RanBP3 enhances nuclear export of active β-catenin independently of CRM1

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β-Catenin is the nuclear effector of the Wnt signaling cascade. The mechanism by which nuclear activity of β-catenin is regulated is not well defined. Therefore, we used the nuclear marker RanGTP to screen for novel nuclear β-catenin binding proteins. We identified a cofactor of chromosome region maintenance 1 (CRM1)–mediated nuclear export, Ran binding protein 3 (RanBP3), as a novel β-catenin–interacting protein that binds directly to β-catenin in a RanGTP-stimulated manner. RanBP3 inhibits β-catenin–mediated transcriptional activation in both Wnt1- and β-catenin–stimulated human cells. In *Xenopus laevis* embryos, RanBP3 interferes with β-catenin–induced dorsoventral axis formation. Furthermore, RanBP3 depletion stimulates the Wnt pathway in both human cells and *Drosophila melanogaster* embryos. In human cells, this is accompanied by an increase of dephosphorylated β-catenin in the nucleus. Conversely, overexpression of RanBP3 leads to a shift of active β-catenin toward the cytoplasm. Modulation of β-catenin activity and localization by RanBP3 is independent of adenomatous polyposis coli protein and CRM1. We conclude that RanBP3 is a direct export enhancer for β-catenin, independent of its role as a CRM1-associated nuclear export cofactor.

Introduction

The Wnt signaling pathway regulates a variety of processes during homeostasis and development, including cellular proliferation, cell fate decision, axis formation, and organ development (Nusse, 1999). Deregulation of the pathway is implicated in many human cancers (Polakis, 2000). The key effector protein of the Wnt pathway is the transcriptional activator β-catenin. Cytoplasmic β-catenin is efficiently trapped in a multiprotein complex containing adenomatous polyposis coli (APC; Groden et al., 1991; Kinzler et al., 1991), Axin (Zeng et al., 1997; Behrens et al., 1998), and glycogen synthase kinase 3β (GSK3β; He et al., 1995). In the absence of a Wnt signal, this complex rapidly phosphorylates β-catenin, targeting it for degradation (Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Sakanaka et al., 1998). Wnt binding to the Frizzled/LRP (low-density lipoprotein receptor–related protein) receptors results in inhibition of the APC–Axin–GSK3β complex by activation of Dishevelled (Boutros and Mlodzik, 1999; Wharton, 2003) and by recruitment of Axin to the plasma membrane by LRP (Mao et al., 2001; Tolwinski et al., 2003). This results in an increase in nonphosphorylated β-catenin that forms active transcriptional complexes in the nucleus with T cell factor (TCF)/lymphocyte enhancer binding factor (LEF) transcription factors (Behrens et al., 1996; Molenaar et al., 1996; Staal et al., 2002).

Nuclear activity of β-catenin is regulated by several mechanisms. In the absence of a Wnt signal, TCF proteins occupy and repress promoters of their target genes by recruiting repressor proteins like Groucho, CtBP (COOH-terminal binding protein), and histone deacetylases (Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998; Waltzer and Bienz, 1998; Brannon et al., 1999; Chen et al., 1999). Interaction of β-catenin with TCF/LEF transcription factors results in activation of these genes. BCL-9/Legless and Pygopus have been shown to be essential components of the β-catenin–TCF transcription complexes (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). β-Catenin also interacts with chromatin remodeling and histone modification proteins such as Brg1 (Brahma-related gene 1) and CBP (CREB binding protein)/p300 to promote target gene activation (Hecht and Kemler,

2000; Takemaru and Moon, 2000; Barker et al., 2001). Furthermore, ICAT (inhibitor of β-catenin and TCF4) and Chibby are identified as nuclear proteins that repress Wnt signaling by competing with TCF for binding to β-catenin (Tago et al., 2000; Takemaru et al., 2003).

In this study, we aimed to identify new modulators of β-catenin in the nucleus. We used the nuclear marker RanGTP to select for nuclear factors that directly bind β-catenin and identified Ran binding protein 3 (RanBP3). We show that RanBP3 inhibits β-catenin–TCF4–mediated transactivation in human cell lines by relocalization of active β-catenin from the nucleus to the cytoplasm. In addition, we show that RanBP3 causes ventralization and inhibits β-catenin–induced double axis formation in *Xenopus laevis* embryos. Loss of *Drosophila melanogaster* RanBP3 results in cuticle defects and expands the Engrailed protein expression domain. We conclude that RanBP3 functions as a novel type of inhibitor of β-catenin and identify its gene as a candidate human tumor suppressor in the commonly deleted chromosomal region 19p13.3.

Results

RanBP3 interacts directly with β-catenin in a RanGTP-stimulated way

To study the interaction between β-catenin and nuclear transport factors, we used GST-tagged β-catenin to pull down interacting proteins from *X. laevis* egg extracts. Interacting proteins were initially analyzed by Western blot using mAb414, which recognizes a phenylalanine glycine (FG)–rich epitope present in multiple nucleoporins. FG repeat–containing nucleoporins Nup62, Nup153, and Nup358 were specifically bound by full-length β-catenin and by the central armadillo (ARM) repeat region (unpublished data). Interestingly, we found a strong interaction between β-catenin and two unknown proteins of ∼80 and 90 kD that were recognized by mAb414 (Fig. 1 A, lanes 3 and 4). These proteins interacted with full-length β-catenin and to a lesser extent with the ARM repeats (ARM 1–12). The mAb414 reactivity indicated that these two proteins contained FG repeats. Two isoforms of RanBP3 stood out as possible candidates for these two unknown proteins because they contain FG repeats and have the correct sizes. Indeed, recombinant human RanBP3-a comigrated with the p90 protein and was recognized by mAb414 (Fig. 1 A, lane 5). To confirm that RanBP3 was one of these new β-catenin–interacting proteins, we repeated the pull-down experiment using HeLa nuclear extracts and an mAb recognizing human RanBP3. The b isoform of RanBP3 was more abundant in HeLa nuclear extracts and copurified with GST-tagged full-length and the ARM repeats of β-catenin (Fig. 1 B). To mimic nuclear conditions, 2 μM of a nonhydrolysable mutant of the small GTPase Ran (RanQ69L-GTP) was added, resulting in increased interaction between β-catenin and RanBP3 (Fig. 1 B, lanes 2 and 4). In the presence of RanQ69L-GTP, the less abundant a isoform of human RanBP3 also bound to full-length β-catenin (Fig. 1 B, lane 2).

To investigate whether the binding between β-catenin and RanBP3 was direct, we performed pull-down assays with GST-tagged β-catenin and recombinant RanBP3. Human
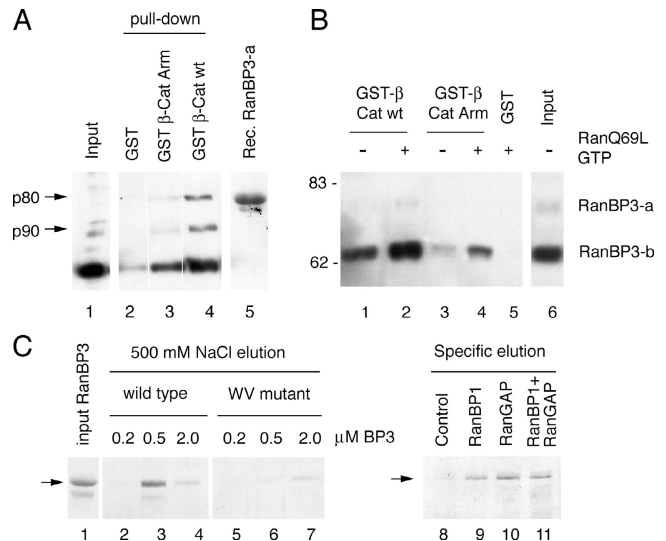

Figure 1. **Identification of RanBP3 as an interaction partner of β-catenin.** (A) Pull-down experiment using immobilized GST (lane 2), GST-tagged β-catenin ARM repeats 1–12 (lane 3), and full-length β-catenin (lane 4) incubated with *X. laevis* egg extract (input; lane 1). Bound proteins were analyzed by Western blot using mAb414 recognizing a subset of nucleoporins. Two unknown proteins, p80 and p90, are marked with arrows. (B) Identification of p80 and p90 as the b and a isoforms of RanBP3. Pull-down experiment as in A, incubated with HeLa nuclear extracts and analyzed using RanBP3 antibody. (C) RanBP3 binds directly to β-catenin. GST-tagged full-length β-catenin (lanes 2–11) was incubated with 2 μM RanGTP and 0.2 μM (lanes 2 and 5), 0.5 μM (lanes 3, 6, and 8–11), or 2.0 μM (lanes 4 and 7) wt (lanes 2–4 and 8–11) or wv mutant (lanes 5–7) RanBP3-b. Bound proteins were eluted as indicated above the lanes and visualized with silver (lanes 1–7) or Coomassie (lanes 8–11) staining.

