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

Lilia Topol, Xueyuan Jiang, Hossoon Choi, Lisa Garrett-Beal, Peter J. Carolan, and Yingzi Yang

Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892

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.

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-
ulatory 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. 1A). 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 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.](https://jcb.rupress.org/content/162/5/900.full)

---

**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 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.
The Journal of Cell Biology

Wnt-5a promotes β-catenin degradation

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.

Wnt-5a promotes β-catenin degradation does not require CaM KII 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 CaM KII (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. Wnt-5a promotes 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.

activity 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.
AT or CaMKII may play a role in promoting 3 D). Together, our results indicate that activation of NF-AT resulted in the up-regulation of CaMKII by a dominant negative CaMKII or KN93 also required in the Wnt-5a signaling pathway for degradation. Interestingly, in the absence of Wnt-5a, inhibition of PKC and CaMKII in 293 cells (unpublished data). Moreover, a dominant negative Siah2 was normally expressed at very low levels as Siah2 up-regulated the TOPFLASH 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 Siah1 and Siah2 (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 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 decreased 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 then 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

---

**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.
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 secretable Wnt antagonist (Wnt-5a) and transduced the 293 cell line with a CMV promoter to overexpress Siah2 or APC. The 293 cells were then co-transfected with TOPFLASH and FOPFLASH plasmids, and the RLU assay 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 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.
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 interdigital area of wild-type limb bud or the distal mesenchyme of Wnt-5a−/− limb bud at 11.5 dpc and the limb buds were cultured for 3 d in vitro. We found that Sfrp-2 expressing cells (red), but not control cells (blue), induced ectopic expression of CollII (arrows), a marker for chondrocyte differentiation in both wild-type and Wnt-5a−/− limbs.

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
cancer cell line SW480, which contains wild-type β-catenin but no APC activity, expression of Wnt-5a did not lead to inhibition of TOPFLASH 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–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 signals inhibiting 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–β-TrCP pathway. Wnt-5a may act through the Siah/APC pathway to mediate canonical Wnt pathway activation so that inappropriate high activation of the canonical Wnt pathway is prevented.

Wnt-5a–induced β-catenin degradation and Ca2+ 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 Ca2+ 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-3β-dependent 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 Coll1 (Lee et al., 1996).
Plasmids

The coding regions of Wnt-5a and Wnt-3a were inserted into pIRESHRFP-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 pIRESHRFP-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 pIRESHRFP-1a. A dominant negative human Siah2 (ΔSiah) was constructed by PCR using the primers Siah2-268 and Siah2-691 (5′-CCTTATGCAGCTGCAAAGCTG-3′ and 5′-CTCTTTGGTCCATCCGAC-3′). The plasmids containing the full length of β-TrCP or a dominant negative β-TrCP were provided by Z. Chen (The University of Texas Southwestern Medical Center, Dallas, TX). The constructs for constitutive active and dominant negative CaMKII were provided by R. Moon (Texas Southwestern Medical Center, Dallas, TX). The constructs for constitutively active and dominant negative CaMKII were provided by R. Moon (Texas Southwestern Medical Center, Dallas, TX). The constructs for constitutively active and dominant negative CaMKII were provided by R. Moon (Texas Southwestern Medical Center, Dallas, TX).

Luciferase assays

In luciferase assays, cells were transfected with a reporter plasmid (TOPFLASH, FOPFLASH, or NF-AT reporter), an internal control plasmid (pRLCMV, Promega), and an empty vector DNA. Luciferase activity was measured 24–48 h after transfection.

Western blot analysis

Western blots were performed as described previously (Storm and Kingsley, 1999). Total proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were probed with primary antibodies, followed by HRP-conjugated secondary antibodies (1:30,000) (Amersham Biosciences), and developed using chemiluminescent substrates (Pierce Chemical Co.). Primary antibodies used in this work were: anti-β-catenin (Transduction Labs), anti-α-tubulin (ICN Biomedicals) anti-Phospho-PKC (Cell Signaling Technology), anti-Phospho-Akt (Transduction Labs), and anti-Phospho-serine (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.

