Intracellular routing of β-catenin

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On the cover: "routing in a mystical world" picture taken by author in Ngorongoro Crater, Tanzania

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"Een dag niet gelachen is een dag niet geleefd"
In liefdevolle herinnering aan mijn moeder Marian.

Contents

Chapter 1

General introduction and scope of this thesis

General introduction

The Nuclear Pore Complex

In eukaryotes, the nuclear envelope separates the nucleus from the cytoplasm and thereby fundamental cellular processes like transcription and translation. This compartmentalization allows additional regulation of essential processes like gene expression and signaling during nuclear-cytoplasmic transport. As a consequence, numerous transport events are needed in order to relocate all RNA, proteins and small molecules from one compartment to the other. Transport between the nucleus and cytoplasm occurs via the Nuclear Pore Complexes (NPC), which are large supramolecular assemblies of ~125 kDa embedded in the nuclear membrane (Reichelt et al., 1990). The NPCs mediate both passive diffusion of small molecules and metabolites and active transport of larger molecules. The transport capacity of NPCs is high, with 500 to 1,000 molecules actively passing a single NPC each second (Ribbeck and Gorlich, 2001; Smith et al., 2002).

Proteomics have shown that despite its huge size, the NPC consists of only 29 different NPC proteins, called nucleoporins or nups. The small number of nups is compensated by a high copy number of the proteins, so that between 8 and 48 copies per nucleoporin are required for the assembly of the vertebrate NPC (Cronshaw et al., 2002). The NPC consists of a central spoke-ring that displays an 8-fold rotational symmetry around a channel that spans the nuclear envelope (Unwin and Milligan, 1982; Maul, 1971). Connected to this central ring are a cytoplasmic ring from which 50 nm filaments extend into the cytoplasm and a nuclear ring, from which a 120 nm basket structure extends into the nucleus (Figure 1A) (Franke and Scheer, 1970a; Franke and Scheer, 1970b; Stoffler et al., 1999). These peripheral structures of the NPC have been visualized by electron microscopy (Figure 1B). Much of the current knowledge of the NPC is derived from studies using yeast. A major difference between the yeast and vertebrate NPC is that the yeast NPC is mobile in the nuclear envelope while the vertebrate NPCs are anchored and therefore static. (Goldberg and Allen, 1995; Belgareh and Doye, 1997; Bucci and Wente, 1997). Nevertheless, despite the smaller size of the NPC in yeast,

the overall structure of the complex is very similar to that of vertebrates (Rout and Blobel, 1993).

There is little homology in the protein sequences of the nucleoporins between different species. This is surprising, as large protein complexes tend to depend on critical protein-protein interactions and, therefore, would be expected to reveal conservation at the primary protein sequence level. The most homologous sequences are the phenylalanine glycine repeats that are present in about one third of all nucleoporins (Rout et al., 2000; Cronshaw et al., 2002). These so-called FG repeats are thought to play an important role in nuclear transport through direct interaction with nuclear transport receptors. Many different models for nuclear translocation have been proposed, but all suggest that FG repeats may be involved in the translocation process (Ribbeck and Gorlich, 2001; Rout et al., 2003; Ben-Efraim and Gerace, 2001). One of these models is the selective phase model, in which nucleoporins interact with their FG repeat regions to each other to form a hydrophobic meshwork that fills the inner channel of the pore. The FG repeat meshwork would function as a selective sieve that restricts passage of inert molecules but that allows passage of molecules capable of facilitated translocation. Transport receptors are molecules that interact directly with the FG repeats. As a result of this binding, the meshwork opens transiently to allow translocation of the receptor-cargo complex that dissolves in the meshwork. After uptake of the receptor-cargo complex by the meshwork, the FG interactions restore immediately (Ribbeck and Gorlich, 2001). *In vitro* data from yeast suggest that the hydrophobic meshwork could be a gel, as phenylalanine interactions cross-link FG repeats into elastic hydrogels that are essential for viability of yeast (Frey et al., 2006). Interestingly, these hydrogels mediate rapid influx of nuclear transport receptors as well as importin-ß-dependent cargo (Frey and Gorlich, 2007).

