

Cell autonomous regulation of multiple Dishevelled-dependent pathways by mammalian Nkd

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Genetic studies have identified *Drosophila* Naked Cuticle (Nkd) as an antagonist of the canonical Wnt/ β -catenin signaling pathway, but its mechanism of action remains obscure [Zeng, W., Wharton, K. A., Jr., Mack, J. A., Wang, K., Gadbaw, M., et al. (2000) *Nature (London)* 403, 789–795]. Here we have cloned a cDNA encoding a mammalian homolog of *Drosophila* Nkd, mNkd, and demonstrated that mNkd interacts directly with Dishevelled. Dishevelled is an intracellular mediator of both the canonical Wnt pathway and planar cell polarity (PCP) pathway. Activation of the c-Jun-N-terminal kinase has been implicated in the PCP pathway. We showed that mNkd acts in a cell-autonomous manner not only to inhibit the canonical Wnt pathway but also to stimulate c-Jun-N-terminal kinase activity. Expression of mNkd disrupted convergent extension in *Xenopus*, consistent with a role for mNkd in the PCP pathway. These data suggest that mNkd may act as a switch to direct Dishevelled activity toward the PCP pathway, and away from the canonical Wnt pathway.

Dishevelled, an intracellular modular protein, is required for the activation of several Wnt signaling pathways, including the canonical Wnt/ β -catenin pathway and the planar cell polarity (PCP) pathway (1). These pathways are activated by a variety of Wnt ligands and receptors, and they signal downstream to at least two different cascades (1–3). One pathway, the canonical Wnt pathway, is mediated by GSK-3 β and β -catenin and controls normal cell growth and cell-fate specification. Aberrant regulation of this pathway causes diverse developmental defects in organisms ranging from *Drosophila* to mammals. Notably, aberrant regulation of the pathway has also been found in a variety of cancer types, implicating the involvement of this pathway in tumorigenesis (4–7). Another Wnt signaling pathway, the PCP pathway, signals via small GTPases and activates c-Jun-N-terminal kinase (JNK) to control epithelial cell polarity in *Drosophila* (8, 9). Recently, the PCP pathway has also been shown to regulate cell polarity during convergent extension movements in developing *Xenopus* and zebrafish embryos (10–13).

Dishevelled is at the branchpoint of these pathways, and different conserved domains within Dishevelled are required for the specific activation of each pathway (10–12, 14–16). To understand how the Dishevelled protein works as a common component in multiple pathways, we screened for mouse Dishevelled (mDvl)-associated proteins in a yeast two-hybrid assay. Among those identified in this assay is a mouse homolog of *Drosophila* Naked Cuticle (Nkd). This finding is particularly interesting because loss-of-function mutation of *nkd* produces a defect that mimics the phenotype caused by excess Wingless, and *nkd* has recently been shown to encode an antagonist of Wingless signaling (17). However, the mechanism by which Nkd functions is unclear. The genetic data in flies suggest that Nkd could function non-cell-autonomously by regulating Wingless production and/or Wingless binding to its receptor, or it could function cell-autonomously by regulating the intracellular cascade of Wingless signaling (17). Here we show that the mammalian Nkd (mNkd) interacts directly with Dishevelled and functions as an

intracellular, cell-autonomous regulator of multiple Dishevelled-mediated Wnt pathways.

Materials and Methods

Two-Hybrid Screen. Yeast two-hybrid screening was carried out by using the complete coding region of mouse Dishevelled 3 (mDvl3) as a bait. A prey cDNA library made from 9.5- to 10.5-day-old (E9.5–E10.5) mouse embryo (18) was screened in the presence of 10 mM 3-amino-1,2,4-triazole (3-AT, Sigma).

Northern Blotting. A mouse embryo RNA blot (CLONTECH) was hybridized with the radioactively labeled coding region of mNkd cDNA. A mouse multiple tissue RNA blot (CLONTECH) was hybridized with a radioactively labeled cDNA fragment of mNkd containing nucleotides 319–690, which corresponds to the Dishevelled-binding domain of mNkd isolated from the yeast two-hybrid screen.

