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–mediating 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; Molemaar 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 Brahma-related gene 1 (Brahma-related gene 1) and CBP (CREB binding protein)/p300 to promote target gene activation (Hecht and Kemler,
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 relocation 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 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 (Engelmeier 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; Engelmeier 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, 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 NH2-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 NH2-terminal GSK3β phosphorylation sites (β-cateninΔGSK3β, 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-cateninΔGSK3β (Fig. 2 D). Again, the RanBP3 RanGTP-binding mutant was less able to repress β-cateninΔGSK3β-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 (∆45 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 (Fig. 3 B). To determine whether RanBP3 depletion also acts on NH2-terminally dephosphorylated β-catenin, we cotransfected β-cateninΔGSK3β with anti–RanBP3 shRNA expression constructs (Fig. 3 C). Reduction of RanBP3 increased reporter activity, confirming that RanBP3 can act on the NH2-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
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 via stimulation 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 APC1–1417, 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 vv 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 experiment 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
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.

**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.

**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.
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-catenin3ΔN35 (Figs. 2 and 4). If RanBP3 is an inhibitor of nuclear transcriptional activity of a TCF reporter gene in human cell lines 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.

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 (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 function of ranbp3 results in a naked cuticle embryo (Fig. 9, A and B). 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 and C). 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) A box plot showing the distribution of nuclear/cytoplasmic 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-ΔGSK-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 relocates 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 F. 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
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
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. 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 NH2-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-cateninΔGSK3 that is resistant to NH2-terminal phosphorylation and degradation. As shown in Fig. 7, RanBP3 causes a clear and significant shift of β-cateninΔGSK3 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 export. 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 between the germ band and the cephalic region (Dundr et al., 1998). These successful 19–nt target sequences were as follows: RanBP3 2 (AAGGCCGAGAATGTCGACA), 3 (AAAGAGCCCCAGAAAAATGAG), 4 (AAGGACCCCGAGAAAAATGAG), 5 (AAGGCCGCGATGGAATGTCG), 6 (AAGGCCGACATGGAATGTCG), 7 (AAACCGCAAGACTTCTGCA), 8 (AAGGCCGACATGGAATGTCG), 9 (AAACCGCAAGACTTCTGCA), and 10 (AAGGACACAGCAGAAATGAGT). These sequences were cloned into the pBluescript vector (Stratagene, La Jolla, CA). We used many of these sequences to inhibit expression of targeted genes in cultured mammalian cells. For injection studies, we used HA–β-catenin (Funayama et al., 1995) and β-galactosidase in pC72+ (gift from R. Rupp, Adolph Butenandt Institute, Munich, Germany). pcP2 + MT–RanBP3 wt and wv mutants were constructed by inserting PCER fragment spanning the ORF derived from pKS-SK-β-catenin/GSK (a gift from R. Nussel) into the BglII and SacII sites of mRFP (Campbell et al., 2002).

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 BioReagents, 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 (Wienchens and Fogotto, 2001), pET14b-h–RanBP3-b (Mueller et al., 1998), pET14b-h–RanBP3-b wt mutant (Engleman et al., 2001), and pRev1.4–RevNES–GFP (Henderson and Elefteriou, 2000). pQ32-Ran and pQ32-RanGap69, 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). This successful 21–nt target sequences were as follows: RanBP3 2 (AAGGCCGAGAATGTCGACA), 3 (AAAGAGCCCCAGAAAAATGAG), 4 (AAGGCCGCCAGAAAAATGAG), 5 (AAGGCCGACATGGAATGTCG), 6 (AAGGCCGACATGGAATGTCG), 7 (AAACCGCAAGACTTCTGCA), 8 (AAGGCCGACATGGAATGTCG), 9 (AAACCGCAAGACTTCTGCA), and 10 (AAGGACACAGCAGAAATGAGT). These sequences were cloned into the pBluescript vector (Stratagene, La Jolla, CA). We used many of these sequences to inhibit expression of targeted genes in cultured mammalian cells. For injection studies, we used HA–β-catenin (Funayama et al., 1995) and β-galactosidase in pC72+ (gift from R. Rupp, Adolph Butenandt Institute, Munich, Germany). pcP2 + MT–RanBP3 wt and wv mutants were constructed by inserting PCR fragments into the EcorI and XbaI sites of pC195. G418-RanBP3 was constructed by introducing a tandem repeat of the 1.2-kb G418 selection cassette into a HindIII–BamHI fragment of pcDNA3-RanBP3-b. 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). This successful 21–nt target sequences were as follows: RanBP3 2 (AAGGCCGAGAATGTCGACA), 3 (AAAGAGCCCCAGAAAAATGAG), 4 (AAGGCCGCCAGAAAAATGAG), 5 (AAGGCCGACATGGAATGTCG), 6 (AAGGCCGACATGGAATGTCG), 7 (AAACCGCAAGACTTCTGCA), 8 (AAGGCCGACATGGAATGTCG), 9 (AAACCGCAAGACTTCTGCA), and 10 (AAGGACACAGCAGAAATGAGT). These sequences were cloned into the pBluescript vector (Stratagene, La Jolla, CA). We used many of these sequences to inhibit expression of targeted genes in cultured mammalian cells. For injection studies, we used HA–β-catenin (Funayama et al., 1995) and β-galactosidase in pC72+ (gift from R. Rupp, Adolph Butenandt Institute, Munich, Germany). pcP2 + MT–RanBP3 wt and wv mutants were constructed by inserting PCR fragments into the EcoRI and XbaI sites of pcDNA3 + Myc. mRFP–ΔGSK3–β-catenin was constructed by inserting a BamHI–SacI-digested PCR fragment spanning the ORF derived from pKS-SK-β-catenin/GSK (a gift from R. Nussel) into the BglII and SacI sites of mRFP (Campbell et al., 2002).

Cell culture, transfection, and reporter assays

Cells were cultured in DMEM or in RPMI (NCH128) 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 (NCH128) 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 (48 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 (Wienchens and Fogotto, 2001) were expressed in Escherichia coli strain BL21 (DE3) 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 RanQ69L, RanBP1, and RanGAP were expressed as previously described (Izaurralde et al., 1997; Englemier et al., 2001). 6XHis-tagged RanBP3a/b wt and vv mutant proteins were gifts from L. Englemier 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® 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 1:50 fluorescein 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 mM 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 μg GST–β-catenin beads with 0.2, 0.5, or 2 μM wt or vv 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 NaVO4, 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 short 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 into 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 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-bp 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: B3 sense primer 1 (AGAACACATGCAGAATTG-TCCAG), B3 antisense primer 1 (GAGGCCGTTTTCTGCTTCCT), B3 sense primer 2 (GAGAAAACGCAAATACGAGGAG), and B3 antisense primer 2 (GGGCCGGCTTTAATTAGTGT), pb5(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 1 (GAGAGATTTGCTACGGCAGAAGA) and Daxin antisense primer (GGCTTGACAGACCCATCGCTT). For β-galactosidase dsRNA, nucleotides spanning from 1296 to 1921 of the lac operon (available from GenBank/EMBL/DBJ 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 μg 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 (Invitrogen), both according to the manufacturer’s protocol. Samples for the RFP-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 (AGTGACACGGCATCAAAC- CAGCGATAAA) and reverse (GCGAAACCGGATTACGAGCAG) and RFP forward (ATGACATCCCGCAGCA) and reverse (TGGGGTTG TGGAGCCGCAG). 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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