RanBP3-b interacted directly with GST–β-catenin, with optimum binding at 0.5 μM RanBP3 (Fig. 1 C, lane 3). These binding characteristics resemble the interaction of RanBP3 with chromosome region maintenance 1 (CRM1), which shows optimal binding at 0.2 μM RanBP3 (Englmeier et al., 2001). Furthermore, we used a RanBP3 mutant that cannot bind to RanGTP because of a point mutation in its RanGTP binding domain (RanBP3 "wv" mutant; Englmeier et al., 2001). This mutant interacted only very weakly with β-catenin and lost its ability to bind at an optimum concentration (Fig. 1 C, lanes 5–7). These data suggest that RanGTP increases the affinity of RanBP3 for β-catenin. To confirm the RanGTP dependency, RanBP3 was bound to β-catenin columns at the optimal concentration of 0.5 μM in the presence of RanGTP and eluted in either the absence or presence of the recombinant Ran cofactors RanBP1 and RanGAP (Fig. 1 C, lanes 8–11). Although virtually no RanBP3 was eluted with buffer only, significant amounts were detected after elution in the presence of 0.5 μM RanBP1, 0.2 μM RanGAP, or a combination of these.

RanBP3 inhibits transcription of a TCF-responsive reporter

Wnt signaling ultimately results in the stabilization of β-catenin, which forms active transcriptional regulation complexes with transcription factors of the TCF/LEF family. A well-established functional readout of Wnt signaling makes use of TCF-responsive luciferase reporter constructs (Korinek et al., 1997).

To test the functional relevance of the interaction between β-catenin and RanBP3, we transfected human embryonic kidney (HEK) 293 cells with reporter constructs that contain either three optimal TCF binding sites (TCF optimal promoter [TOP]) or three mutated binding sites (fake optimal promoter [FOP]). Transfection of a Wnt1 plasmid resulted in a strong activation of the TOP reporter but not of the FOP control (Fig. 2 B). Cotransfection of increasing amounts of RanBP3 repressed Wnt1/β-catenin transactivation dose dependently (Fig. 2 B). A mutant of RanBP3 that cannot interact with RanGTP and binds β-catenin with less affinity (Fig. 1 C) was less active than wild-type (wt) RanBP3 (Fig. 2 B). To investigate whether RanBP3 inhibits Wnt signaling downstream or upstream of β-catenin, we mimicked Wnt signaling in HEK293 cells by expressing β-catenin. RanBP3 could still specifically inhibit activation of the TOP reporter (Fig. 2 C), whereas the RanBP3 wv mutant was less effective. These experiments show that RanBP3 inhibits TCF-dependent transcription by acting on either β-catenin itself or regulators downstream of β-catenin. We confirmed that the expression levels of our wt and wv mutant RanBP3 constructs were equal by analyzing cell lysates from transfected HEK293 cells on Western blot (Fig. 2 A).

The interaction of recombinant β-catenin with RanBP3 (Fig. 1 C) implies that RanBP3 can bind NH_2-terminally unphosphorylated β-catenin, which is thought to be the signaling-competent form of the protein. To determine whether this is the case in vivo, we used a β-catenin mutant that contains alanines in all four NH_2-terminal GSK3β phosphorylation sites (β-catenin$^{\Delta GSK3\beta}$; Barth et al., 1999) and therefore is constitutively active. This mutant stimulated expression of the TCF reporter to levels that were two to three times higher than wt β-catenin (unpublished data). Coexpression of wt RanBP3 leads to a significant reduction in transactivation by β-catenin$^{\Delta GSK3\beta}$ (Fig. 2 D). Again, the RanBP3 RanGTP-binding mutant was less able to repress β-catenin$^{\Delta GSK3\beta}$–mediated transactivation.

To address whether RanBP3 could also affect expression of endogenous target genes of β-catenin–TCF, we expressed RanBP3 in human colon carcinoma cell line HCT116. This cell line harbors an activating mutation in β-catenin (Δ45 catenin) and therefore expresses increased levels of the target gene *c-myc* (He et al., 1998). Expression of wt RanBP3 decreased c-Myc protein levels compared with control cells (Fig. 2 E, lanes 2 and 3). Although expressed in higher levels, the wv mutant RanBP3 was less capable of decreasing c-Myc levels.

Reduction of RanBP3 results in increased transactivation of a TCF-responsive reporter

In addition to studying the effects of RanBP3 overexpression, we studied the effects of RanBP3 depletion. We expressed short hairpin RNAs (shRNAs) directed against unique parts of RanBP3 that are present in all isoforms of RanBP3. We obtained several shRNA RanBP3 constructs that down-regulate RanBP3 protein levels in HEK293 cells (Fig. 3 A).

When we coexpressed Wnt1 and RanBP3 shRNAs, we observed significant increases in TCF/LEF reporter activity compared with the GFP–RNA interference (RNAi) control
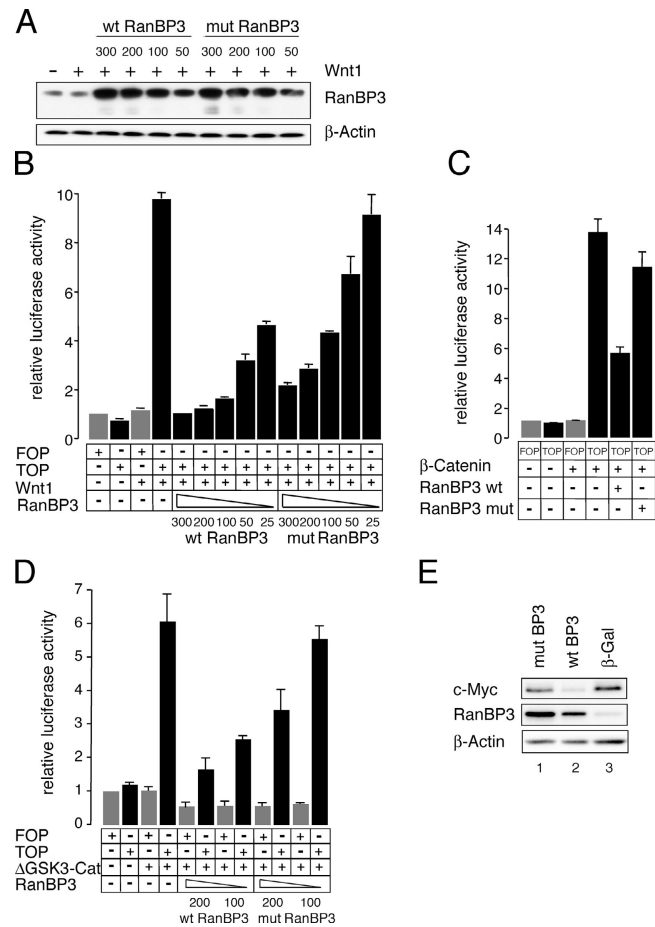
Figure 2. **Expression of RanBP3 inhibits β-catenin/TCF–mediated transcriptional activation.** (A) Wt and wv mutant RanBP3 are expressed at equal levels. HEK293 cells were transfected with the indicated constructs (ng), and lysates were analyzed 48 h after transfection by Western blot with the indicated antibodies. (B) RanBP3 represses Wnt1-induced β-catenin/TCF–mediated transcriptional activation dose dependently. HEK293 cells were transfected with TOP (black bars) or the control FOP (gray bars), Wnt1, and decreasing amounts of RanBP3 wt or wv mutant as indicated (ng), and luciferase activity was measured after 48 h. (C and D) RanBP3 represses transcriptional activation induced by wt β-catenin (C) or ΔGSK3–β-catenin (D). HEK293 cells were transfected with the indicated constructs, and luciferase activity was measured 48 h after transfection. In all experiments, normalized relative luciferase values are shown as corrected with pRL-CMV Renilla. Bars represent SEMs of independent experiments. (E) RanBP3 inhibits the expression of the endogenous Wnt target c-Myc. HCT116 colon carcinoma cells expressing Δ45–β-catenin were transfected with GFP and β-galactosidase, RanBP3 wt, or mutant plasmids. 2 d after transfection, GFP-positive cells were sorted using flow cytometry, lysed in sample buffer, and analyzed by Western blot using the indicated antibodies.

(Fig. 3 B). To determine whether RanBP3 depletion also acts on NH_2-terminally dephosphorylated β-catenin, we cotransfected β-catenin$^{\Delta GSK3\beta}$ with anti–RanBP3 shRNA expression constructs (Fig. 3 C). Reduction of RanBP3 increased reporter activity, confirming that RanBP3 can act on the NH_2-terminally dephosphorylated or "activated" form of β-catenin. In the absence of Wnt signaling, depletion of RanBP3 did not result in increased reporter activity (Fig. 3 D), arguing for a specific effect on β-catenin. The direct binding of RanBP3 to β-catenin that we observed (Fig. 1 C) indicated that RanBP3 may act on