Cloning of mNkd. Full-length mNkd cDNA was cloned by a combination of mouse fetal cDNA library screening, reverse transcription-PCR (RT-PCR), and rapid amplification of cDNA ends (RACE) methodologies. A fetal mouse cDNA library (OriGene Technologies) was screened by PCR using oligo pairs: 5'-CCTCCAAGAAGCAGCTCAAGTT-3' and 5'-TTGTGCTCTGCAGATCGGTATGG-3'. A Marathon-ready cDNA library from mouse lung (CLONTECH) was then used to obtain the 5' end sequence of mNkd cDNA by 5' RACE. The oligo sequence of mNkd cDNA used in the RACE was 5'-CCCCTCAGGAGCCACGGTGAGCTTCAC-3'.

Mammalian Cell Cultures and Luciferase Reporter Assay. HEK293 and Cos7 cells were grown in DMEM supplemented with 10% (vol/vol) FBS. NIH 3T3 cells were grown in DMEM supplemented with 10% (vol/vol) calf serum. Transfections were performed by using Lipofectamine Plus (Life Technologies, Grand Island, NY) according to the manufacturer's protocols. Luciferase reporter assays were carried out in 12-well plates as described (19). The LEF-1 luciferase reporter activity of each sample was determined and normalized by using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Immunoblots and Immunoprecipitations. Cell lysates were prepared with lysis buffer (150 mM NaCl/20 mM Tris-HCl, pH 7.5/1 mM

Abbreviations: PCP, planar cell polarity; JNK, c-Jun-N-terminal kinase; Nkd, *Drosophila* Naked Cuticle; RT-PCR, reverse transcription-PCR; GST, glutathione S-transferase; EF-hand, common helix-loop-helix calcium binding motif; mNkd, mammalian homolog of Nkd; Dsh, *Drosophila* Dishevelled; En, embryo *n* days old.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF343352).

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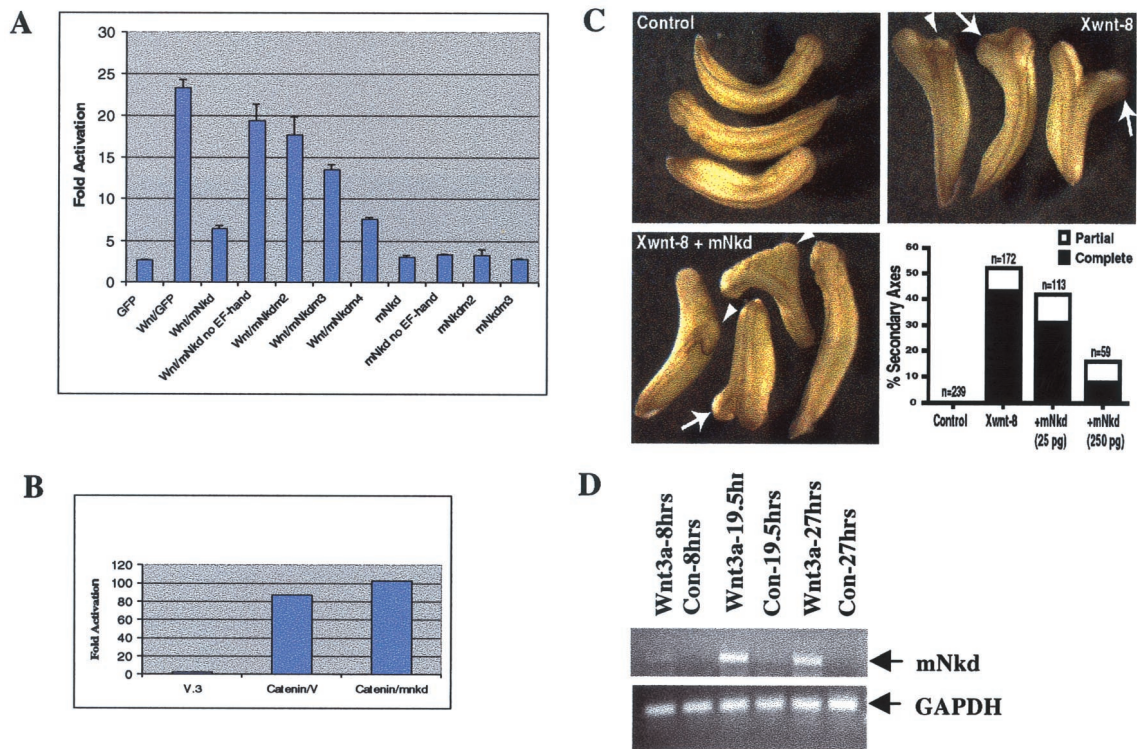