A second nuclear transport model which contributes to a broad concept of nucleocytoplasmic transport, is the virtual gating model (Rout et al., 2003). It proposes that the NPC channel creates an energy barrier by Brownian movements of FG repeats, that decorate the entrance of both sides of the pore channel. Large neutral mol-
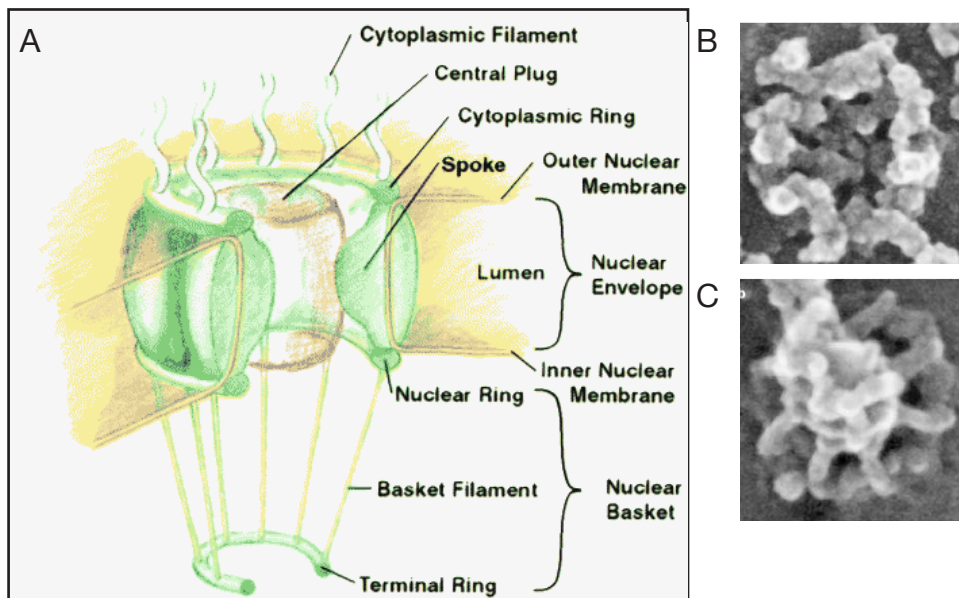
Figure 1. A. Schematic representation of a nuclear pore complex. Indicated are the spoke, nuclear and cytoplasmic ring structures and the nuclear basket and cytoplasmic filaments. From Ohno et al., 1998. B and C. Scanning electron microscopy images of the cytoplasmic (B) and nuclear (C) side of a NPC, adapted from Allen et al., 2000.

ecules cannot overcome the entropic barrier of the channel, whereas transport factors such as the importins, are able to lower the energy barrier by interacting with the FG repeats, catalyzing the transport reaction.

Nuclear Transport

Small metabolites and molecules up to 30 kDa can freely pass the NPC, yet larger cargo can only pass by active transport that requires energy and soluble receptors that can interact with both cargo and NPC (Mattaj and Englmeier, 1998; Gorlich and Kutay, 1999; Frey and Gorlich, 2007). Most of these transport receptors belong to the ß-karyopherin family that has approximately 20 members functioning as a carrier in either import (importins) or export (exportins) (Fornerod et al., 1997; Kudo et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997; Gorlich and Kutay, 1999; Strom and Weis, 2001). Transport receptors recognize their cargo by target signals. The best studied cargo signals are the classical lysine-rich nuclear localisation signals (NLS) and the leucine-rich nuclear export signals (NES). Examples of classical NLS and NES are the NLS of SV40 large T that is recognized by the importin-α/ß dimer, and the NES of HIV-1 that is exported by the export receptor

CRM1 (Gorlich and Kutay, 1999; Fischer et al., 1995; Wen et al., 1995).

Directionality of nucleocytoplasmic transport is provided by the asymmetrical distribution of the GTPase Ran (Figure 2). Like other small GTPases, Ran can either be bound to GDP or GTP and needs cofactors to accelerate the conversion between the two states. The guanine exchange factor of Ran (RanGEF) is called RCC1 and is localised to the nucleus. As cellular levels of GTP exceed those of GDP, RCC1 will load Ran in the nucleus with GTP (Ohtsubo et al., 1989; Bischoff and Ponstingl, 1991). The Ran gradient across the nuclear envelope is steep, because at the cytoplasmic side, RanGTP is quickly hydrolysed by the cytoplasmic GTPase activating proteins (RanGAPs). These proteins are Ran-binding protein 1 and 2 (RanBP1/2) and RanGAP. RanGTP in the nucleus provides directionality to transport as it dissociates imported complexes while stabilizing export complexes (Weis et al., 1996; Izaurralde et al., 1997; Richards et al., 1997). NLS-bearing proteins are recognized by the adaptor molecule Importin-α that binds to the cargo. The Importin-α-cargo dimer is bound by Importin-ß to form a stable import complex that is subsequently translocated into the nucleus. In the nucleus, Ran-GTP binds and dissociates the im-
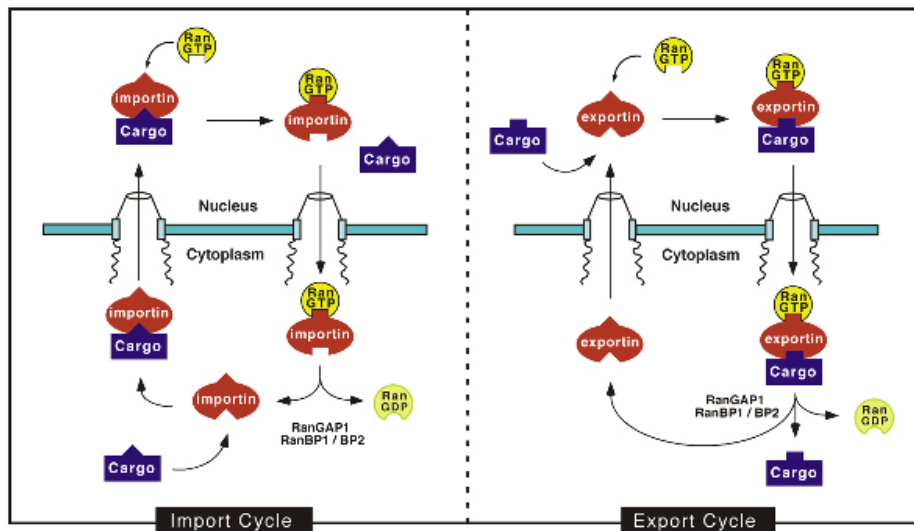
Figure 2. Schematic overview of a nucleocytoplasmic import (left) and export cycle (right). Adapted from (Ohno et al., 1998).

