Wnt Activates the Tak1/Nemo-like Kinase Pathway*

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Genetic studies on endoderm-mesoderm specification in Caenorhabditis elegans have demonstrated a role for several Wnt cascade components as well as for a MAPK-like pathway in this process. The latter pathway includes the MAPK kinase kinase-like MOM-4/Tak1, its adaptor TAP-1/Tab1, and the MAPK-like LIT-1/Nemo-like kinase. A model has been proposed in which the Tak1 kinase cascade counteracts the Wnt cascade at the level of β-catenin/TCF phosphorylation. In this model, the signal that activates the Tak1 kinase cascade is unknown. As an alternative explanation of these genetic data, we have explored whether Tak1 is directly activated by Wnt. We find that Wnt1 stimulation results in autophosphorylation and activation of MOM-4/Tak1 in a TAP-1/Tab1-dependent fashion. Wnt1-induced Tak1 stimulation activates Nemo-like kinase, resulting in the phosphorylation of TCF. Our results combined with the genetic data from C. elegans imply a mechanism whereby Wnt directly activates the MOM-4/Tak1 kinase signaling pathway. Thus, Wnt signal transduction through the canonical pathway activates β-catenin/TCF, whereas Wnt signal transduction through the Tak1 pathway phosphorylates and inhibits TCF, which might function as a feedback mechanism.

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1 The abbreviations used are: RNAi, RNA-mediated interference; MAPK, mitogen-activated protein kinase; MAPKKK, MAPK kinase kinase; Nlk, Nemo-like kinase; APC, adenomatous polyposis coli; HEK, human embryonic kidney; DN, dominant negative; EMS, endoderm-mesoderm; LiCl, lithium chloride; GST, glutathione S-transferase; HA, hemagglutinin; wrn, wormadillo; tab, TGF-β-activated kinase; mom-4 and lit-1. Mom-4 is similar to mammalian TGF-β-activated kinase (tab-1); lit-1 is similar to Nemo-like kinase (nlk) (7, 8).

Wnts are a family of secreted proteins involved in a wide range of developmental processes and, as indicated above, control of asymmetric division and cell polarity. Wnts act by binding to frizzled receptors, which constitute a subfamily of seven-transmembrane-spanning proteins (9). In the canonical Wnt/β-catenin signaling pathway, β-catenin is stabilized in the cytoplasm and forms a nuclear complex with members of the TCF/LEF family of DNA-binding molecules to activate transcription of target genes (10, 11). In colorectal cancer in man, inappropriate activation of the Wnt signaling pathway leads to stabilization of β-catenin (11, 12). In most colorectal tumors, this stabilized β-catenin is the result of truncating mutations in the adenomatous polyposis coli (APC) tumor suppressor or of activating mutations that alter or delete regulatory phosphorylation sites in β-catenin (13, 14). Several target genes of TCF-β-catenin complexes that might function in the process of tumorigenesis have been identified (15). Mutations that increase β-catenin protein levels lead to cell fate changes during development and tumor formation in adult animals (16, 17). Cells use various strategies to combat this condition, including the expression of negative regulators that modulate β-catenin levels such as Naked and Axin2 (18, 19), expression of inhibitory Wnts such as Wnt5A (20), the expression of Siah and subsequent degradation of β-catenin by the proteasome (21), and the activation of the Tak1/Nlk kinase pathway described in this report.

Mammalian Tak1 is related to MAPKKKs and was initially identified by complementation assays in yeast, where it can substitute for the MAPKKK Ste11p (22). The kinase activity of Tak1 is stimulated by treatment of cells with TGFβ, bone morphogenetic proteins, IL1 (22–24), and by one of the ligands for the Eph receptors, ephrinB1 (25). Tab1 was identified as a specific interaction partner of Tak1 and activates Tak1 by directly binding to its catalytic domain (26). Besides Tab1-induced Tak1 activation, Tak1 can be activated by a ubiquitin-dependent mechanism (27). Tak1 activity regulates various downstream signal transduction pathways. It activates the NFκB transcription factor by stimulating the NFκB-activating kinase or the IκB kinase (28, 29). Tak1 might also play a role in the activation of c-Jun N-terminal kinase (30, 31) and the MAP kinase p38α (32). Most important for the current study, Tak1 functions upstream of the MAPK Nlk and enhances Nlk kinase activity (33).