Fig. 4. mNkd inhibits the canonical Wnt pathway and mNkd-mRNA level is up-regulated by Wnt. (A) HEK293 cells were transfected with expression constructs that included 0.02 μ g of LEF-1, 0.2 μ g of luciferase reporter (25), 0.02 μ g of pRL-TK (Promega), 0.1 μ g of pCGWnt-1, and 0.2 μ g of GFP, mNkd, or mNkd-mutant derivatives. LEF-1 luciferase reporter activities were determined. (B) mNkd did not inhibit the β -catenin-activated LeF-1 reporter. HEK293 cells were transfected with expression constructs that included 0.02 μ g of LEF-1, 0.2 μ g of luciferase reporter (25), 0.02 μ g of pRL-TK (Promega), 0.1 μ g of β -catenin, and 0.2 μ g of vector or mNkd. (C) mNkd inhibited Xwnt-8-induced secondary axes formation in *Xenopus*. Control *Xenopus* embryos are shown at Upper Left. Embryos were injected ventrally with 5–10 pg of Xwnt-8 RNA and developed with secondary axes (Upper Right). Secondary axes were scored as complete (arrows) or partial (arrowheads) based on development of anterior structures such as eyes and cement glands. Coexpression of mNkd with Xwnt-8 decreased the frequency of secondary axes formation as well as the percentage of secondary axes that contained anterior structures (Lower Left and Lower Right). This effect was dose-dependent. (D) BALB/c L1 mouse liver epithelial cells were treated with either Wnt-3a or Neo-conditioned medium (34) for indicated hours. PCR was carried out by using primer pairs 5'-TGTTGACCATTCCCCACATCAA-3' and 5'-AAATGGGGTGTCAAGGAGGTGGAA-3'. The PCR products were separated by agarose gel electrophoresis. GAPDH, glyceraldehyde-3-phosphate control.

mNkd that is associated with mDvl in the yeast two-hybrid experiments and is highly conserved between the *Drosophila* Nkd and mNkd, the requirement of the EF-hand in the association with mDvl was investigated. Based on the crystal structure of the third EF-hand of the Recoverin protein (20), we made mutations that either changed the consensus residues in the calcium-binding loop or deleted the entire calcium-binding loop together with the surrounding amino acids (Fig. 3C). These mNkd mutants were expressed in HEK293 or Cos7 cells. Coimmunoprecipitation experiments revealed that none of these mutations significantly impaired the ability of these mNkd proteins to associate with mDvl (Fig. 3A and B). These data showed that mNkd associates with mDvl in mammalian cells and that the intact EF-hand is not required for the association.