port complex, resulting in the release of the NLS cargo (Rexach and Blobel, 1995; Gorlich et al., 1996). Importin-ß and RanGTP remain together and shuttle back into the cytoplasm for a new round of transport. Importin-α is recycled back to the cytoplasm by its own nuclear export receptor, CAS (cellular apoptosis susceptibility gene) (Kutay et al., 1997). Reversely, export complexes are formed in the nucleus and require the association of Ran-GTP to form a stable complex. Nuclear proteins bearing a classical leucine-rich NES are recognized by chromosome region maintenance 1 (CRM1). The presence of Ran-GTP in the nucleus increases the affinity of CRM1 for the NES of its cargo and stabilizes the export complex (Fornerod et al., 1997). After translocation, the complex dissociates in the cytoplasm due to the GTPase activity of RanGAP and RanBP1/2. RanGDP is translocated back into the nucleus by nuclear transport factor 2 (NTF2), where RCC1 catalyses the generation of RanGTP (Ribbeck et al., 1998; Smith et al., 1998).

RanBP3 as a cofactor of CRM1-mediated nuclear export

CRM1 is the export receptor for NES-bearing proteins but it also exports many other cargos through interaction via adaptor proteins. These cargos include small nuclear RNAs and Snurportin 1, which are involved in splicing and editing of preribosomal subunits (Ohno et al., 2000; Para-skeva et al., 1999). Ran-binding protein 3 (Ran-BP3) and its yeast homologue Yrb2 have been identified as cofactors of CRM1 NES export (Taura et al., 1998; Noguchi et al., 1999; Englmeier et al., 2001; Lindsay et al., 2001). RanBP3 is a nuclear protein that contains a Ran binding domain and FG repeats but it is not associated with the NPC (Mueller et al., 1998). RanBP3 promotes the assembly and export of CRM1-NES complexes by direct binding of RanBP3 to CRM1 (Englmeier et al., 2001). Furthermore, RanBP3 has been shown to function as a nuclear retention factor that retains CRM1 inside the nucleus (Sabri et al., 2007) (Figure 3).

In addition to stabilizing CRM1-dependent NES export, RanBP3 binds to and functions as a cofactor of RCC1 (Taura et al., 1997; Mueller et al., 1998; Nemergut et al., 2002). RanBP3 binds RCC1 in the presence of Ran, preferentially in its GTP-bound state and stimulates its enzymatic activity. RanBP3 could therefore be a co-activator of RanGDP/GTP exchange activity. Moreover, CRM1 has been shown to bind to a complex consisting of RanBP3, RCC1 and RanGDP or RanGTP. Therefore, it has been proposed that RanBP3 could act as a scaffolding protein that brings these components together to increase loading and export of such complexes (Nemergut et al., 2002).
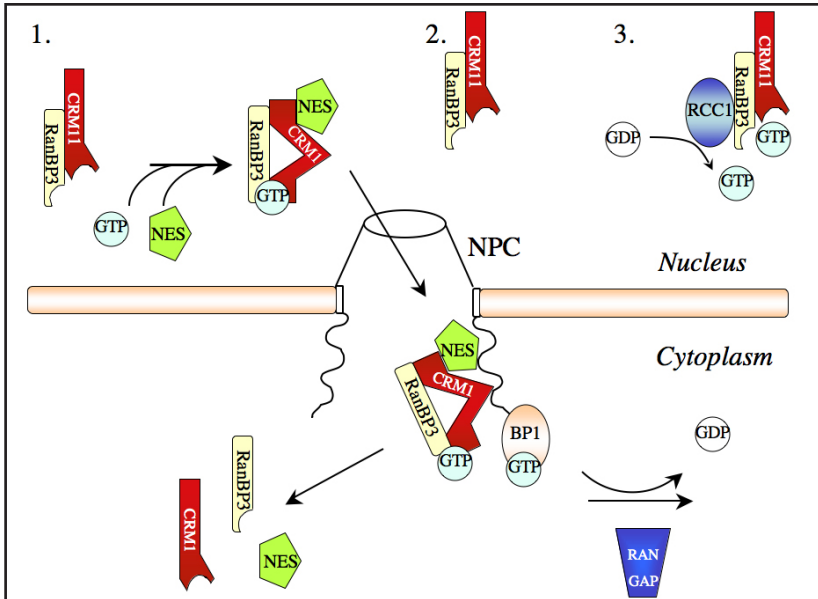
Figure 3. Schematic representation of RanBP3 function. 1. RanBP3 stimulates the stability and export of CRM1-NES export complexes. 2. RanBP3 functions as a nuclear anchor keeping CRM1 concentrations high in the nucleus. 3. RanBP3 stimulates RanGEF activity to increase the efficiency of CRM1 loading with NES cargo.