DNA isolation and RT-PCR

Total RNA was extracted using RNA-Beo (Test-Tel, Inc.) according to the manufacturer’s instructions and analyzed with RT-PCR using one-step RT-PCR kit (IQAGEN). Endogenous mouse Siah1 was detected using the following primers: mSiah1fw, 5′-GCCATATCAATAATTTCGACC-3′; and mSiah1rev, 5′-CCCGATCTCGTACACAGAC-3′. For endogenous mouse Siah2, the following primers were used: 5′-GCTATGGCCTCTCTATGTT-TAATA-3′ and 5′-GGCTAACCCTGGTTATCTTCT-3′. To detect endogenous human Siah1, the following primers were used: huSiah1fw, 5′-CCGGAATTCCACATCAGGGATATTGATATTGCCGG-3′ and huSiah1rev, 5′-GGCCTGAGCTACACAGGGATATTGATATTGCCGG-3′. GAPDH was detected using the following primers: forward, 5′-ACACAGTGCTACGGCATAC-3′ and reverse, 5′-TCCACACCTGTTGCTGCTA-3′.

We thank Drs. Robert Nussbaum, Igor Dawid, Alan Kimmel, Lee Niswander, and Bill Pavan for critical reading of the manuscript and members of the Genetic Disease Research Branch for helpful discussion. We thank Dr. Diane Slusarski for communicating results before publication. We also thank Darryl Leja and Mike Cichanowski for helping with the manuscript preparation.

Submitted: 25 March 2003
Accepted: 23 June 2003

References

Bienz, M., and H. Clevers. 2003. Armadillo-beta-catenin signals in the nucleus—proof beyond a reasonable doubt? Nat. Cell Biol. 5:179–182.

Chan, S.K., and G. Struhl. 2002. Evidence that Armadillo transduces wingless by mediating nuclear export or cytosolic activation of Pangolin. Cell. 111:265–280.

Chen, R.H., W.V. Ding, and F. McCormick. 2000. Wnt signaling to beta-catenin involves two interactive components. Glycogen synthase kinase-3beta inhibition and activation of protein kinase C. J. Biol. Chem. 275:17894–17899.

Crabtree, G.R., and E.N. Olson. 2002. NFAT signaling: choreographing the social lives of cells. Cell 109(Suppl):S67–S79.

DasGupta, R., and E. Fuchs. 1999. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development. 126:4557–4568.

Frew, I.J., R.A. Dickins, A.R. Caddihi, M. Del Rosario, C. Reinhard, M.J. O’Connell, and D.D. Bowtell. 2002. Normal p53 function in primary cells deficient for Siah genes. Mol. Cell. Biol. 22:8155–8164.

Habas, R., Y. Kato, and X. He. 2001. Wnt/Frizzled activation of Rho regulates vertebral joint formation in the developing appendicular skeleton. Mol. Cell. Biol. 21:8643–8654.

Hartmann, C., and C.J. Tabin. 2001. Wnt-14 plays a pivotal role in inducing syновial joint formation in the developing appendicular skeleton. Cell. 104:341–351.

Hu, G., and E.R. Fearon. 1999. Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. Mol. Cell. Biol. 19:724–732.

Huelsken, J., and W. Birchmeier. 2001. New aspects of Wnt signaling pathways in higher vertebrates. Curr. Opin. Genet. Dev. 11:547–553.

Hughes, S.H., J.J. Greenhouse, C.J. Petropoulos, and P. Suntrave. 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. 61:3004–3012.

Ishitani, T., S. Kishida, J. Hyodo-Miura, N. Ueno, J. Yasuda, M. Waterman, H. Shiruya, R.T. Moon, J. Ninomiya-Tsuji, and K. Matsumoto. 2003. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+)-pathway to antagonize Wnt/beta-catenin signaling. Mol. Cell. Biol. 23:131–139.

Jho, E.H., T. Zhang, C. Domon, C.K. Joo, J.N. Freund, and F. Costantini. 2002. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol. Cell. Biol. 22:1172–1183.

Klein, P.S., and D.A. Melton. 1996. A molecular mechanism for the effect of lithium...
i um on development. Proc. Natl. Acad. Sci. USA 93:8485–8489.

Korinik, V., N. Barker, P.J. Morin, D. van Wichen, R. de Weger, K.W. Kinzler, B. Vogelstein, and H. Clevers. 1997. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC–/– colon carcinoma. Science. 275: 1784–1787.