Figure 3. **Reduction of RanBP3 by RNAi results in increased β-catenin/TCF–mediated transcription activation.** (A) Western blot showing that different shRNAs against RanBP3 reduce RanBP3 protein levels in HEK293 cells. Cells were transfected with shRNAs, and pHA262-PUR was cotransfected to introduce puromycin resistance. 24 h after transfection, cells were grown on puromycin medium for 48 h and cell lysates were prepared and analyzed on Western blot with the indicated antibodies. (B) RNAi against RanBP3 increases Wnt1-induced β-catenin/TCF–mediated transcription. HEK293 cells were transfected with the indicated constructs, and activity of TOP (black bars) and FOP (gray bars) was measured 72 h after transfection. Error bars represent SDs of technical replicates of a representative experiment. (C) RNAi against RanBP3 increases β-catenin/TCF–driven transcription in HEK293 cells that transiently express an active form of β-catenin (ΔGSK3–β-catenin). Cells were transfected with indicated constructs, and luciferase activity was measured after 72 h. (D) Coexpression of CRM1 with RanBP3 shRNA constructs does not affect β-catenin/TCF–mediated transcription in Wnt1-transfected cells. HEK293 cells were transfected with the indicated constructs, and 72 h after transfection luciferase activity was measured. For all experiments, relative luciferase levels are shown as corrected with CMV-Renilla-luc. Error bars in C and D represent SEMs of independent experiments.
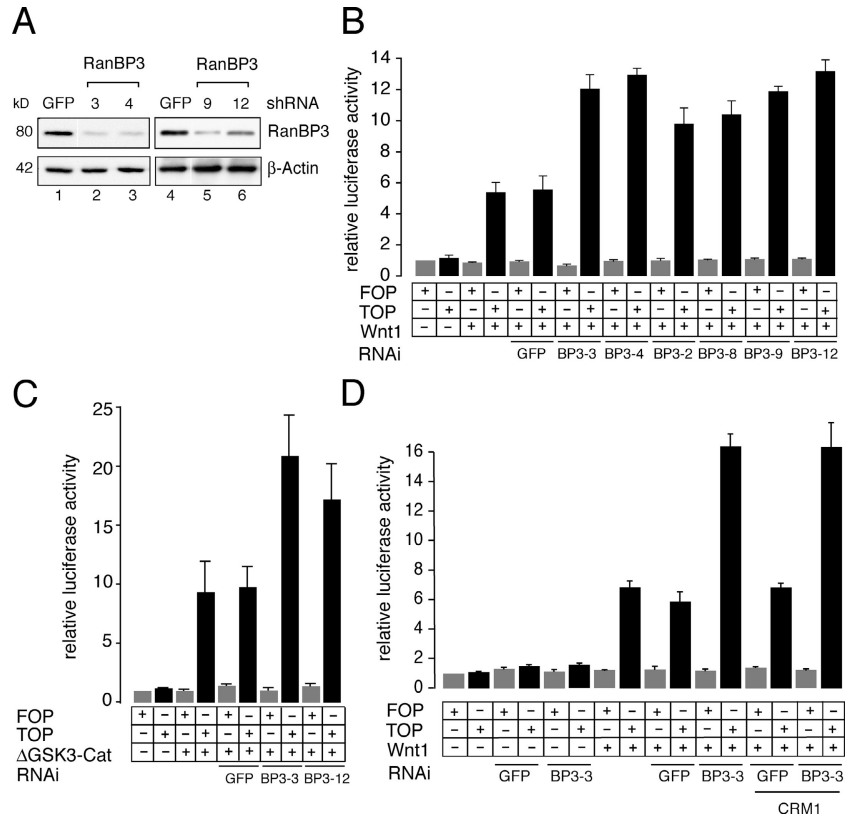
the Wnt signaling pathway independently of CRM1, which has been reported to play a role in β-catenin nuclear export via interaction with APC (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). Increased expression of CRM1 is able to compensate for reduction of CRM1 nuclear export at reduced RanBP3 levels (Taura et al., 1998; Noguchi et al., 1999). Therefore, we expressed CRM1 in combination with Wnt1 and RanBP3 shRNAs. As shown in Fig. 3 D, CRM1 overexpression did not reverse the effects of RanBP3 depletion, indicating that the mechanism by which Wnt signaling is modulated by RanBP3 is independent of CRM1-mediated nuclear export of β-catenin.

RanBP3 down-regulates β-catenin–mediated transactivation independently of APC

To further address the question of whether RanBP3 represses β-catenin transcriptional activation by stimulating export of β-catenin via the APC–CRM1 pathway, we expressed RanBP3 in human colorectal cancer cell lines that express COOH-terminal truncations of APC. First, we tested DLD1 cells, which express APC[1–1417], which retains some β-catenin binding sites but lacks all COOH-terminal nuclear export signals (NESs), the most highly conserved APC NESs in evolution. As shown in Fig. 4 A, β-catenin/TCF activity is already high in these cells. Expression of a RanBP3 wt or wv mutant could still dose-dependently down-regulate transcriptional activity, with the mutant again being a less potent inhibitor (Fig. 4 A). Because APC in DLD1 cells can still bind to β-catenin and NESs have also been reported in the NH₂ terminus of APC, we repeated the experi-

ment in COLO320 cells. These cells express a very short APC truncation (1–811) that lacks all β-catenin binding sites. β-catenin/TCF activity was much higher in these cells than in DLD1 cells, a finding that correlates with the severity of the APC mutation (Fig. 4 B; Rosin-Arbesfeld et al., 2003). Nevertheless, transfection of the RanBP3 expression constructs caused a significant down-regulation of transcription (Fig. 4 B). Therefore, the mechanism by which RanBP3 inhibits β-catenin is independent of a nuclear export function of APC.

RanBP3 influences subcellular localization of active β-catenin

To study the mechanism by which RanBP3 inhibits Wnt signaling, we tested the possibility that RanBP3 influences the stability of β-catenin. We transfected HEK293 cells with or without Wnt1 in combination with shRNA constructs. Total β-catenin levels were virtually unchanged after expression of Wnt1 alone or in combination with shRNA against RanBP3 (Fig. 5 A). When the same blot was probed with anti–active β-catenin, recognizing NH₂-terminally dephosphorylated β-catenin, we observed an increase in Wnt1-transfected cells but no effects on RanBP3 (Fig. 5 A). From this data, we conclude that RanBP3 depletion does not affect β-catenin degradation.

We next prepared nuclear and cytoplasmic extracts from HEK293 cells transfected with or without Wnt1 and RNAi against GFP or RanBP3. Total β-catenin was mostly detected in the cytosol fraction (Fig. 5 B). No change in total β-catenin levels was observed in the nucleus or cytoplasm after transfection with Wnt1 (Fig. 5 B, lane 2) or treatment with RNAi (lanes 3 and 4). When we stained for active β-catenin, a clear increase
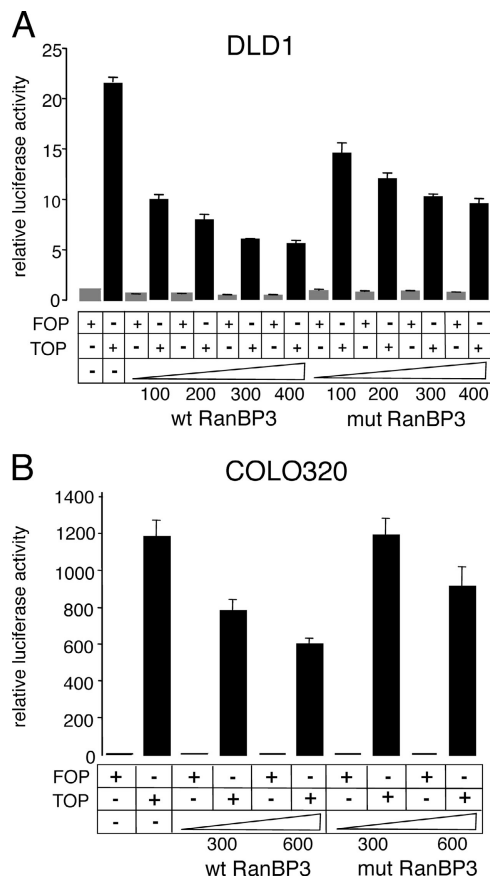
A

DLD1

B

COLO320

Figure 4. **RanBP3 antagonizes Wnt/β-catenin transactivation in APC-mutated colon carcinoma cells.** Luciferase assay showing that RanBP3 inhibits β-catenin–mediated transactivation in colon carcinoma cell lines DLD1 and COLO320. (A) APC type I truncated human colon carcinoma cell line DLD1 (APC 1–1417) was transfected with luciferase reporter constructs and increasing amounts of RanBP3 expression constructs as indicated. DLD1 cells express a truncated APC protein that lacks all its COOH-terminal NESs. (B) Luciferase reporter assay as in A, performed in the APC type I truncated human colon carcinoma cell line COLO320 (APC 1–811). These cells express a short APC protein that lacks all β-catenin binding and regulatory sites. Relative luciferase activity was measured 48 h after transfection. Error bars show SDs of a representative experiment.

A

Whole cell extracts

B

Nuclear extracts

Cytoplasmic extracts

Figure 5. **Depletion of RanBP3 results in nuclear accumulation of active β-catenin.** (A) Depletion of RanBP3 does not alter the levels of both total and active dephosphorylated β-catenin. HEK293 cells were transfected with or without Wnt1 and shRNA constructs against GFP or RanBP3. 72 h after transfection, whole cell lysates were analyzed by Western blot with the indicated antibodies. (B) RNAi against RanBP3 results in increased levels of active β-catenin in the nucleus. HEK293 cells were transfected with the indicated constructs, and 72 h after transfection, nuclear and cytoplasmic extracts were prepared and analyzed by Western blot. TCF4 and tubulin staining are shown as markers for purity of the nuclear and cytoplasmic fractions. As a loading control in the nuclear fractions, TCF4 and a nonspecific reaction of the antibody recognizing active β-catenin are shown.

was evident after stimulation with Wnt1 (Fig. 5 B, lane 2). Interestingly, when cells were transfected with RNAi against RanBP3, active β-catenin significantly increased in the nuclear fraction and decreased in the cytosolic fraction (Fig. 5 B, lane 4), suggesting that RanBP3 relocates active β-catenin from the nucleus to the cytoplasm. As controls for fractionation, TCF4 was used as a nuclear marker and α-tubulin as a cytoplasmic marker. Both proteins were strongly enriched in the proper compartments.