Ran and transport receptor independent translocation

A few molecules have been identified that are larger than 30 kDa, yet pass the pore independently of Ran and transport receptors. These are importin-α, ß-catenin and p120, all members of the armadillo (arm) repeat family that is characterized by superhelical arm repeats involved in protein-protein interactions (Miyamoto et al., 2002; Fagotto et al., 1998; Eleftheriou et al., 2001; Wiechens and Fagotto, 2001; Roczniak-Ferguson and Reynolds, 2003). The mechanism these proteins use to translocate into and out of the nucleus is exceptional, as they do not rely on the normal nuclear transport pathways. Structurally, the arm repeats are related to the HEAT repeats of the transport receptors that are critical for interaction with the nucleoporins (Malik et al., 1997). A possible transport mechanism of importin-α, ß-catenin and p120 is therefore that they use their arm repeats to translocate through the nuclear pore. In this way they resemble the nuclear transport receptors, rather than being cargo molecules. Indeed, the arm repeats of p120 and ß-catenin have been shown to be essential for nuclear import and export. Moreover, importin-α and ß-catenin have been shown to mediate nuclear import of Ca^{2+}/calmodulin-dependent kinase type IV and Lef1 respectively, showing that they can take along some cargo as they pass the NPC (Roczniak-Ferguson and Reynolds, 2003; Funayama et al., 1995; Kotera et al., 2005; Asally and Yoneda, 2005). Although most proteins rely on the nuclear transport receptors to enter and exit the nucleus, few exceptions have now been identified that seem to mediate their own transport through the NPC.

The Wnt signaling cascade

Wnt proteins are secreted ligands that mediate signaling from cell to cell and regulate a wide variety of processes during animal development and tissue homeostasis. These include axis determination and organ development during embryogenesis, self-renewal of stem cells and regulation of proliferation and differentiation of progenitor cells in adult life (Clevers, 2006). Interestingly, continuous stimulation of the Wnt pathway may contribute to the depletion of stem cells, which as has been shown in mice lacking the secreted Wnt antagonist Kloto, results in increased ageing (Brack et al., 2007; Liu et al., 2007). Not surprisingly, malfunction of the Wnt signaling cascade is implied in many diseases including cancer, bone density defects, diabetes and Alzheimer's (Moon et al., 2004).

The output of the secreted Wnt signal is cell type

specific and is determined by the expression of Wnt receptors on the receiving cell and not by the Wnt ligand. Wnt signaling regulates a large number of target genes and many of them contribute to feedback loops regulating the Wnt pathway itself (for a complete list of target genes see http://www.stanford.edu/~rnusse/wntwindow.html). There are 19 different Wnts in humans and they are extensively conserved between different species (for a review; Nusse, 2005). Wnts are cysteine-rich proteins that are very hydrophobic due to palmitoylation, a modification that is essential for their activity (Willert et al., 2003). There are two types of Wnt receptors, the classical Frizzleds (Fz) and LRP5/6 (Arrow in *D. melanogaster*) (Bhanot et al., 1996; Wehrli et al., 2000). Frizzleds are large transmembrane proteins that span the plasma membrane 7 times and directly interact with the extracellular cystein-rich domain of Wnt (Hsieh et al., 1999; Dann et al., 2001). LRP5/6 are single spanning transmembrane receptors that can also interact with Wnt, yet do so with much lower affinity compared to Fz (Tamai et al., 2000).