We also identified the domain on Dishevelled that associated with mNkd. Dishevelled is a highly conserved protein and contains three distinct domains (1). The N-terminal region has a DIX domain that is required for canonical Wnt signaling. The middle region of Dishevelled contains a PDZ domain that is known to bind GBP/Frat1 (21, 22) and CK1 ϵ (23, 24), both positive regulators of the canonical Wnt pathway. The C-terminal region contains a DEP domain that is crucial for regulating the PCP pathway. Fragments corresponding to different regions of *Drosophila* Dishevelled (Dsh; Fig. 3DI) were expressed in *E. coli* as GST-fusion proteins. Equal amounts of each fragment (Fig. 3DII) were mixed with *in vitro*-translated mNkd in the binding buffer, precipitated with glutathione beads

and separated by SDS/PAGE. mNkd associated with the DM fragment of Dsh that encompasses the PDZ domain with the adjoining N-terminal basic amino acid stretch. Notably, the PDZ domain alone is not sufficient for the association. Also, there is no association of mNkd with the DIX domain in the N-terminal region or the DEP domain in the C-terminal region of Dsh (Fig. 3DIII). Thus mNkd is associated with a region within Dsh shared with GBP/Frat 1 and CK1 ϵ .

mNkd Antagonizes Canonical Wnt Signaling. Because Dishevelled is a known positive regulator of the canonical Wnt pathway and mNkd is found here to be directly associated with Dishevelled, the role of mNkd in the canonical Wnt pathway was tested in mammalian cell culture by using a Wnt-1 ligand-responsive luciferase reporter assay (25). In multiple experiments, activation of the reporter by Wnt-1 was inhibited 75% by coexpression of mNkd in mammalian cells (Fig. 4A). Expression of wild-type mNkd, in the absence of Wnt-1, had no effect on the activity of the reporter. These data suggest that mNkd negatively regulates the canonical Wnt pathway and are consistent with the inhibitory effect that *Drosophila* Nkd has on Wingless signaling in genetic studies (17). Interestingly, the EF-hand mutants of mNkd showed an impaired ability to inhibit the canonical Wnt pathway (Fig. 4A), although these mutants were all capable of binding to Dishevelled (Fig. 3A and B). These data suggest that the association of mNkd with Dishevelled alone is not sufficient to inhibit the canonical Wnt pathway, and that the intact EF-hand

is required for the inhibitory function. Importantly, mNkd failed to inhibit the gene response elicited by overexpression of β -catenin, a result that placed mNkd upstream of β -catenin in the canonical Wnt pathway (Fig. 4B).

We also used the induction of secondary axes by ectopic expression of Xwnt-8 in *Xenopus* embryo as an assay for the role of mNkd in the canonical Wnt pathway *in vivo* (26–28). Ventral blastomere injection of 5–10 pg of Xwnt-8 RNA induced secondary axes in over 50% of the embryos; most of these secondary axes contained anterior structures (Fig. 4C). Coinjection of 25 pg of mNkd RNA suppressed the effect of Xwnt-8 and resulted in fewer secondary axes (Fig. 4C). Coinjection of higher doses of mNkd (250 pg) resulted in even fewer secondary axes (Fig. 4C), only half of which contained anterior structures, indicating that mNkd inhibited the canonical Wnt pathway *in vivo*. The promoter of the *Xenopus* nodal-related-3 (*Xnr-3*) gene has been shown to be directly activated by the canonical Wnt/ β -catenin signaling in the *Xenopus* embryo (29), and expression of Xwnt-8 RNA into *Xenopus* animal caps activated transcription from a coinjected *Xnr-3*-luciferase reporter plasmid *in vivo* (data not shown). Consistent with the results from mammalian cell culture (Fig. 4A) and the secondary axis assay (Fig. 4C), coinjection of mNkd RNA suppressed Xwnt-8 activation of the *Xnr-3* promoter (data not shown). These data indicate that mNkd is an inhibitor of the canonical Wnt/ β -catenin pathway both *in vitro* and *in vivo*. Together, our findings that mNkd interacted directly with Dishevelled and inhibited the canonical Wnt pathway upstream of β -catenin suggest that mNkd is an intracellular antagonist of the Wnt pathway.