Nuclear/cytoplasmic fractionation data does not always reflect the subcellular localization in living cells because pools of proteins that are not tightly bound to nuclear or cytoplasmic structures and are relatively small may leak through nuclear pore complexes of permeabilized cells. We therefore assayed the effect of RanBP3 overexpression on active β-catenin in situ using the anti–active β-catenin antibody. In our hands, this antibody did not visualize endogenous dephosphorylated β-catenin in Wnt1-transfected HEK293 cells (unpublished data). We therefore tested two colon carcinoma cell lines (SW480 and
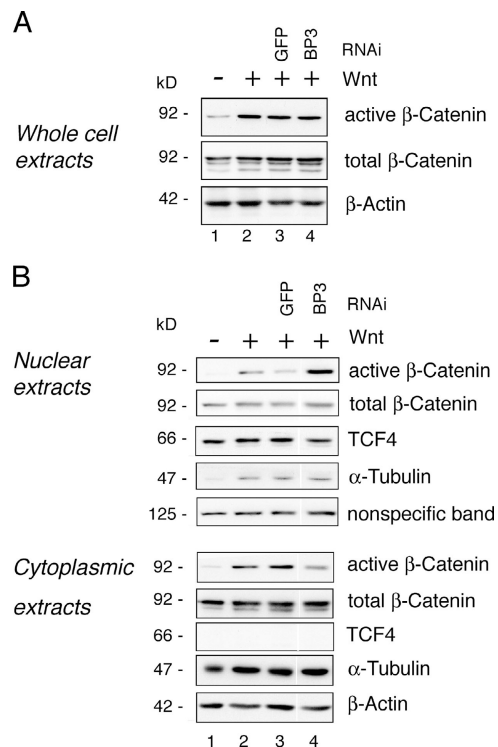
DLD1) that have a constitutively activated β-catenin because of a mutation in APC (Rosin-Arbesfeld et al., 2003). In SW480, but not in DLD1, the anti–dephosphorylated β-catenin antibody recognizes a clear nuclear signal above background (Fig. 6, A and C). The presence of this signal correlates with the exceptionally high β-catenin activity as measured in luciferase assays (Fig. 6 D), i.e., ∼30-fold higher than in DLD1. Importantly, RanBP3 overexpression leads to a clear reduction of active β-catenin signal from the SW480 nuclei (Fig. 6 A) but has no influence on total β-catenin localization (Fig. 6 B). This indicates that, even in the extremely active SW480 cell line, only a very small proportion of total β-catenin is properly dephosphorylated and active, and that this is the pool RanBP3 acts on.

RanBP3 enhances nuclear export of active β-catenin independently of CRM1

Reduction of active nuclear β-catenin by RanBP3 in SW480 cells was not accompanied by an increase in cytoplasmic signal, raising the question of whether RanBP3 induces enhanced nuclear export of active β-catenin or its increased phosphorylation. However, enhanced nuclear export would result in dilution in a

Figure 6. **RanBP3 induces specific depletion of endogenous nuclear active β-catenin.** SW480 (A and B) or DLD1 (C) colon carcinoma cells were transfected with RanBP3 expression plasmids and stained after 45 h for dephosphorylated β-catenin (A and C) or total β-catenin (B). RanBP3 expression was visualized in the same cells using a RanBP3 polyclonal (A and C) or mAb. (D) Luciferase reporter assay as in Figs. 2–4 measuring relative β-catenin activity. Cells were transfected as in A and C. Error bars represent SDs of technical replicates.
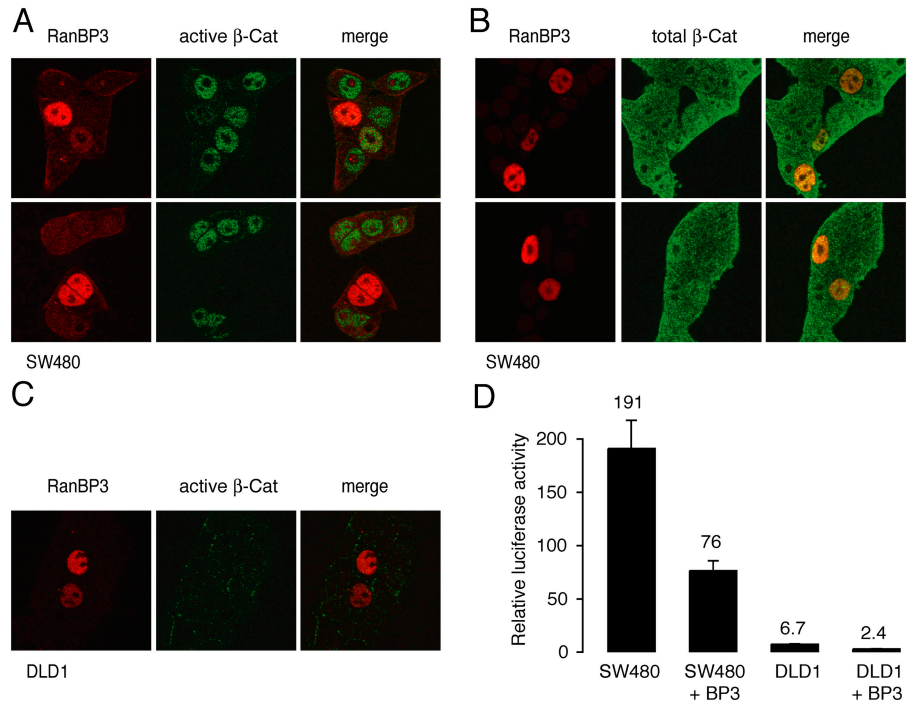
cytoplasmic volume that is ∼10-fold larger than that of the nucleus, precluding detection by the anti–dephosphorylated β-catenin antibody. To discriminate between the two possibilities, we mimicked the active state of β-catenin using a monomeric red fluorescent protein (mRFP)–tagged, constitutively active form of β-catenin, the previously used β-catenin^{ΔGSK3β}. To determine whether this fusion protein was biologically active, we performed a TCF reporter assay in the malignant mesothelioma cell line NCI-H28, which carries a homozygous deletion of the β-catenin gene (Calvo et al., 2000). This prevented possible activating effects of this mutant on endogenous β-catenin. mRFP–β-catenin^{ΔGSK3β} activated the very low endogenous TCF activity of these cells to a great extent (Fig. 7 B). We next compared the subcellular localization of this protein in the presence or absence of exogenous RanBP3 (Fig. 7 A). Care was taken to record cells of similarly low expression levels (Fig. 7 C). In control cells, more mRFP–β-catenin^{ΔGSK3β} was present in the nuclei than in the cytoplasm (median nuclear to cytoplasmic ratio of 1.38, n = 37). In contrast, cells expressing exogenous RanBP3 showed higher cytoplasmic than nuclear mRFP–β-catenin^{ΔGSK3β} levels (median nuclear to cytoplasmic ratio of 0.77, n = 41). Importantly, addition of 50 mM of the CRM1 inhibitor leptomycin B (LMB; Wolff et al., 1997) did not significantly change the effect of RanBP3 (median nuclear to cytoplasmic ratio of 0.80, n = 52), even though photobleaching experiments show that mRFP–β-catenin^{ΔGSK3β} rapidly shuttles between the nucleus and cytoplasm (unpublished data). Identical LMB treatment dramatically relocalized the NES-containing reporter protein Rev(1.4)-NES-GFP (Henderson and Eleftheriou, 2000) to the nucleus (Fig. 6 D). We conclude that RanBP3 enhances nuclear export of active β-catenin and that this export is independent of CRM1. To confirm that endogenous activated β-catenin relocalizes from the nucleus to

the cytoplasm upon overexpression of RanBP3 in HEK293 cells, we transfected these cells with Wnt1 and RanBP3. Indeed, we observed increased active β-catenin levels in both nuclear and cytoplasmic fractions, with the nuclear pool being more sensitive than the cytoplasmic pool to RanBP3 overexpression (Fig. 7 E). The decrease in cytoplasmic active β-catenin is consistent with increased nuclear export of β-catenin and subsequent degradation in the cytoplasm.

RanBP3 suppresses dorsal-ventral axis formation in *X. laevis* embryos

To study the role of RanBP3 in Wnt signaling in a physiological context, we used an *X. laevis* axis duplication assay. During *X. laevis* embryonic development, Wnt signaling determines patterning along the dorsal-ventral axis. Ectopic ventral injection of β-catenin mRNA in four-cell embryos resulted in clear axis duplication (Fig. 8, A and B). The majority (75%) of the embryos showed a complete duplication of the dorsal-ventral axis. 22% of the embryos showed a partial duplication, i.e., a secondary axis without duplicated cement gland. However, coinjection of β-catenin mRNA with RanBP3 mRNA resulted in a strong suppression of the double axis phenotype in the majority (63%) of the embryos. Few partial or very partial secondary axis phenotypes (24 and 13%, respectively) were observed in these embryos (Fig. 6 B). We also coinjected β-catenin mRNA with mRNA of the RanBP3 wv mutant that is defective in RanGTP binding. This mutant suppressed the double axis phenotype but was not as potent of an inhibitor as the wt RanBP3 (Fig. 8, A and B; P = 4e-8). This data correlates with our findings that this RanBP3 mutant binds β-catenin with less affinity (Fig. 1) and that it is less active in repressing the transcriptional activity of a TCF reporter gene in human cell lines (Figs. 2 and 4). If RanBP3 is an inhibitor of nuclear β-catenin

function, dorsal injection of RanBP3 mRNA is expected to result in ventralization of the embryo. We therefore injected four-cell embryos dorsally with either RanBP3 or control mRNA and scored ventralization after 3 d of development using the dorsoanterior index (DAI; Kao and Elinson, 1988). Mild to severe ventralization was observed (DAI 1–4) in 80% of RanBP3-injected embryos (Fig. 8 C), whereas <10% of control-injected embryos showed these phenotypes. Complete ventralization (DAI 0) was not observed. An important direct downstream target of dorsal nuclear β-catenin activity is the early Wnt-inducible homeobox gene *Siamois* (Brannon et al., 1997). We therefore tested to determine whether expression levels of this gene were reduced in the RanBP3-injected embryos by RT-PCR. In four independent experiments, we detected an approximately twofold decrease in *Siamois* levels in late stage 9 embryos (Fig. 8, D and E). This decrease is rather mild, consistent with the incomplete ventralization phenotypes observed. Based on these findings, we conclude that RanBP3 not only is a repressor of Wnt signaling in human cell lines but also functions as an antagonist of Wnt signaling in *X. laevis* embryos.