It has been proposed that the C. elegans MOM-4/LIT-1 pathway converges with the Wnt cascade to down-regulate POP-1 in the posterior daughter cell E (8, 34). POP-1 is a substrate for TGFβ, and phosphorylation appears to relocalize POP-1 from the nucleus to the cytoplasm (8). Similarly, Tak1 (the mammalian homologue of MOM-4) functions upstream of Nlk and TCF down-regulation is accomplished through the phosphorylation...
of TCFs by Nlk (35). The negative regulation of TCF/β-catenin by this Tak1/Nlk pathway raises the possibility that these kinases might control tumorigenesis in Wnt cascade-driven tumors.

Tak1 can be activated by several stimuli, but the upstream cues that induce MOM-4/Tak1 to activate LIT-1/Nlk in worms or vertebrates remain largely unknown (Fig. 1A). In the present study, we analyzed whether the MOM-4/LIT-1 and the Tak1/Nlk signaling pathways are directly regulated by Wnts as suggested by genetic data from C. elegans (Fig. 1B). We find that Wnt stimulation results in Tak1 activation followed by the activation of Nlk. We provide evidence that Wnt can activate a MAPK signal transduction pathway that might function as an inhibitory feedback mechanism involved in fine-tuning the canonical Wnt signaling pathway.

EXPERIMENTAL PROCEDURES

Reagents—Human embryonic kidney (HEK293T) cells were grown in RPMI 1640 with standard supplements. C57MG cells with the Wnt1 transgene were grown in Dulbecco’s modified Eagle’s medium with standard supplements and 50 ng/ml tetracycline. Generation of C57MG/Wnt1 cells has been previously described (35). The following antibodies were used: 9E10 (mouse) for Myc-tagged proteins, anti-FLAG monoclonal antibody M2 (mouse) (Sigma), 12CA5 (mouse) for HA-tagged proteins, anti-Tak1 (rabbit) (Upstate Biotechnology), anti-T7 (mouse) (Novagen), and anti-Wnt1 clone Mc123 (mouse) (Upstate Biotechnology). cDNAs encoding MOM-4 and TAP-1 were cloned by PCR from a C. elegans cDNA library based on published sequences (7, 8) and verified by sequencing. The MOM-4 and TAP-1 primers used were: 9.5-AGACGTGCAATGCACAATTTTGCACAAGTCCAG; MOM-4, 5'-AGACGGTGACACGAGATTCGTCACAG; MOM-4, 5'-ACAGGTGATCCTACATCTTCCATTTCA; TAP-1, 5'-AGACGGACCATGCGGACGCGGTTTC; TAP-1, 3'-AGACGGACCATGCGGACGCGGTTTC; TAP-1, 3'-CTACCTTTTTCATCTGCAGAGATGACATC, 9S192A, 5'-CCAATAATAGGGGGCTGCTGCTTGG; TAP-1, 3'-GCACCCCGCTGCAAGGACAGCCGCTTATTTGG; TAP-1, 3'-CTACCTTTTTCATCTGCAGAGATGACATC, 9S192A, 5'-CCAATAATAGGGGGCTGCTGCTTGG; TAP-1, 3'-GCACCCCGCTGCAAGGACAGCCGCTTATTTGG; TAP-1, 3'-CTACCTTTTTCATCTGCAGAGATGACATC, 9S192A, 5'-CCAATAATAGGGGGCTGCTGCTTGG; TAP-1, 3'-GCACCCCGCTGCAAGGACAGCCGCTTATTTGG. Mouse cDNA encoding MKK6 was cloned into pRP259 (GST expressed as a fusion with the C terminus). MKK6-GST construct was transformed in BL21 (DE3) and by a mobility shift, indicative of phosphorylation (Fig. 2, compare A and C). Surface plasmon resonance was used to determine whether the MOM-4 mobility shift was caused by phosphorylation of MOM-4 in HEK293T cells. In the absence of Wnt signals, MOM-4 could be activated by overexpression of TAP-1 as evidenced by increased abundance and by a mobility shift, indicative of phosphorylation (Fig. 2A, lanes 1 and 2, and 2D). Lower amounts of the MOM-4-binding protein TAP-1 did not induce the mobility shift of MOM-4 (Fig. 2A, lane 2). We then examined the effect of Wnt1 stimulation in the presence of these limiting amounts of TAP-1. Co-expression of MOM-4 and TAP-1 with increasing amounts of Wnt1 resulted in increased amounts and a mobility shift of MOM-4 in a dose-dependent manner (Fig. 2A, lanes 4–6, and 2B). In the absence of TAP-1 (Fig. 2A, lanes 7–9), Wnt1 stimulation did not induce the mobility shift of MOM-4. The Wnt1-induced increase in abundance of the MOM-4 protein varied somewhat between experiments (Fig. 2, compare A with D).