The key event in Wnt signaling is the cytoplasmic stabilization and accumulation of ß-catenin that subsequently delivers the signal to the nucleus where it regulates the expression of target genes (Figure 4), (van de Wetering et al., 1997). In Wnt unstimulated cells, ß-catenin is mainly present in the adherens junctions while free cytoplasmic ß-catenin is rapidly degraded by the action of a degradation complex, consisting of the scaffolding proteins Axin and APC, and the kinases GSK3α/ß and CK1. The complex marks ß-catenin for degradation by phosphorylating the protein on its N-terminus (Hart et al., 1998). CK1 acts as a priming kinase by phosphorylating Ser 45 on ß-catenin after which GSK3 phosphorylates Ser 33, 41 and Thr 37 (Liu et al., 2002; Amit et al., 2002; Yanagawa et al., 2002). Phosphorylated ß-catenin is subsequently recognized by the E3 ligase ßTrCP (ß-transducin repeat-containing protein) that poly-ubiquitinates the protein, after which it is degraded by the proteasome (Hart et al., 1999; Aberle et al., 1997).

In the presence of a Wnt signal, Wnt binds to the Frizzled and LRP5/6 receptors thereby initiating a downstream signaling process. For a long time, Dishevelled was known as an activator of the Wnt pathway and was placed between the receptor and the degradation complex. It was shown to interact with the Fz receptor and to be phosphorylated in response to Wnt stimulation (Wong et al., 2003; Wallingford and Habas, 2005). Recently, a

study implied that Dishevelled oligomers cluster the Wnt/Fz/LRP complexes into large multimeric protein complexes at the plasma membrane, shortly after stimulation of cells with Wnt3a (Bilic et al., 2007). These clusters are termed LRP signalosomes and contain multiple components of the Wnt cascade, including the kinases involved in the phosphorylation of LRP6.

Wnt stimulation results in the phosphorylation of the intracellular tail of the LRP6 receptor by a membrane-tethered CK1, called CK1γ, and GSK3ß (Davidson et al., 2005; Zeng et al., 2005). This seems a paradox as CK1α,δ,ε and GSK3ß are antagonists of the Wnt pathway by degrading ß-catenin. Yet, these kinases are known to act as both agonists and antagonists in different signaling cascades and their activity is most likely determined in a spatial and temporal manner (Price, 2006). GSK3ß phosphorylates LRP6 on multiple PPPSP consensus sites, and CK1γ phosphorylates LRP6 on sites in close proximity of the PPPSP residues. Both kinases are essential for Wnt signal transduction and it remains to be elucidated whether Wnt signaling regulates the activity of GSK3, CK1 or both. The five PPPSP motifs in LRP5/6 cooperatively promote phosphorylation of each other which amplifies Wnt signaling (MacDonald et al., 2008). Phosphorylated PPPSP sites serve as docking sites for Axin that is recruited to the receptor complex. This is an important step in Wnt signaling as it results in the degradation of Axin by an as yet unidentified mechanism (Mao et al., 2001). Axin is present in limiting amounts in the cell (Lee et al., 2003) and without this scaffolding protein, the destruction complex is disabled, allowing ß-catenin to stabilize and transduce the signal to the nucleus. A relatively novel component of the destruction complex is the microtubule-associated factor MACF1 (microtubule actin crosslinking factor) that is implicated in recruiting Axin to the plasma membrane upon Wnt stimulation (Chen et al., 2006).

Wnt signaling in the nucleus

ß-Catenin is the signal transducing member of the Wnt cascade. In the nucleus, ß-catenin associates with HMG box transcription factors of the TCF/Lef family to regulate the expression of Wnt target genes (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). In a complex with TCF, ß-catenin can regulate transcription, as TCF contains the DNA binding site and ß-catenin the transactivation domain (van de Wetering et al., 1997). Without Wnt, TCF/Lef pro-

teins are already bound to target genes, but their association with repressor proteins like Groucho, CtBP and histone deacetylases, results in silencing of the genes (Cavallo et al., 1998; Roose et al., 1998; Brannon et al., 1999). When ß-catenin enters the nucleus, it physically replaces Groucho on TCF/Lef (Daniels and Weis, 2005) and recruits chromatin remodelling proteins, like Bgr1 (Barker et al., 2001), and histone modification proteins like the acetylase CBP/p300 (Hecht et al., 2000; Takemaru and Moon, 2000) to its C-terminus to promote target gene activation. B-cell lymphoma-9 (Bcl-9, Legless in *D. melanogaster*) and Pygopus are two essential co-activators in the TCF/ß-catenin complex. Bcl9 interacts directly with the N-terminus of ß-catenin and acts as a linker protein to attach Pygopus to the transcription complex. It has been suggested that Bcl-9/Pygopus activate Wnt signaling by nuclear import or retention of ß-catenin (Townsley et al., 2004) or, alternatively, by acting as transcriptional activators (Hoffmans et al., 2005). Other nuclear regulators of ß-catenin include Chibby, which inhibits transactivation by competing with TCF for ß-catenin binding, and ICAT, a polypeptide that binds to ß-catenin and prevents its interaction with TCF (Tago et al., 2000; Takemaru et al.,

2003). APC and Axin function in the degradation complex in the cytoplasm but can enter the nucleus and could therefore compete with TCF for ß-catenin binding (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000; Wiechens et al., 2004). A role for APC in export followed by cytoplasmic degradation of ß-catenin has been described (Henderson, 2000). Furthermore, APC may function as a transcriptional inhibitor by facilitating CtBP-mediated repression of Wnt target genes (Sierra et al., 2006).