Loss of function of *ranbp3* results in a naked cuticle phenotype in *D. melanogaster*

Wnt signaling is highly conserved between different species. We identified the *D. melanogaster* RanBP3 homologue and used RNAi to study its role in *D. melanogaster* development. At the end of embryogenesis, the ventral epidermis is covered by a cuticle that is built up by a repeating pattern of naked cuticle and denticles (Fig. 9 A). Wingless (Wg; *D. melanogaster* Wnt) signaling increases levels of ARM (β-catenin) that specifies the fate of epidermal cells responsible for secreting naked cuticle. Therefore, loss of *wg* expression results in an embryo that is covered with denticles lacking naked cuticle (Nusslein-Volhard and Wieschaus, 1980) and overexpression of *wg* results in a naked cuticle embryo (Noordermeer et al., 1992). Likewise, loss of an inhibitor of Wnt signaling also results in naked cuticle embryos as shown by RNAi against Daxin (Willert et al., 1999). As a control, we injected embryos with β-galactosidase double-stranded RNA (dsRNA) and observed that the majority (97%) developed into larvae that were indistinguishable from noninjected wt larvae (Fig. 9 A). 3% of these control embryos showed some very weak effects on denticle belt formation (Fig. 9 G). RNAi against Daxin resulted in a significant increase in naked cuticle phenotype in 24% of the Daxin dsRNA–injected embryos (Fig. 9 G), with phenotypes varying from partial loss of denticles to completely naked embryos (Fig. 9, B and C). Injection of dsRNA against the *D. melanogaster* RanBP3 caused a partial or complete transformation of denticles into naked cuticle in 14% of the embryos (Fig. 9, D–F). The most severe phenotypes of the RanBP3 RNAi embryos showed deformation of both the head and spiracles (Fig. 9 F), resembling Daxin RNAi (Fig. 9 C). In addition, almost all RanBP3 RNAi embryos showing a strong naked cuticle phenotype were shorter than the embryos injected with Daxin dsRNA. To confirm that the RanBP3 dsRNA injections resulted in decreased RanBP3 levels, we performed RT-PCR on buffer and RanBP3 dsRNA–injected embryos.
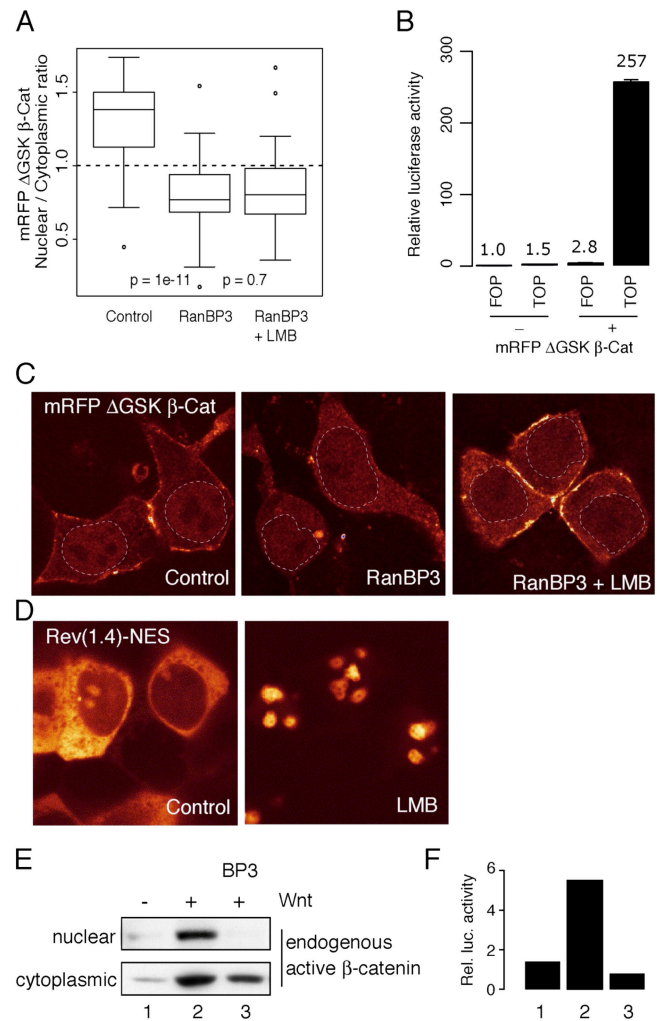

Figure 7. **RanBP3 enhances nuclear export of active β-catenin independently of CRM1.** (A and C) Effect of RanBP3 on mRFP–ΔGSK–β-catenin nucleocytoplasmic distribution in HEK293 cells in the presence or absence of 50 nM LMB for 3 h. (A) Box plot showing the distribution of nuclear/cytoplasmic ratios of mRFP–ΔGSK–β-catenin of two independent experiments. P values are according to Mann-Whitney tests. Representative mRFP fluorescence images are shown in C. Highlighted nuclear borders are drawn on the basis of accompanying phase-contrast images. (B) Functionality of mRFP–ΔGSK3–β-catenin. NCI-H28 cells (lacking endogenous β-catenin) were transfected with indicated constructs, and 48 h after transfection, luciferase activity was measured. Relative luciferase levels as corrected for transfection efficiency (Renilla luciferase activity) are shown. Error bars represent SDs. (D) Representative fluorescence images of HEK293 cells expressing GFP-Rev(1.4)-NES in the presence or absence of 50 nM LMB for 3 h. (E and F) Endogenous activated β-catenin relocalizes from the nucleus to the cytoplasm upon overexpression of RanBP3. HEK293 cells were transfected with Wnt and RanBP3 as indicated together with TOP-TK-luc and Renilla transcription reporter plasmids and fractionated after 48 h as in Fig. 5. Localization of active β-catenin was monitored using anti–active β-catenin antibody. Amounts of protein loaded were normalized on transfection efficiency (Renilla luciferase activity). Normalized β-catenin/TCF–dependent luciferase activity is depicted in F.

Fig. 9 H shows that RanBP3 mRNA levels were indeed decreased in RanBP3 dsRNA–injected embryos, whereas RP49 control mRNA levels remained unaffected. We then assayed the effects of RanBP3 dsRNA injection on *wg* target gene induction. For this, stage 10 RanBP3 or Daxin dsRNA–injected embryos were stained with anti-Engrailed antibody. Normal *engrailed*