To determine whether the MOM-4 mobility shift was caused by phosphorylation of MOM-4, Wnt1-stimulated samples were incubated with λ-phosphatase. This treatment decreased the intensity of the shifted MOM-4 (Fig. 2D), indicating that the mobility shift of MOM-4 was caused by phosphorylation. To determine whether the MOM-4 mobility shift induced by Wnt1 stimulation coincided with increased MOM-4 kinase activity, we performed an in vitro kinase assay (Fig. 2C). Overexpression of TAP-1 stimulated the autophosphorylation activity of MOM-4 (Fig. 2C, lanes 1–3). Interestingly, when Wnt1 was co-expressed with MOM-4 and limiting amounts of TAP-1, Wnt1 stimulation could induce the catalytic activity of the MOM-4 kinase (Fig. 2C, lanes 4–7). In the absence of the TAP-1 adaptor, no autophosphorylation activity of MOM-4 was observed (Fig. 2C, lanes 8–11). These observations led us to hypothesize that Wnt1 stimulation could activate autophosphorylation and, hence, the catalytic activity of MOM-4. However, upon stimulation with Wnt1, MOM-4 accumulates in cells, which made it difficult to distinguish between enhanced
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Wnt1 activates Tak1

Fig. 1. (A) Current model for interaction between the Wnt and MOM-4/Tak1-LIT-1/Nlk pathways. The Tak1/Nlk-MOM-4/Lit1 pathway counteracts the Wnt signaling pathway at the level of TCF/POP-1 phosphorylation. The adaptor proteins TAP-1 and Tab1 were identified as MOM-4/Tak1 interacting proteins and function as activators of MOM-4 and Tak1, respectively. The upstream signal that activates MOM-4/Tak1 and Nlk is unknown (B). Our results suggest a novel model whereby Wnt directly activates the MOM-4/Tak1 kinase pathway in a TAP-1/Tab1-dependent manner, resulting in the activation of Nlk and TCF phosphorylation.

activation versus increased abundance of MOM-4. We therefore pursued the analysis of equal amounts of the MOM-4 complex from transfected HEK293T cells. Several transfections were performed to define conditions under which equal amounts of MOM-4 were produced in the various combinations (Fig. 2E). The amount of lysate was adjusted accordingly to allow equal amounts of the protein complexes to be subjected to an in vitro kinase assay. Upon Wnt1 stimulation, we thus found an increase in MOM-4 autophosphorylation (Fig. 2E). Additionally, we investigated whether Wnt1 stimulation could enhance phosphorylation of the exogenous substrate MKK6 by MOM-4. These combined results indicated that Wnt1 induced the activation and autophosphorylation of MOM-4 in a TAP-1-dependent fashion.

Wnt1 Stimulation Results in Autophosphorylation and Increased Kinase Activity of Tak1—We then asked whether the mammalian homologue of the MOM-4 kinase, Tak1 and its adaptor, Tab1, could also be activated by Wnt1 (Fig. 3). Tak1 was expressed together with increasing amounts of Wnt1 in the presence or absence of exogenous Tab1. Subsequently, Tak1 was immunopurified and an in vitro kinase assay was performed. Expression of increasing amounts of the adaptor Tab1 resulted in an increase in abundance and phosphorylation of Tak1 (Fig. 3A, lanes 1 and 3). Co-expression of Tak1, limiting amounts of Tab1, and increasing amounts of Wnt1 resulted in phosphorylation of Tak1 in a dose-dependent manner (Fig. 3A, lanes 4–6). We also observed a mobility shift of the Tak1 protein (Fig. 3A). To determine whether this was caused by phosphorylation of Tak1, Wnt1-stimulated samples were incubated with λ-phosphatase. This treatment decreased the intensity of the shifted Tak1, indicating that this was indeed the case (Fig. 3B). Tak1 could still induce some increase in phosphorylation of Tak1 in the absence of exogenous Tab1 (Fig. 3A, lanes 7–9). Although we cannot formally rule out that Tab1 is dispensable for the Wnt-induced activation of Tak1, we believe that endogenously expressed Tab1 in HEK293T cells suffices for activation of Tak1 by Wnt1. To determine whether there is an increase in Tak1 kinase activity after Wnt1 induction, we performed an in vitro kinase assay. Wnt1 induced the catalytic activity of Tak1 as visualized by autophosphorylation of Tak1 (Fig. 3C, anti-Ha) and phosphorylation of Tab1 (Fig. 3B, anti-T7).