ß-Catenin nuclear transport in more detail

ß-Catenin functions with its family members α-catenin and p120 in cell adhesion as a structural component of adherens junctions. In this complex, ß-catenin connects E-cadherin to α-catenin that dynamically interacts with the actin cytoskeleton (McCrea et al., 1991; Kemler, 1993; Drees et al., 2005; Yamada et al., 2005). Besides its structural role at the plasma membrane, ß-catenin functions as a key player in the Wnt signal transduction pathway. In this signaling cascade, the protein translates the extracellular Wnt signal into a transcriptional response by regulating transcription of target genes in a com-
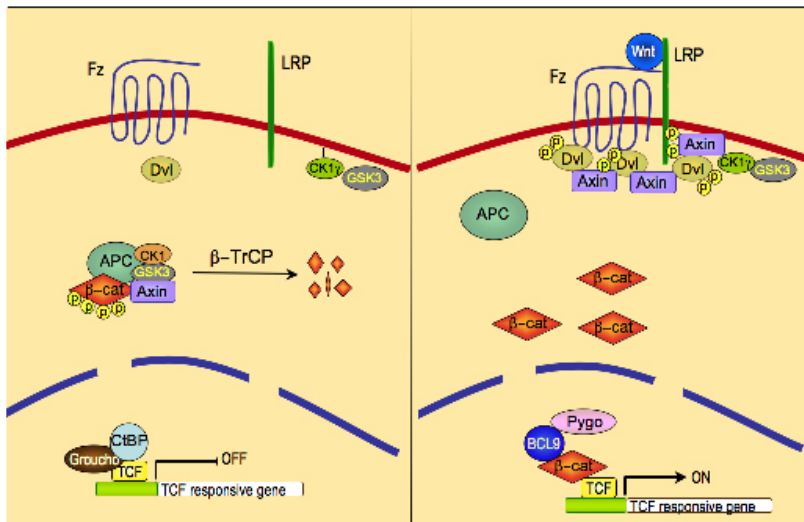
Figure 4. Schematic representation of the canonical Wnt signaling cascade. Left panel; without Wnt, ß-catenin is trapped by the degradation complex and phosphorylated by GSK3α/ß and CK1. Phosphorylated ß-catenin is recognized by the E3 ubiquitin ligase ßTrCP and rapidly degraded by the proteasome. In the nucleus, binding of Groucho and CtBP inhibit TCF-mediated transcription of Wnt target genes. Right panel; Wnt binds to Fz and LRP5/6, bridging these two receptors, regulating the phosphorylation of Dvl that is recruited to Fz. Dvl oligomers cluster Fz/LRP complexes which could activate the kinases CK1γ and GSK3 to phosphorylate the intracellular domain of LRP5/6. This acts as a docking site for Axin, which is sequestered from the degradation complex, allowing ß-catenin to accumulate and enter the nucleus. In the nucleus, ß-catenin replaces Groucho on TCF and interacts with co-activators Bcl9 and Pygo to activate the transcription of Wnt target genes.

plex with TCF/Lef transcription factors (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). Nuclear import and export of ß-catenin are therefore crucial regulatory events in the Wnt signaling cascade.

With a molecular weight of 92 kDa, ß-catenin nuclear import would be expected to rely on the importin-α/ß pathway. However, ß-catenin does not contain an NLS. Since the protein was found to localize to the nucleus of cells with over-expressed TCF/Lef, it was originally hypothesized that ß-catenin would enter the nucleus with Lef1 in a piggy-back mechanism (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). A ß-catenin mutant that lacks the TCF/Lef binding domain can however, still enter the nucleus, refuting the piggy-back mechanism (Orsulic and Peifer, 1996; Prieve and Waterman, 1999). In *in vitro* transport assays, where digitonin is used to permeabilize the plasma membrane while keeping the nuclear envelope intact, ß-catenin was able to enter the nucleus without the need to add back washed out nuclear transport receptors or RanGTP (Fagotto et al., 1998; Yokoya et al., 1999; Suh and Gumbiner, 2003). These studies show that in principle, ß-catenin only relies on itself and its interactions with the nucleoporins to translocate into the nucleus. As nuclear translocation of ß-catenin is an important step in the Wnt signaling cascade, the unusual nuclear import mechanism of ß-catenin is both surprising and intriguing. It is therefore expected that under physiological conditions ß-catenin import is subject to Wnt signaling-dependent regulation, most likely through retention of the protein in the cytoplasm or nucleus. Mapping of the ß-catenin domains that are necessary for nuclear import showed that arm repeats 10-12 and the C-terminus are required for nuclear import and that these domains largely overlap with those required for export (Koike et al., 2004).