Figure 8. RanBP3 rescues β-catenin–induced double axis formation in *X. laevis* embryos. (A) *X. laevis* embryos were injected ventrally at the four-cell stage with β-catenin mRNA in the presence or absence of control β-galactosidase or *X. laevis* RanBP3-b mRNA. (top) Wt noninjected embryos. (middle) Double axis phenotype as induced by the injection of β-catenin mRNA. (bottom) Embryos that are rescued from the double axis phenotype by coexpression of RanBP3 and β-catenin mRNA. (B) Quantification of the different phenotypes of two independent experiments in four categories: complete secondary axis (with cement gland), partial secondary axis (i.e., any secondary axis lacking the cement gland), vestigial axis (very small posterior protrusion or pigmented line), and normal (only one axis). P values are according to Pearson's χ^2 test for count data. (C) Dorsal injection of RanBP3 results in ventralization of *X. laevis* embryos. Four-cell stage embryos were injected dorsally with RanBP3 or control (β-galactosidase) mRNA and analyzed 3 d later for ventralization using the standardized DAI. This scale runs from 0 (complete ventralization) to 5 (normal development). Frequencies are derived from three independent experiments. P values as in B. (D) The β-catenin downstream target *siamois* is significantly down-regulated in RanBP3-injected embryos. Embryos were injected as in C and analyzed for *siamois* or ornithine decarboxylase (ODC) mRNA using RT-PCR. Amplified ethidium bromide–stained DNA of four experiments was quantified and normalized to mean signals from β-galactosidase–injected embryos and represented in a box plot. P values are according to Mann-Whitney tests. (E) Representative signals from RT-PCR reactions visualized by ethidium bromide staining.
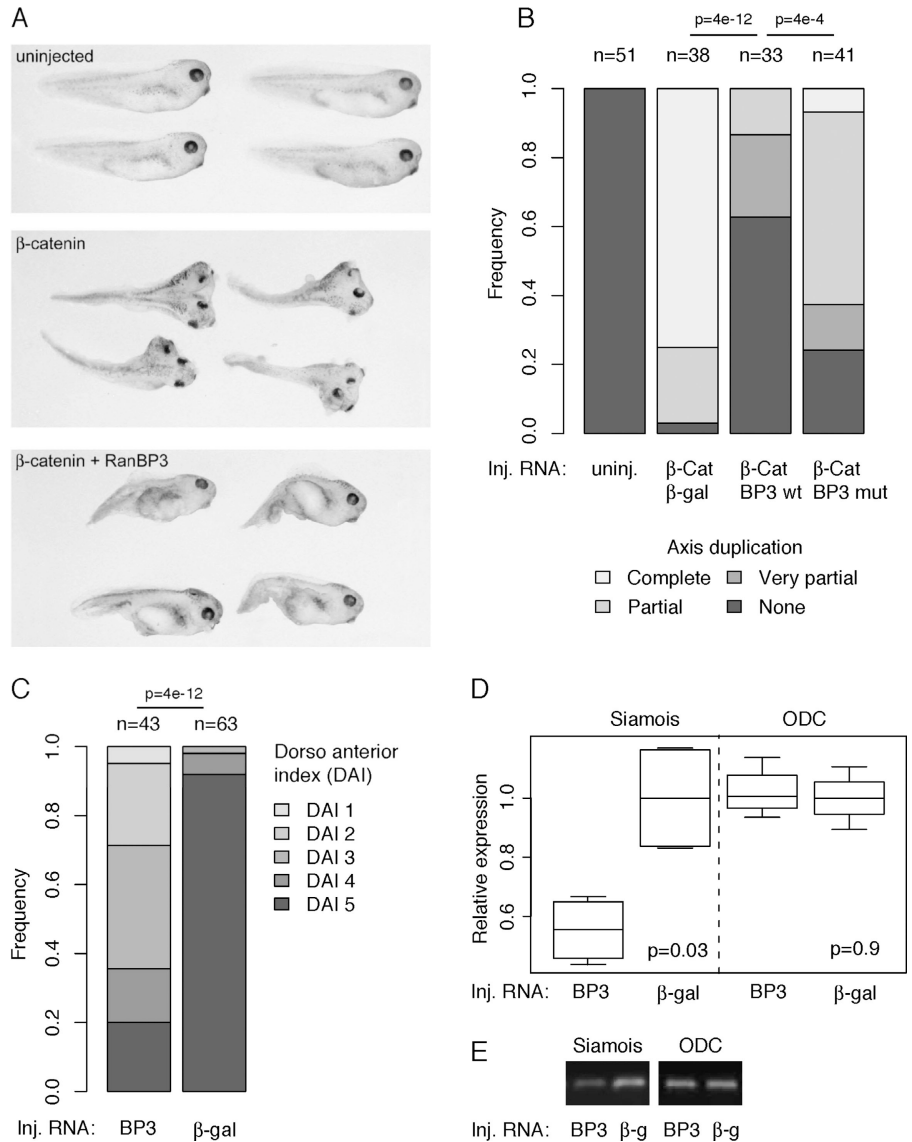
expression is present in segmental stripes that are two cells wide (Fig. 9 I, left). Removal of the Wnt signaling inhibitor Daxin by dsRNA injection resulted in a broader Engrailed expression pattern that extended from two to four rows of cells (Fig. 9 I, middle). In RanBP3 dsRNA–injected embryos, Engrailed expression expanded by one row of cells (Fig. 9 I, right). These in vivo data show that removal of RanBP3 leads to a phenotype that is associated with Wnt signaling activation, suggesting that RanBP3 also acts as negative regulator of Wnt signaling in *D. melanogaster*.

Discussion

In this study, we identify RanBP3 as a novel inhibitor of Wnt signaling that acts on β-catenin directly by enhancing nuclear export of its active form. We show that RanBP3 binds directly to β-catenin and that the interaction is increased in the presence of RanGTP. Expression of RanBP3 represses Wnt signaling both in vitro and in *X. laevis* embryonic development. Inhibition of RanBP3 by RNAi causes overactivation of Wnt signaling in

tissue culture cells and in *D. melanogaster* embryos. In addition, expression of RanBP3 in human cells specifically reduces active β-catenin levels in the nucleus and relocates ΔGSK3–β-catenin from the nucleus to the cytoplasm, independently of CRM1.

RanBP3 was originally identified as a nuclear protein that contains FG repeats and a RanGTP-binding domain (Mueller et al., 1998). RanBP3 can directly bind the nuclear export receptor CRM1, stimulating the formation of nuclear export complexes and increasing the export rate of certain CRM1 substrates (Englmeier et al., 2001; Lindsay et al., 2001). One mechanism by which RanBP3 could influence β-catenin activity would therefore be increased nuclear export via the CRM1 pathway. Although the nuclear export mechanisms of β-catenin are not fully understood, two pathways have been proposed (Henderson and Fagotto, 2002). In the first, β-catenin exits the nucleus independently of nuclear export receptors by interacting directly with proteins of the nuclear pore complex (Eleftheriou et al., 2001; Wiechens and Fagotto, 2001). In the second pathway, β-catenin exits the nucleus via the CRM1 pathway, but
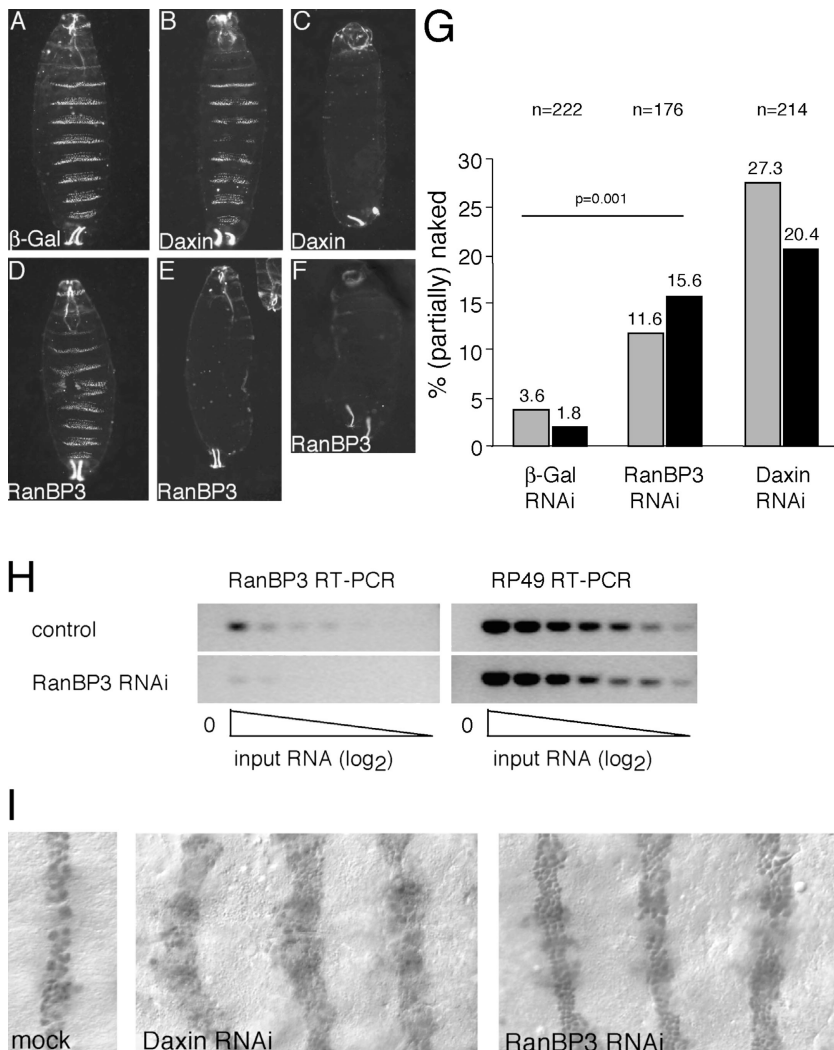
Figure 9. Loss of RanBP3 by RNAi results in a naked cuticle phenotype in *D. melanogaster*. Shown are dark field images of cuticle preparations of control (β-galactosidase; A), *D. melanogaster* Daxin (B and C), and *D. melanogaster* RanBP3 dsRNA–injected embryos (D–F). Loss of Daxin and RanBP3 results in increased Wnt signaling and replacement of denticles by naked cuticle. Partially naked cuticles (B and D), nearly naked cuticles (E), and naked cuticles (C and F) are shown. All views are ventral, top is posterior. (G) Quantification of two representative experiments showing the frequency of the cuticle phenotype. P values are calculated as in Fig. 7 B. Note that the contribution of the completely naked phenotype in the RanBP3 RNAi embryos is relatively high (not depicted). (H) RT-PCR showing reduction in RanBP3 mRNA levels in RanBP3 dsRNA–injected embryos. Embryos were injected as in A, and RNA was extracted after 15 h of development. RT-PCRs specific for RanBP3 or control (ribosomal protein RP49) were performed using nothing (0) or a series of twofold dilutions of extracted RNA. (I) Loss of RanBP3 function by dsRNA injection results in increased expression of the wg target gene *engrailed*. Shown are an Engrailed antibody staining of a buffer-injected embryo (left), a Daxin dsRNA–injected embryo (middle), and an RanBP3 dsRNA–injected embryo (right). Note that the buffer-injected embryo developed until late stage 11, whereas the Daxin and RanBP3 RNAi embryos shown are stage 10 embryos, which explains the larger cells in the former embryo. The number of Engrailed-positive cell rows between stages 10 and 11 is identical. Ventral-lateral view is shown, posterior is left.

because β-catenin does not contain NESs of its own, it uses binding to APC to exit the nucleus. The APC tumor suppressor does contain functional NESs and has been shown to be exported by CRM1 (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). Therefore, RanBP3 could inhibit β-catenin by stimulating its export via APC and CRM1. However, four lines of evidence argue against this. First, in a CRM1 export complex, RanBP3 would bind to the complex via CRM1. Instead, we find that RanBP3 interacts directly with β-catenin. Second, β-catenin activity is RanBP3 sensitive in the colon carcinoma cell line COLO320 (Quinn et al., 1979) that expresses a short type I APC truncation lacking all β-catenin interaction sites (Rosin-Arbesfeld et al., 2003). We cannot formally exclude the possibility that the neuronal APC-like protein APC2 (van Es et al., 1999), which is expressed in certain colon carcinoma cell lines, compensates for the loss of APC. However, in luciferase reporter assays, CRM1 overexpression does not reverse stimulation of β-catenin activity caused by depletion of RanBP3. Finally, RanBP3-mediated relocalization of active β-catenin is insensitive to LMB, a potent CRM1 inhibitor (Wolff et al., 1997). Therefore, we conclude that the mechanism by which RanBP3 inhibits β-catenin is independent of CRM1 and APC.