These observations led us to hypothesize that Wnt1 stimulation could activate autophosphorylation and, hence, the catalytic activity of Tak1. However, like MOM-4, Tak1 accumulates in cells upon stimulation with Wnt1, which makes it difficult to distinguish between enhanced activation and abundance of Tak1. Therefore, we again adjusted transfection conditions to generate comparable amounts of Tak1 (Fig. 3D). The amount of lysate was adjusted to analyze identical amounts of the protein complexes in an in vitro kinase assay. Thus, we found that Wnt stimulation led to a strong increase in Tak1 autophosphorylation (Fig. 3D). Additionally, we investigated whether Wnt1 stimulation could enhance phosphorylation of the exogenous substrate MKK6 by Tak1. Indeed, the phosphorylation of MKK6-GST by Tak1 was enhanced, compared with phosphorylation in the absence of Wnt1 stimulation (Fig. 3D). These combined results indicated that Wnt1 induced the activation and autophosphorylation of Tak1.

Wnt1 Stimulates the Kinase Activity of Nlk via Tak1—To address whether endogenous Tak1 kinase is connected to the Wnt1 signal transduction machinery, we performed an in vitro kinase assay on immunopurified Tak1 from C57MG cells that were induced to express Wnt1 (35). Induction of Wnt1 by removal of tetracycline indeed induced the kinase activity of endogenous Tak1 in C57MG cells (Fig. 4A). We were not able to immunopurify endogenous Tak1 from HEK293T cells for in vitro kinase assays because of the low expression level of Tak1 in HEK293T cells.

Nlk is a 60-kDa threonine/serine protein kinase that can be activated by Tak1 (33, 36). Nlk can phosphorylate TCFs, thereby down-regulating TCF transcriptional activity (33). To test whether Nlk activity is modulated by Wnt1 stimulation via Tak1, we co-expressed Nlk-FLAG with Wnt1 in HEK293T cells. Cell extracts were subjected to immunoprecipitation with anti-FLAG antibody (Nlk), and the immunopurified Nlk was subjected to an in vitro kinase assay. We observed that Wnt1 induced an increase in Nlk kinase activity (Fig. 4B, lanes 1 and 2), presumably through the activation of endogenous Tak1. Co-expression of wild type Tak1 with Nlk also resulted in an increase in Nlk kinase activity (Fig. 4B, lanes 1 and 3), suggesting that both Wnt1 and Tak1 are upstream activators of Nlk. We next examined whether Wnt1-induced activation of Nlk was indeed mediated via endogenous Tak1. To that end, we
co-transfected Nlk with Wnt1 and either of two dominant negative (DN) Tak1 constructs (Tak1-K63W and Tak1-S192A). The K63W-Tak1 mutant lacks kinase activity and is not phosphorylated following Wnt1 stimulation or when coexpressed with Tab1 (data not shown). The activation of many protein kinases, including MAPKKKs, involves the phosphorylation of serine and/or threonine residues in a region termed the kinase activation loop between subdomains VII and VIII (37). Mutation to alanine of serine 192 in this activation loop abolishes both phosphorylation and activation of Tak1 (37). Both mutant Tak1 proteins (K63W and S192A) inhibited Nlk activation induced by Wnt1 (Fig. 4B, lanes 4 and 5). These results implied that Wnt1 activates Nlk via Tak1.