Nuclear export of ß-catenin is somewhat similar to its import because the protein does not contain a recognizable NES. In micro-injection and permeabilized cell experiments, ß-catenin was shown to exit the nucleus on its own, independently of CRM1 nuclear export and RanGTP (Prieve and Waterman, 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). In addition to the model in which ß-catenin can enter and exit the nucleus on its own, it has been proposed that ß-catenin exits the nucleus by riding along with the Wnt signaling components APC, Axin or the protein LZTS2, that depend on their NES sequences to be exported via the CRM1 nuclear

export pathway (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000; Wiechens et al., 2004; Cong and Varmus, 2004; Thyssen et al., 2006). Especially, published data suggest a role of APC in nuclear export of ß-catenin (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). These data are based upon co-localisation experiments in which ß-catenin was found to mimic the localisation of APC in cells containing either active or inactive NESs (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). However, the studies suggesting a role of APC, Axin and LZTS2 in ß-catenin nuclear export ignore the observations that ß-catenin is capable of nuclear export on its own, and that it is not influenced by inhibition of the CRM1 pathway (Yokoya et al., 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001; Krieghoff et al., 2006). In addition, mutant ß-catenin lacking its APC binding domain is capable of nuclear export, and a complex between APC/Axin, ß-catenin, CRM1 and RanGTP has not been identified (Prieve and Waterman, 1999). Yet, the discussion whether ß-catenin exits the nucleus by itself or via the CRM1 pathway has not ended, as data from Eleftheriou et al. (2001) show that APC might contribute to a minor proportion of ß-catenin nuclear export, as a small fraction of endogenous ß-catenin was sensitive to leptomycin B (LMB) treatment in semi-permeabilized SW480 cells (Eleftheriou et al., 2001; Henderson and Fagotto, 2002). Measuring the nuclear transport kinetics of ß-catenin in living cells will aid to our understanding of ß-catenin nuclear transport.

Crosstalk between ß-catenin at the plasma membrane and Wnt signaling

ß-Catenin has a dual role in cell-cell adhesion and Wnt signaling transduction (Nusse, 2005). As such, it can interact with many proteins at different locations in the cell. At the plasma membrane, ß-catenin binds E-cadherin and α-catenin, in the cytoplasm it binds to the proteins of the destruction complex, and in the nucleus it interacts with many transcriptional regulators. The arm repeats mediate most of these complex interactions. It is interesting that the binding domains of E-cadherin, APC, Axin and TCF all overlap, suggesting that there is competition between these proteins for binding to ß-catenin (Ha et al., 2004; Huber and Weis, 2001; Graham et al., 2000; Cox et al., 1999). How is the interaction between ß-catenin and its multiple binding partners regulated? This is an important question as it relates to the issue of whether ß-catenin at the plasma membrane is

communicating with ß-catenin in the Wnt signaling cascade.

For a long time, it was thought that the pools of ß-catenin for cell-cell adhesion and transcription were separated. Experiments in fruit flies have shown that when armadillo/ß-catenin was limiting, ß-catenin was integrated into the adherens junctions (Cox et al., 1996). It was therefore thought that newly synthesized ß-catenin mostly associated with E-cadherin because this binding affinity was highest. The remaining ß-catenin molecules in the cytoplasm would be trapped by the degradation complex in the cytoplasm and degraded (Hart et al., 1998; Aberle et al., 1997). Only in the presence of Wnt, ß-catenin would escape degradation and signal to the nucleus. However, some recent studies have revealed the existence of molecular switches that determine whether ß-catenin interacts with adherens junctions or transcriptional complexes (Gottardi and Gumbiner, 2004; Brembeck et al., 2004). Gottardi and Gumbiner have shown that different molecular forms of ß-catenin control the interactions between ß-catenin and its binding partners. Biochemistry shows that in the absence of Wnt, ß-catenin binds equally well to E-cadherin and TCF. However, Wnt stimulated cells generate a monomeric form of ß-catenin that preferentially binds TCF. Furthermore, the C-terminus of ß-catenin can fold back and interact with its own arm repeats. This conformation preferentially interacts with TCF rather than with E-cadherin (Cox et al., 1999; Piedra et al., 2001; Castano et al., 2002; Gottardi and Gumbiner, 2004). Thus, Wnt signaling generates a form of ß-catenin that favours the interaction with TCF to stimulate transcription. The molecular details of this model are unclear, although posttranslational modifications are likely candidates (Gottardi and Gumbiner, 2004).