Kuhl, M., L.C. Sheldahl, C.C. Malbon, and R.T. Moon. 2000a. Ca(2+/-)calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fate changes. J. Biol. Chem. 275: 12701–12711.

Kuhl, M., L.C. Sheldahl, M. Park, J.R. Miller, and R.T. Moon. 2000b. The Wnt/ Ca+2 pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet. 16:279–283.

Lee, C.S., L.A. Burritt, N.R. May, A. Kispert, and C.M. Fan. 2000. SHH-N LDL-receptor-related protein mediates Wnt signalling in mice. Curr. Biol. 10:10–17.

Lescher, B., B. Haenig, and A. Kispert. 1998. sFRP-2 is a target of the Wnt-4 signaling pathway in the developing metanephric kidney. Dev. Dyn. 213:440–451.

Lickert, H., A. Kispert, S. Kutsch, and R. Kemler. 2001. Expression patterns of Wnt genes in mouse gut development. Mech. Dev. 105:181–184.

Liu, J., J. Stevens, C.A. Rote, H.J. Yost, Y. Hu, K.L. Neufeld, R.L. White, and N. Matsunami. 2001. Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. Mol. Cell. 7:927–936.

Matsuzawa, S.I., and J.C. Reed. 2001. Siah-1, SIP, and Ebi collaborate in a novel Wnt pathway linking p53 to the adenomatous polyposis coli protein. Mol. Cell. 7:915–926.

Meng, L., R. Mohan, B.H. Kwok, M. Elfson, N. Sin, and C.M. Crews. 1999. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. Proc. Natl. Acad. Sci. USA. 96:10403–10408.

Moon, R.T., B. Bowerman, M. Boutros, and N. Perrimon. 2002. The promise and peril of Wnt signaling through beta-catenin. Science. 296:1644–1646.

Niehrs, C. 2001. Developmental biology. Solving a sticky problem. Nature. 411:349–354.

Ohayon, E. Schejter, A. Tomlinson, and S. DiNardo. 2000. arrow encodes an LDL-receptor-related protein essential for Wingless signalling. Nature. 407:527–530.

Olson, D.J., and D.M. Gibo. 1998. Antisense wnt-5a mimics wnt-1-mediated C57MG mammary epithelial cell transformation. Exp. Cell Res. 241:134–141.

Parr, B.A., M.J. Shea, G. Vassileva, and A.P. McMahon. 1993. Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. Development. 119:247–261.

Peifer, M., and P. Polakis. 2000. Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. Science. 287:1606–1609.

Pimou, K.L., J. Brennan, S. Monnley, B.J. Avery, and W.C. Skarnes. 2000. An LDL-receptor-related protein mediates Wnt signaling in mice. Nature. 407:535–538.

Rich, R.C., and H. Schulman. 1998. Substrate-directed function of calmodulin in autophosphorylation of Ca2+/calmodulin-dependent protein kinase II. J. Biol. Chem. 273:28424–28429.

Rossi, F., H.E. MacLean, W. Yuan, R.O. Francis, E. Semenova, C.S. Lin, H.M. Kronenberg, and D. Colombini. 2002. p107 and p130 Coordinately regulate proliferation, Cbf1 expression, and hypertrophic differentiation during endochondral bone development. Dev. Biol. 247:271–285.

Rudnicki, J.A., and A.M. Brown. 1997. Inhibition of chondrogenesis by Wnt gene expression in vivo and in vitro. Dev. Biol. 185:104–118.

Ryu, J.H., S.J. Kim, S.H. Kim, C.D. Oh, S.G. Hwang, C.H. Chun, S.H. Oh, J.K. Seong, T.L. Huh, and J.S. Chun. 2002. Regulation of the chordocyte phenotype by beta-catenin. Development. 129:5541–5550.

Sanyety, T., S. Kume, Y. Amasaki, and K. Mikoshiba. 2002. The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos. Nature. 417:295–299.

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

Shukunami, C., Y. Ohita, M. Sakuda, and Y. Hiraki. 1998. Sequential progression of the differentiation program by bone morphogenetic protein-2 in chondrogenic cell line ATDC5. Exp. Cell Res. 241:1–11.