**Wnt1 Stimulation Results in Phosphorylation of TCF4 via Tak1**—Nlk has been shown to phosphorylate LEF-1/TCF on two serine/threonine residues located in its central region (38). We examined therefore whether Wnt1 stimulation, like Tak1 and Nlk overexpression, resulted in the phosphorylation of TCF4. HEK293T cells were transfected with FLAG-TCF4 together with various combinations of Wnt1, Tak1, and Tab1 expression plasmids (Fig. 5A). TCF4 was immunopurified and subjected to an *in vitro* kinase assay performed in the presence of 1 μg of MKK6-GST and separated by SDS-PAGE. Phosphorylated proteins were visualized by autoradiography. The intensity of the MKK6-GST- and MOM-4-phosphorylated proteins were measured, and the results are expressed as activity.
way downstream of Frizzled by treatment with an inhibitor of GSK3β, lithium chloride (LiCl) (Fig. 5, C and D). HEK293T cells were transfected with Ha-tagged Tak1 and a low amount of Tab1 and were stimulated with LiCl. Cell extracts were subjected to immunoprecipitation or immunoblotting with anti-Ha or anti-T7, and the immunopurified Tab1-Tak1 complexes were subjected to an in vitro kinase assay. In contrast to Wnt1 stimulation, LiCl did not result in the induction of a

and an experiment was performed using equal amounts of purified Tak1 from HEK293T cells in the presence or absence of Wnt1 stimulation. The protein complexes were subjected to an in vitro kinase assay in the presence of 1 μg of MKK6-GST and separated by SDS-PAGE. Phosphorylated proteins were visualized by autoradiography. The intensity of the MKK6-GST and Tak1-phosphorylated proteins was measured, and the results are expressed as activity.
mobility shift of Tak1 or in the phosphorylation of Tab1 by Tak1 (Fig. 5, C and D). However, like Wnt1, LiCl activated the canonical Wnt pathway as measured in a TCF reporter gene assay (Fig. 5C).

Wnt5A Does Not Induce Activation of Tak1 or Phosphorylation of TCF4—Wnt5A suppressed the β-catenin/TCF transcriptional activity in various colon carcinoma cell lines such as LS174T (Fig. 6A). Ishitani et al. (41) suggested that Wnt5A-induced inhibition of transcriptional activity is the result of the activation of the Tak1/Nlk kinase pathway. In contrast, it was found by Topol et al. (20) that Wnt5A-induced inhibition of transcriptional activity is caused by the degradation of β-catenin, is independent of phosphorylation of GSK3β, and involves the induction of Siah2 expression. We considered the possibility that Wnt5A might suppress canonical Wnt pathway activity through activation of Tak1/Nlk and phosphorylation of TCFs. To investigate this, we analyzed the effect of Wnt5A stimulation on the phosphorylation status of Tak1 in HEK293T cells. In the absence of Wnt signals, Tak1 could be activated by overexpression of Tab1 as evidenced by increased abundance and by a mobility shift, indicative of phosphorylation (Fig. 6B,
Both involved in the generation of endoderm in Nlk. Mutational screens and RNAi have identified Wnt signaling of upstream inputs that activate MOM-4/LIT-1 or Tak1/Tab1 and TCF (33). We therefore analyzed the effect of Wnt1, Wnt5A did not result in increased abundance or a mobility shift of Tak1 and Tab1 with increasing amounts of Wnt5A. We then examined the effect of Wnt5A stimulation, like Wnt1 stimulation, resulted in the phosphorylation of TCF4. HEK293T cells were transfected with FLAG-TCF4 together with Wnt1 in the presence or absence of Tak1 or Wnt5A (Figs. 6D and 5A, lanes 1, 3, and 5). TCF4 was immunopurified, and an in vitro kinase assay was performed. Like co-expression of Tak1 and Tab1 (Fig. 5A, lane 4), expression of Wnt1 alone (Fig. 6D, lane 3) or in combination with Tak1 (Fig. 6D, lane 2) resulted in the phosphorylation of TCF4 (Figs. 5A and 6D). However, Wnt5A stimulation did not result in phosphorylation of TCF4 (Fig. 6D, lane 4).

