a mutant human β-catenin with a Ser to Ala point mutation at the amino acid 37 (Zorn et al., 1999), provided by Y. Li (Baylor College of Medicine, Houston, TX), were inserted into the pIRES-hGFP-1 expression vector. A mutant form of β-catenin that lacks amino acids 29–48 (Δβ-catenin; provided by F. McCormick, University of California, San Francisco, CA) was PCR amplified and cloned into pIRES-hGFP-1a. The dominant negative human APC (2543–2843) was constructed by PCR as described previously (Liu et al., 2001) and subcloned into pIRES-hrGFP-1a. The human Siah1 and 2 expression constructs were purchased as EST clones (I.M.A.G.E. 947650 and 5749927). The coding region of *Sfrp-2* was subcloned into the *RCAS* vector (Hughes et al., 1987) to generate *Sfrp-2-RCAS*. The dominant negative mouse *Siah-1* (ΔSiah 297–298) was amplified by PCR with primers MΔN-SIAH1F (CGGAATTCACCATGCCTTGTAAATATGCCTCTTCTG) and MΔN-SIAH1R (CCGCTCGAGTCAACACATGGAAATAGTTACATTG), and cloned into pIRES-hGFP-1a. A dominant negative human Siah2 (ΔSiah2) was constructed by PCR using the primers Siah2–268 and Siah2–691 (5′-CCTATTCTGCAGTGCCAGGC-3′ and 5′-CTCTTGTTTCTCCAGCACCAG-3′). The plasmids containing the full length of β-TrCP or a dominant negative β-TrCP were provided by Z.J. Chen (The University of Texas Southwestern Medical Center, Dallas, TX). The constructs for constitutively active and dominant negative CaMKII were provided by R. Moon (University of Washington School of Medicine, Seattle, WA). D. Bohmann provided the c-Jun expression vector (University of Rochester, Rochester, NY).

Limb cultures

Mouse limbs were dissected from 11.5- or 12.5-dpc embryos in PBS. Control CEF cell pellets or CEF cell pellets transfected with *Sfrp-2-RCAS* DNA were inserted into the interdigit region of wild-type limbs or the distal region of the *Wnt-5a*$^{-/-}$ limbs. The limbs were cultured as previously described (Storm and Kingsley, 1999).

Cell culture, transfection, and nucleofection

NIH3T3, CHO, 293, and HeLa cell lines were obtained from American Type Culture Collection. Rat chondrosarcoma cells were obtained from Y. Yamada (National Institutes of Health, Bethesda, MD). Cells were seeded the day before transfection and transfected with the indicated plasmids using LipofectAMINE PLUS as specified by the manufacturer (Invitrogen). In luciferase assays, cells were transfected with a reporter construct (TOPFLASH, FOPFLASH, or NF-AT reporter), an internal control (pRLSV40 or phRL-null, obtained from Promega) and indicated plasmids in 6-well plates. The total amount of transfected DNA was kept constant by adding empty vector DNA. Luciferase activity was measured at indicated time after transfection according to the Dual-Luciferase Reporter Assay System (Promega). The results are shown as relative luciferase activity. The histograms are presented as the average ± SD from three independent transfections. For Western and gene expression analysis, cells were transfected in 60-mm plates. A special electroporation apparatus with the Nucleofector technology (Amaxa GmbH) was used to achieve high transfection efficiency in the SW 48 and SW480 cells. *Sfrp-2-RCAS* DNA was transfected into CEF cells and the transfected cells were split 2 d later. 2 d after the cells were split, they were harvested and cell pellets were generated according to a procedure described previously (Yang and Niswander, 1995).

Western blot analysis

Mouse embryos were collected at 11.5 d after coitum (dpc). Distal limb bud tissues were dissected from wild-type and *Wnt-5a*$^{-/-}$ embryos, washed in PBS, and frozen on dry ice. The frozen limb samples were boiled for 10 min in 2×NuPAGE LDS sample buffer. Cells were washed in PBS and lysed in boiling 2×NuPAGE LDS sample buffer and treated as described previously (Topol et al., 2000). Total proteins were separated by 4–12% Bis-Tris NuPAGE (Invitrogen) and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membranes were probed with primary antibodies, followed by HRP-conjugated secondary antibodies at 1:30,000 (Amersham Biosciences), and developed using chemiluminescent substrates (Pierce Chemical Co.). Primary antibodies used in this work: anti–β-catenin (Transduction Labs), anti–α-tubulin (ICN Biomedicals) anti–Phospho-PKC kit (Cell Signaling Technology), anti-activated CaMKII (Promega), and anti–phospho-c-jun (Ser 63) and anti–c-Jun (Cell Signaling Technology) antibodies.

Treatment with pharmacological inhibitors

CsA was used to inhibit calcineurin at 1 μM and KN 93 was used to inhibit CaMKII at 25 μM. 24 h after transfection, inhibitors were added. After 16 h of incubation with the inhibitors, cells were lysed for luciferase assay or Western analysis.

RNA isolation and RT-PCR

Total RNA was extracted using RNA-Bee (Test-Tel, Inc.) according to the manufacture's instructions and analyzed with RT-PCR using one-step RT-PCR kit (QIAGEN). Endogenous mouse *Siah1* was detected using the following primers: mSiah1fwd, 5′-GGCAATCAAATATTTCTGGCC-3′; and mSiah1rev, 5′-CACGCATGCGTGCACAGAC-3′. For endogenous mouse *Siah2*, the following primers were used: 5′-GCATAGCGCTCTCTATGTTTAATA-3′ and 5′-GCTCAACTCTGTTGATTCTCT-3′. To detect endogenous human *Siah1*, the following primers were used: huSiah1fwd, 5′-CGGAATTCACCATGCCCTGTAAATATGCGTCTTCTG-3′; and huSiah1rev, 5′-CCGCTCGAGTCAACACATGGAAATAGTTACATT-3′. Human *Siah2* was detected with primers: 5′-GCTAATAAACCCTGCAGCAA-3′ and 5′-ACTTCTGGCGGCATTGGTTA-3′. GAPDH was detected using the following primers: forward, 5′-ACCACAGTCCATGCCATCAC-3′; and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′.

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