It was recently suggested that nuclear β-catenin signaling is performed mainly by β-catenin dephosphorylated at serine 37 and threonine 41, which are main target sites of GSK3β (Staal et al., 2002; van Noort et al., 2002). Depletion of RanBP3 by RNAi specifically increases the amount of dephosphorylated β-catenin in nuclear fractions, whereas RanBP3 overexpression has the opposite effect. No concomitant increase, but rather a small decrease, of cytoplasmic endogenous active β-catenin was observed by overexpression of RanBP3. We attribute this to cytoplasmic phosphorylation and subsequent degradation of wt β-catenin.

Endogenous active β-catenin was visualized in situ, using the anti–active β-catenin antibody recognizing dephosphorylated β-catenin. This was only possible in SW480 colon carcinoma cells that contain a high level of active β-catenin because of severely defective APC function (Korinek et al., 1997). RanBP3 overexpression reduced active β-catenin levels in the nucleus but had no effect on total β-catenin. This suggests that only a small proportion of total β-catenin is active in SW480 cells and confirms the specificity of RanBP3 for active β-catenin. Apparently, absence of proper β-catenin phosphorylation and degradation is not sufficient for β-catenin to be in an active, dephosphorylated state. Also, we infer that the modulation by

RanBP3 of β-catenin activity as measured in our luciferase reporter assays acts on a small dephosphorylated pool, explaining why RanBP3 modulates wt and ΔGSK3–β-catenin to a similar extent (Figs. 2 and 4).

To determine whether RanBP3 enhances β-catenin NH$_2$-terminal phosphorylation or nuclear export, we have visualized both nuclear and cytoplasmic distribution of active β-catenin. For this, we used a fluorescently tagged β-catenin^{ΔGSK3} that is resistant to NH$_2$-terminal phosphorylation and degradation. As shown in Fig. 7, RanBP3 causes a clear and significant shift of β-catenin^{ΔGSK3} from the nucleus to the cytoplasm. We therefore conclude that RanBP3 directly enhances nuclear export of active β-catenin. How does RanBP3 perform this task? Recent studies have indicated that the interactions of nuclear factors with chromatin or with each other are dynamic (Dundr et al., 2002; Phair et al., 2004). This suggests that RanBP3 does not need to actively remove β-catenin from the TCF/LEF–chromatin complexes. We therefore favor the possibility that association with RanBP3 prevents association of active β-catenin with chromatin and keeps it in a more soluble state. In itself, this would be sufficient to allow CRM1-independent nuclear exit. We do not know whether RanBP3 accompanies β-catenin to the cytoplasm and acts as a true nuclear export factor. The stimulatory effect of RanGTP on the β-catenin–RanBP3 interaction and the consistently weaker inhibitory effects on β-catenin of a RanBP3 mutant unable to bind RanGTP would argue in favor of this possibility. Hydrolysis of RanGTP in the cytoplasm would increase the efficiency of release of β-catenin for subsequent interactions with the cytoplasmic interacting proteins, such as E-cadherin or the APC–Axin–GSK3β complex.

We studied the effect of RanBP3 in *X. laevis* and *D. melanogaster* embryogenesis. Overexpression of the *X. laevis* homologue of RanBP3 during early embryogenesis inhibits β-catenin–dependent dorsoventral axis formation. RNAi of the *D. melanogaster* homologue of RanBP3 causes naked cuticle phenotypes and a broader Engrailed expression domain because of overactivation of the Wnt signaling pathway. Therefore, the results obtained in these two model organisms support the results obtained in cultured human cell lines and indicate that the inhibitory function of RanBP3 is highly conserved in metazoan evolution.

Wnt signaling plays an important role in tumor initiation and progression in a variety of human solid tumors, including colon carcinomas, hepatocellular carcinomas, and melanomas (Bienz and Clevers, 2000; Polakis, 2000). As a negative modulator of Wnt signaling, RanBP3 is a novel candidate tumor suppressor protein. Interestingly, the RanBP3 gene is located in 19p13.3, a region that is commonly deleted in several types of cancer and in which multiple tumor suppressor genes are likely to be present (Lee et al., 1998; Oesterreich et al., 2001; Tucci et al., 2001; Yanaihara et al., 2003; Kato et al., 2004; Miyai et al., 2004; Yang et al., 2004). Further work is required to determine whether the loss of the RanBP3 gene contributes to these or other types of cancer.

In conclusion, we have identified an unexpected role for RanBP3 as a novel inhibitor of Wnt signaling that enhances nuclear export of active β-catenin. This function is separate from its role in CRM1-mediated nuclear export. The structural similarities between CRM1 and β-catenin suggest that RanBP3 may be a more general cofactor for nuclear export of ARM repeat proteins.

Materials and methods

Data analysis
Statistical analysis was done using the R software package (R Development Core Team, 2005).

Reagents
Antibodies used were β-catenin (Transduction Laboratory and Santa Cruz Biotechnology, Inc.), RanBP3 (Transduction Laboratory and Affinity Bio-Reagents, Inc.), active β-catenin, TCF4 (Upstate Biotechnology), 414 (Eurogentec/Babco), α-tubulin (European Collection of Cell Cultures), actin (Oncogene Research Products), and c-Myc (Santa Cruz Biotechnology, Inc.). The 4D9 anti-Engrailed/invected mAb was a gift from C. Goodman (University of California, Berkeley, Berkeley, CA; Patel et al., 1989).

Plasmids
The following plasmids were used: GST–β-catenin and GST-ARM (Wiechens and Fagotto, 2001), pET14b-h-RanBP3-b (Mueller et al., 1998), pET14b-h–RanBP3-b wv mutant (Englmeier et al., 2001), and pRev(1.4)-RevNES-GFP (Henderson and Eleftheriou, 2000). pQE32-Ran and pQE32-RanQ69L were gifts from D. Görlich (Center for Molecular Biology Heidelberg, Heidelberg, Germany). TOP/FOP-Tk, Wnt1, GFP–β-catenin, and pSUPER plasmid were gifts from H. Clevers (Hubrecht Laboratory, Utrecht, Netherlands), R. Kypta (University of California, San Francisco, San Francisco, CA), and R. Agami (Netherlands Cancer Institute, Amsterdam, Netherlands). pcDNA3-RanBP3-b wt and pcDNA3-RanBP3-b wv mutants were constructed by generating a blunt NdeI–EcoRV fragment from pET14b-h–RanBP3-b wt and wv mutants and by inserting these fragments into the EcoRV site of pcDNA3 (Invitrogen). shRNAs were expressed from the pSUPER vector (Brummelkamp et al., 2002). The successful 19-nt target sequences were as follows: RanBP3 2 (AAG-GCGGAGAAGATTCTGACA), 3 (AAAGAGCCCCAGAAAAATGAG), 4 (AAGAGCCCCAGAAAAATGAGT), 8 (AAGCCGACATGGAGAATG-CTG), 9 (AACCGCAACGAACTATTTCCT), and 12 (AAGGACACAG-GTCAGTTGTAT). pSUPER-GFP was a gift from S. Nijman (Netherlands Cancer Institute), and pBS(SK-)-Daxin-Myc was a gift from R. Nusse (Stanford University, Stanford, CA). For *X. laevis* injection studies, we used HA–β-catenin (Funayama et al., 1995) and β-galactosidase in pCS2+ (gift from R. Rupp, Adolph Butenardt Institute, Munich, Germany). pCS2 + MT–RanBP3 wt and wv mutants were constructed by inserting PCR fragments into the EcoRI and XbaI sites of pCS + Myc. mRFP–ΔGSK3–β-catenin was constructed by inserting a BamHI–SacII–digested PCR fragment spanning the ORF derived from pRK5-SK-catenin-GSK (a gift from R. Nusse) into the BglII and SacII sites of mRFP (Campbell et al., 2002).