In Colon Carcinoma Cell Lines, Wnt1 Inhibits TCF Transcriptional Activity Downstream of β-catenin and Independent of APC Function—Tak1/Nlk activation has been shown to down-regulate transcriptional activation mediated by β-catenin and TCF (33). We therefore analyzed the effect of Wnt1 stimulation on constitutive β-catenin/TCF transcriptional activity in colorectal cancer cells. In such cells, the Wnt receptors are essentially disconnected from the downstream canonical Wnt effectors β-catenin and TCF. The colon carcinoma cell line LS174T (which carries a β-catenin mutation) was co-transfected with luciferase reporter constructs containing either multimeric TCF-binding sites (TOPFLASH) or mutated TCF-binding sites (FOPFLASH) together with Wnt1. This resulted in −80% reduction of the TCF transcriptional activity (Fig. 7A), comparable with what has been published for Nlk (34) (Fig. 7, A and B) and Wnt5A (Fig. 6A). This observation was extended by co-transfecting TOPFLASH or FOPFLASH together with Wnt1 in the APC mutant cell lines SW480 and DLD1, which resulted in, respectively, 50 and 85% reduction in constitutive TCF transcriptional activity (Fig. 7B). We next examined whether the Wnt1-induced inhibition of TCF transcriptional activity was mediated through Tak1 and/or Nlk. Therefore, LS174T was co-transfected with TOPFLASH or FOPFLASH, Wnt1 and DN-Tak1 (K63W-Tak1), or DN-Nlk (K155M-Nlk) (Fig. 7C). Both mutants largely abolished the inhibition of TCF transcriptional activity that is induced by Wnt1. These results imply that Wnt1 inhibited TCF transcriptional activity in the colon carcinoma cell line LS174T via Tak1 and Nlk.

**DISCUSSION**

Genetic data from *C. elegans* provides clues to the identification of upstream inputs that activate MOM-4/LIT-1 or Tak1/Nlk. Mutational screens and RNAi have identified Wnt signaling components and proteins of the Tak1/Nlk pathway that are both involved in the generation of endoderm in *C. elegans* (3–8). We have explored whether Tak1 is directly activated by Wnt. In this study, we demonstrated that Wnt1 activates the MOM-4/Tak1 MAPKKK and, subsequently, the Nlk MAPK.

Wnt proteins have previously been shown to activate at least three different signaling cascades. First, the canonical Wnt pathway as described above has been extensively defined by biochemical and genetic studies in vertebrates and invertebrates (10, 11). Second, the planar polarity pathway is largely defined by genetic studies in invertebrates. The planar polarity pathway branches off from the canonical Wnt pathway at the level of Frizzled/Dishevelled to activate a Jun kinase pathway (39). A third Wnt-triggered pathway has predominantly been defined biochemically. Its prototype activator is Wnt5A, which together with Frizzled-2 mobilizes Ca^{2+} and activates protein kinase C (PKC) (40). Our study builds on existing genetic data in *C. elegans* showing that Wnt (the homologue of Mom2) activates Tak1/Nlk and provides biochemical evidence for a fourth Wnt signaling pathway, which we propose to term the Wnt-Tak1 pathway. A minimal outline of this pathway is given in Fig. 1B.

Wnt1 signaling stabilizes cytosolic β-catenin, which in turn forms a complex with one of the TCF transcription factors and thereby activates expression of specific target genes (15). The Tak1/Nlk kinase pathway ultimately leads to phosphorylation of TCFs and the down-regulation of TCF transcriptional activity. Wnt5A is considered a noncanonical Wnt because it does not signal by stabilizing β-catenin. Rather, Wnt5A has been suggested to antagonize canonical Wnt activity (20, 41). The mechanism whereby Wnt5A leads to inhibition of the canonical Wnt pathway might involve the stimulation of intracellular Ca^{2+} release and activation of protein kinase C and CaMKII (41). In contrast to this, it has been suggested that Wnt5A leads to degradation of β-catenin, which is dependent on APC and does not require CaMKII (20). First, we examined the activation of the Tak1/Nlk kinase pathway by the inhibitory Wnt, Wnt5A. In contrast to the study of Ishitani et al. (41), we find that Wnt5A does not activate Tak1 or induce phosphorylation of TCF4. Rather, our data support the notion that Wnt5A acts through degradation of β-catenin and independent of the Tak1/Nlk signaling pathway, as proposed by Topol et al. (20). In colorectal cancer cells with constitutively active TCF-β-catenin complexes, we find that Wnt1 signaling results in decreased TCF transcriptional activity. We propose that Wnt1 can also exert an inhibitory effect on the canonical Wnt cascade. Because this inhibitory activity of Wnt1 does not require APC, it is likely that it functions in a different way than Wnt5A. Up-regulation of Wnt1 may prevent tumor formation in cells that have both stabilized β-catenin or mutated APC. Like the inhibitory function of Wnt1, Golan et al. (48) recently showed that human Frizzled 6 acts as a negative regulator of the canonical TCF/β-catenin pathway by activation of the Tak1/Nlk pathway.