mNkd mRNA Is Induced by Wnt Ligand in Mammalian Cells. Recently, a number of genes have been identified that are induced by Wnt expression in mammalian cells (30–33). We also tested whether mNkd-mRNA levels changed when cells were treated with Wnt expression in mammalian cells (30–33). We also tested whether mNkd-mRNA levels changed when cells were treated with Wnt ligands. BALB/c LI mouse liver epithelial cells were treated with Wnt-3A-conditioned medium or control medium (34) for 8 h, 19.5 h, or 27 h, respectively. Levels of mNkd transcripts increased significantly in cells treated with Wnt-3a-conditioned medium for 19.5 h and 27 h compared with control treatments (Fig. 4D). Levels of the glyceraldehyde-3-phosphate dehydrogenase transcript remained constant in all conditions (Fig. 4D). The results of quantitative PCR recorded in the linear range using a LightCycler corroborated the PCR data (data not shown). Wnt-3a-conditioned medium also caused an increase in mRNA levels of mNkd in L cells (data not shown). The mRNA levels of mNkd also increased in BALB/c LI mouse liver epithelial cells treated with lithium chloride (data not shown), a known inhibitor of GSK-3 (35). Thus, the ability of mNkd to inhibit the intracellular signaling of the canonical Wnt pathway, in conjunction with the result that mNkd is itself a downstream target of the canonical Wnt signaling, suggests that mNkd is an intracellular cell-autonomous negative-feedback regulator of the canonical Wnt pathway.

mNkd Stimulates JNK Activity. Genetic and biochemical studies have shown that Dishevelled controls cell polarity by acting as an upstream activator of the JNK pathway both *in vivo* and *in vitro* (15). Because mNkd is directly associated with Dishevelled, we tested whether mNkd participated in the JNK pathway. NIH 3T3 cells were transfected with expression constructs of mNkd and c-Jun, in which c-Jun served to monitor JNK activities. In this assay, expression of mNkd or mDvl alone induced a strong phosphorylation of c-Jun which was detected by blotting with an antibody specific for phosphoserine-63 (Fig. 5A). These data showed that mNkd had an effect similar to Dishevelled in activating the JNK pathway in mammalian cell culture assays.

mNkd Expression Inhibits Convergent Extension. Activation of JNK seems to be an important step in the PCP pathway (8, 15), and