Cell culture, transfection, and reporter assays
Cells were cultured in DME or in RPMI (NCI-H28) supplemented with 10% fetal calf serum and penicillin/streptomycin (GIBCO BRL) and were transfected using Fugene 6 (Roche) as instructed by the manufacturer. For reporter assays, cells were cultured in 12-well plates and transfected with 100 ng TOP/FOP-Tk-luc, 0.5 ng pRL-CMV, 10 ng Wnt1, 30 ng GFP–β-catenin, 20 (HEK293) or 100 (NCI-H28) ng ΔGSK3–β-catenin, 100 ng GFP-CRM1, and 100 ng RanBP3 wt/mutant or as indicated. Luciferase activity was measured 48 h after transfection using the Dual-luciferase reporter assay system (Promega). Reporter assays using shRNAs were performed as the aforementioned reporter assays using 200 ng shRNA constructs, and luciferase activity was measured 72 h after transfection. HCT116 cells were grown to 50% confluency in 10-cm dishes and transfected with 5 µg of β-galactosidase or RanBP3 wt or wv mutant expression constructs and 0.5 µg EGFP-N3 plasmid to select for transfected cells. 40 h after transfection, GFP-positive cells were collected using flow cytometry. Cells were lysed in sample buffer, and 200,000 cells were resolved on a 10% SDS-PAGE gel and analyzed by Western blotting.

Protein expression and purification
GST, GST-ARM (amino acids 144–665), and GST–β-catenin (Wiechens and Fagotto, 2001) were expressed in *Escherichia coli* strain BL21-pLysS and lysed by sonication in 500 mM NaCl, 20 mM Hepes-KOH, pH 7.9,

8.7% glycerol, and 2.5 mM 2-mercaptoethanol supplemented with Complete protease inhibitor cocktail tablets (Roche). GST–β-catenin fusion proteins were purified from postribosomal supernatants using protein G–Sepharose (GE Healthcare). His-tagged Ran RanQ69L, RanBP1, and Ran-GAP were expressed as previously described (Izaurralde et al., 1997; Englmeier et al., 2001). 6× His-tagged RanBP3a/b wt and wv mutant proteins were gifts from L. Englmeier and I. Mattaj (European Molecular Biology Laboratory, Heidelberg, Germany).

Western blotting
Proteins were analyzed by SDS-PAGE (25 μg per lane) and Western blotting using Immobilon-P transfer membrane (Millipore). Aspecific sites were blocked with 5% nonfat milk at RT for 1 h. Primary antibodies were incubated in 1% nonfat milk overnight at 4°C or 1–3 h at RT in the following dilutions: β-catenin, 1:5,000; ABC, 1:500; RanBP3, 1:5,000; TCF4, 1:500; 414, 1:1,000; tubulin, 1:20; actin, 1:5,000; and c-Myc, 1:1,000. Blots were washed with PBS/0.05% Tween 20. Enhanced chemiluminescence (GE Healthcare) was used for detection of proteins.

Immunofluorescence and confocal microscopy
SW480 and DLD1 cells were transfected with 600 ng RanBP3 per six wells using Fugene 6. 45 h after transfection, cells were fixed for 10 min in 3.7% formaldehyde in PBS, permeabilized for 5 min in 0.2% Triton/PBS, and incubated for 1 h at RT with primary antibodies diluted in 0.05% BSA/PBS. Cells were washed in PBS, incubated in fluorescently conjugated secondary antibody (Invitrogen), and mounted in Vectashield (Vector Laboratories). Images were recorded using a confocal microscope (NT; Leica). HEK293 cells were transfected with 40 ng mRFP–GSK3–β-catenin, 200 ng RanBP3, and/or 200 ng GFP-Rev-NES per six wells using Fugene 6. After 40 h, cells were either treated or not treated with 50 nM LMB for 1 h. Cells were fixed for 10 min in 3.7% formaldehyde in PBS and mounted in Vectashield. In each condition, cells with equally low expression were recorded with a confocal microscope (TCS SP2 AOBS; Leica). Nuclear and cytoplasmic regions of confocal images were quantified and background subtracted, and nuclear/cytoplasmic ratios were calculated using Image J software.

In vitro binding studies
In pull-down assays, 750 pmol GST, GST–β-catenin, or GST-ARM were incubated for 1 h at 4°C with X. laevis extracts (Hetzer et al., 2000) and 1:1 diluted in 200 mM NaCl, 20 mM Hepes-KOH, pH 7.9, 8.7% glycerol, and 2.5 mM 2-mercaptoethanol (buffer A). RanQ69L was added at 2 μM. In binding assays using HeLa nuclear extracts (4C Biotech), RanQ69L was used at 1 μM. Proteins were eluted with buffer A supplemented with 300 mM NaCl. After TCA precipitation, proteins were analyzed by Western blot. Pull-down assays using all recombinant proteins were performed by incubating for 1 h at 4°C; 1.5 μM GST–β-catenin beads with 0.2, 0.5, or 2 μM wt or wv mutant RanBP3 and 2 μM RanGTP in PBS, 8.7% glycerol, and 2 mM MgCl₂. Proteins were eluted with 500 mM NaCl, 8.7% glycerol, 2 mM MgCl₂, and 2.5 mM 2-mercaptoethanol in the presence or absence of RanBP1 or RanGAP in PBS and prepared for analysis on SDS-PAGE.

Cell fractionation
For cell fractionation, we used the protocol of Andrews and Faller (1991) with the following adaptations. Cells and nuclei were spun down at 4°C for 3 min at 500 and 300 g, respectively. 10 mM NaF, 2 mM NaVO₃, and protease inhibitors (Complete protease inhibitor cocktail tablets minus EDTA) were added to the lysis buffers. After incubation in hypotonic buffer, NP-40 was added at a concentration of 10% and samples were vortexed shortly and passed through a 25G needle. Whole cell extracts were reconstituted by mixing nuclear and cytosolic extracts.

X. laevis injection studies
mRNAs were synthesized in vitro using SP6 polymerase (Promega). mRNAs were injected in the subequatorial region of a dorsal or ventral blastomere at the four-cell stage as described previously (Fagotto et al., 1996, 1997). Embryos were raised in 0.1× MBSH (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.33 mM Ca[NO₃]₂, 0.41 mM CaCl₂, 10 mM Hepes, pH 7.4, 10 mg/ml benzylpenicillin, and 10 mg/ml streptomycin) until tail bud stage and scored. RNA was prepared from late stage 9 embryos as previously described (Schohl and Fagotto, 2003).

dsRNA synthesis D. melanogaster
β-galactosidase, Daxin, and RanBP3 dsRNAs were synthesized according to Kennerdell and Carthew (1998) and purified using S400 Spin Columns

(GE Healthcare). PCR products were verified by DNA sequencing. For D. melanogaster RanBP3 dsRNA, two 750-bps fragments that span exon-2 of the D. melanogaster RanBP3 gene (GC10225) were amplified from genomic DNA. Fragment 1 spans the RanBP3 ORF from position 341–1104, and fragment 2 from position 683 to 3′UTR position 1423. The following primers were used: BP3 sense primer 1 (AGAACAACATGCCAAATGT-TCAG), BP3 antisense primer 1 (GACGCCGTTTTTCGCTTCCTCT), BP3 sense primer 2 (AGAAACGCAAATACGAGGAGGT), and BP3 antisense primer 2 (GGCGCGCTTTATTAATTAGTGT). pBS(SK-)-Daxin-Myc (Willert et al., 1999) was used as a template to generate a 750-bp dsRNA Daxin fragment spanning nucleotides 1462–2210. The following primers were used: Daxin sense primer (GAGAAGTTTGCACTGGACGAAGA) and Daxin antisense primer (GGCTTGACAAGACCCATCGCTT). For β-galactosidase dsRNA, nucleotides spanning from 1296 to 1921 of the lac operon (available from GenBank/EMBL/DDBJ under accession no. J01636) were subcloned into pGEMT-easy and T7 RNA polymerase promoters were added by PCR of the linearized plasmid.

Cuticle analysis and immunohistochemistry
Embryos were prepared for injections as previously described (Kennerdell and Carthew, 1998) with minor modifications. Embryos were injected with 3 μM dsRNA, and for RanBP3 RNAi, a 1:1 mixture of two dsRNA fragments was used. After injection, the embryos were covered with oil and incubated for 48 h at 18°C in a humidified chamber. After incubation, the embryos were manually dissected from their viteline membranes and incubated overnight at 65°C in glycerol/acetic acid (1:3). The next day, embryos were mounted in Hoyers mounting medium and incubated for 1–2 d at 55°C and visualized by dark field microscopy. For anti-Engrailed antibody staining, embryos were incubated for 15 h, fixed, manually devitalinized, and processed for antibody staining according to standard procedures (Patel, 1994).

D. melanogaster RT-PCR
Dechorionated wt embryos were injected with buffer or RanBP3 dsRNA and then aged at 16°C for 15 h. RNA was prepared and treated with DNase (RNA-Easy kit; QIAGEN), and randomly primed first-strand cDNA was prepared using SuperScript kit (Invitrogen), both according to the manufacturer's protocol. Samples for the RP49-specific control PCRs were initially diluted 80-fold to compensate for higher expression levels. Subsequently, a series of twofold dilutions was performed for each sample; 1 μl of each dilution was used in a PCR reaction. Primers were chosen to span an intron to allow discrimination of PCR products originating from contaminating genomic DNA from those originating from first-strand cDNA. Primers used were as follows: RanBP3 forward (AGTGACAGCGATAACA-CAGCGATAA) and reverse (GCAGAAACGGATTATTCAGCAGG) and RP49 forward (ATGACCATCCGCCCAGCA) and reverse (TTGGGGTTG-GTGAGGCGGAC). 30-cycle PCRs were performed using SuperTaq Plus polymerase (SpheroQ), and equal volumes of the reaction products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining.

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