Tak1 activation requires the adaptor Tab1, yet the direct connection between Frizzled and Tak1/Tab1 remains unresolved. In a different experimental setting, Tak1 activation has been shown to involve ubiquitination of an unknown component that is complexed with Tak1, Tab1, and a second adaptor, called Tab2 (27). The exact biochemical requirements and the molecular context in which occupation of Frizzled receptors by Wnt1 leads to Tak1 activation remains to be further explored. The co-receptors for Frizzled, LRP5 and LRP6, do not play a role in the Tak1/Nlk pathway (data not shown).

Our data imply that Tak1 participates in more than one signaling pathway. Likewise, components of the canonical Wnt pathway, notably GSK3β and β-TrCP, play key roles in other pathways, *i.e.* the insulin and NFκB signaling pathways, respectively. Specificity of individual pathways that utilize common components may be accomplished by the creation of separate pools of such components. Thus, a pool of GSK3β that is complexed to APC and axin is dedicated to the canonical Wnt pathway and may not participate in insulin signal transduction. In the IL1 signaling pathway, Tak1 activation by polyubiquitination results in the phosphorylation of IkB kinase and MKK6, leading to activation of NFκB and c-Jun N-terminal
kinase-p38 kinase pathways, respectively. In the Wnt1 signaling pathway, a pool of Tak1 that is complexed to Tab1 and several yet unidentified proteins might be dedicated to the Wnt pathway, finally leading to the activation of Nlk, and may not participate in the activation of NFκB or c-Jun N-terminal kinase-p38 kinase pathways.

It is interesting that two opposing Wnt-activated signal transduction cascades would converge on the same transcriptional regulator, TCF. TCF performs a dual transcriptional regulatory function in worms, flies, and vertebrates (49). In the absence of canonical Wnt signaling, TCF is complexed with transcriptional corepressors such as Groucho and as a consequence actively represses TCF target gene transcription. Canonical Wnt signaling induces the formation of β-catenin-TCF complexes, which turns TCF into a transcriptional activator. Our results suggest an additional mechanism of silencing the transcriptional activity of TCF. Here, we show that Wnt1 might play an important role in modulating the Wnt signaling

Fig. 7. Effects of Wnt1 expression on TCF-dependent reporter gene activity in colon carcinoma cell lines. A, LS174T cells were transfected with luciferase reporter plasmid (200 ng), CMV-RNL plasmid (20 ng), Nlk-FLAG (300 or 500 ng), or pcDNA3-Wnt1 (90 ng). After 48 h of incubation, cells were harvested and luciferase activity was measured. Each transfection was performed in duplicate in at least three independent experiments. B, SW480 and DLD1 cells were transfected with the amounts of plasmids as in panel A. Cells were harvested, and luciferase activity was measured. C, LS174T cells were transfected with luciferase reporter plasmids. Effects of Wnt1 expression on TCF-dependent reporter gene activity in colon carcinoma cell lines are shown.
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strength, the time span of activation, and/or the place of activation. The role of Wnt1 early in development will be the determination of the development of a specific region of the central nervous system. To find an additional, inhibitory role of Wnt1 in development it will be informative to study ablation of Wnt1 later in development. Because the activity of Wnt1 can be both activating or inhibitory, it is likely that tumor formation does not arise by mutations in the Wnt1 gene and no tumors are found with this mutation.

In worms, the Wnt-Tak1 cascade, when activated, removes POP-1/TCF from the nucleus (7, 8), possibly by inducing its phosphorylation by Tak1, Tab1, and Mdl2 (49). We thank K. Matsumoto for Tak1, Tab1, and Mdl2, members of the Clevers laboratory for helpful discussions. We thank K. Matsumoto for Tak1, Tab1, and Mdl2, members of the Clevers laboratory for helpful discussions.
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