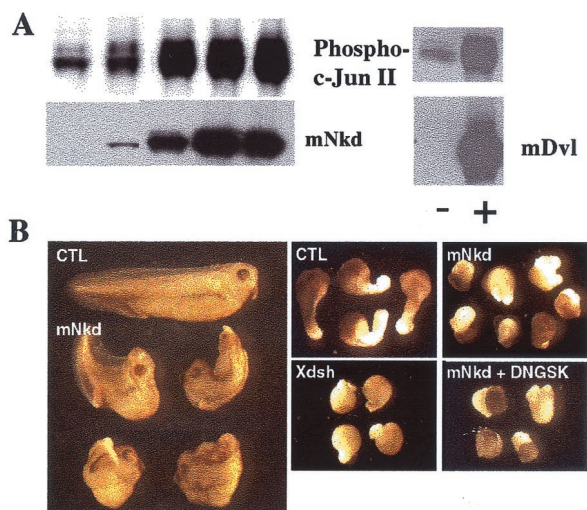


Fig. 5. mNkd activates JNK in mammalian cell culture and alters convergent extension movements in *Xenopus laevis*. (A) The JNK assay was carried out as described (15). NIH 3T3 cells were transfected with plasmids expressing c-Jun and mNkd or c-Jun and mDvl3. Cell lysates were separated by SDS/PAGE and transferred onto a nitrocellulose membrane. The membrane was blotted with PhosphoPlus c-Jun (Ser-63) II antibody (New England Biolabs). The same membrane was then stripped and blotted with Xpress antibody (Invitrogen) to detect the amount of Xpress-tagged mNkd or blotted with Myc antibody to detect Dishevelled. (B) Dorsal expression of mNkd in developing *Xenopus* embryos inhibited the normal elongation and straightening of the anteroposterior axis (Left; control embryo at top, mNkd-injected embryos below). (Center Upper) Control open-face Keller explants of the dorsal marginal zone elongated and changed shape significantly. (Right Upper) Explants expressing mNkd failed to elongate or to change shape. (Center Lower) Overexpression of wild-type Xdsh mimicked overexpression of mNkd. (Right Lower) Downstream activation of the canonical Wnt pathway by coexpression of dominant-negative GSK-3 β did not attenuate the inhibitory effect of mNkd on convergent extension. Expression of dominant-negative GSK-3 β alone had no effect on convergent extension (data not shown).

it has been demonstrated recently that a vertebrate cognate of the *Drosophila* PCP pathway (8) controls convergent extension movements during vertebrate development (10–13). In both *Xenopus* and *Drosophila*, hyperactivation of this pathway disrupts PCP signaling without affecting the canonical Wnt pathway (12–15). Consistent with its ability to activate JNK *in vitro* (Fig. 5A), mNkd overexpression inhibited the normal elongation of *Xenopus* embryos (Fig. 5B, Left). The normal formation of anterior structures in these embryos indicates that the phenotype is not the result of ventralization, suggesting that mNkd inhibits convergent extension. To assess more directly the effects of mNkd on convergent extension, we examined open-face Keller explants of the dorsal mesoderm (12). Such explants made from control embryos elongated and changed shape significantly (Fig. 5B, Center Upper), whereas explants made from embryos expressing mNkd failed to elongate (Fig. 5B, Right Upper). These effects are similar to those elicited by overexpression of other wild-type components of the planar cell polarity cascade, such as Xdsh (Fig. 5B, Center Lower; ref. 12) and Xfz-8 (13), indicating a role of mNkd in controlling the PCP pathway.

Because mNkd is an inhibitor of the canonical Wnt pathway (Fig. 4A), it was important to test whether the effects of mNkd on convergent extension were simply a consequence of the effects of mNkd on the canonical Wnt pathway. Expression of dominant-negative GSK-3 β , a strong activator of the canonical Wnt pathway in *Xenopus* (36, 37), did not attenuate the inhibitory effects of mNkd on convergent extension (Fig. 5B, Right Lower). Taken together, these data suggest that mNkd inhibits convergent extension by overstimulating the PCP-signaling cas-

cade, and that this effect is independent of its inhibitory role on the canonical Wnt pathway.

Discussion

Previous genetic studies identified *Drosophila* Nkd as an important negative regulator of Wnt signaling. However, the mechanism by which Nkd effects this regulation has not been elucidated. In this study, we showed that mNkd, a mammalian homolog of Nkd, also negatively regulates canonical Wnt signaling. Furthermore, we have demonstrated that mNkd associates with Dishevelled directly and functions as an intracellular cell-autonomous Wnt-inducible negative regulator of the canonical Wnt pathway at a step upstream of β -catenin. Our data also revealed the importance of the intact EF-hand calcium-binding domain in regulating Wnt signaling, a result that is particularly intriguing in light of other studies that reported stimulation of intracellular calcium release by Wnt ligands (38–40). In addition, we have shown here that mNkd not only suppresses the canonical Wnt pathway but also activates JNK, a component of the PCP pathway. Furthermore, mNkd expression inhibits con-

vergent extension in *Xenopus* in a manner similar to overexpression of other PCP pathway components.

Because mNkd binds to Dishevelled and Dishevelled is a known regulator of both the canonical Wnt pathway and the PCP pathway (1), the data described here suggest the possibility that mNkd could act as a switch that directs Dishevelled toward the JNK/PCP pathway and away from the canonical Wnt/ β -catenin pathway. Finally, as an intracellular negative regulator of the canonical Wnt pathway, mNkd shares properties with the tumor suppressors Axin and APC.

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