Direct communication between the adherens junctions and the signaling pool of ß-catenin has also been reported. During epithelial-to-mesenchymal transitions (EMT), cadherin complexes fall apart, thereby releasing high levels of ß-catenin that could theoretically contribute to Wnt signaling. The structural and functional integrity of the cadherin-catenin complex is regulated by phosphorylation (Lilien et al., 2002). Phosphorylation of ß-catenin (or E-cadherin) on serine/threonine results in increased stability (Bek and Kemler, 2002; Lickert et al., 2000), while phosphorylation of ß-catenin on tyrosine results in its release from E-cadherin. Tyrosine phosphorylation of ß-catenin initiates EMT, which plays important roles during embryonic development and tumour metastasis (Behrens et al., 1993; Fujita et al., 2002; Piedra et al., 2003). A subset of protein kinases that are not members of the Wnt pathway have been shown to phosphorylate ß-catenin on tyrosine. These include c-Src, c-MET, ErbB2 and RTK (Roura et al., 1999; Danilkovitch-Miagkova et al., 2001). Birchmeier's lab has shown that BCL9-2, a transcriptional activator of TCF/ß-catenin, functions as a molecular switch between ß-catenin's adhesive and transcriptional functions. They have shown that ß-catenin interacts directly with BCL9-2 and that this interaction is increased after ß-catenin phosphorylation on Tyr142. (Brembeck et al., 2004). This phosphorylation event disrupts binding of ß-catenin to α-catenin, blocks its interaction with the destruction complex (Danilkovitch-Miagkova et al., 2001), and increases transcriptional activation (Brembeck et al., 2004). Interestingly, increasing BCL9-2 levels shifts ß-catenin from the cadherin complex to the nucleus and induces EMT, while knockdown of BCL9-2 has opposite effects (Brembeck et al., 2004). BCL9-2 thus acts as a switch that the cell can use to shift the balance between ß-catenin in cell adhesion and Wnt signaling. Recently, the structure of zebrafish ß-catenin showed that there is a significant hinge motion at Arg151 which overlaps the α-catenin and BCL9-2 binding site (Xing et al., 2008). Whether phosphorylation of Tyr142 affects this dynamic hinge motion and or the choice between α-catenin or BCL9-2 binding should be subject of future studies. It is clear that cross-talk between ß-catenin at the adherens junctions and signaling pool is possible, and that the processes that regulate these decisions are very complex (for reviews see (Nelson and Nusse, 2004; Harris and Peifer, 2005)). Knowledge about the processes that regulate the balance between cell adhesion and cell signaling is important for our understanding of embryonic development and tumourigenesis.

Wnt signaling and cancer

The development of cancer is a multistep process that involves mutations in both oncogenes and tumour suppressor genes resulting in uncontrolled cell division, resistance to apoptosis, invasion of surrounding tissues, metastasis and stimulation of angiogenesis. It is important to identify the biological pathways affected by these mutations to design specific anti-cancer drugs. The Wnt pathway is the driving force of many cancers and mutations in this pathway are found in both sporadic and hereditary forms of cancer, including colon, breast and hepatocellu-

lar carcinomas (Polakis, 2000). The role of Wnt signaling in cancer is best described for colorectal cancer. The normal physiological function of the Wnt pathway in the colon is to regulate the number of stem cells in the crypts, the area where cell division occurs. In these crypts, Wnt signaling is active and ß-catenin/TCF4 complexes transactivate target genes that trigger a cell proliferative program. From the crypts, epithelial cells differentiate by shutting down Wnt signaling and gradually move up along the villi, where they eventually shed into the gut lumen (Korinek et al., 1998). Inhibition of Wnt signaling in the villi coincides with increased cellular APC levels and decreased levels of nuclear ß-catenin, which allows the cells to differentiate (Smith et al., 1993; Midgley et al., 1997).

Over 90% of all cases of human colorectal cancers show activating mutations in the Wnt pathway, mostly truncating mutations in APC. Interestingly, when APC is found to be intact in colorectal cancers, the tumour contains activating mutations in ß-catenin (Morin et al., 1997). These mutations alter the N-terminal phosphorylation of ß-catenin and thereby its stability and activity. Other mutations in the Wnt pathway that are linked to cancer occur in Axin or its homologue Axin2/Conductin, which also affect the degradation of ß-catenin (Satoh et al., 2000; Clevers, 2000; Liu et al., 2000). Transcriptional regulation of ß-catenin/TCF4 target genes is an important mechanism by which Wnt signaling leads to colorectal cancer as it allows growth advantage for initial expansion. Although the transcriptional output of Wnt signaling is cell type specific and highly diverse, many TCF target genes repress differentiation and thus could stimulate tumorigenesis (van de Wetering et al., 2002; Willert et al., 2002). There are also some well-known TCF targets with clear roles in tumourigenesis, such as the cell cycle regulator c-Myc and the matrix metalloproteinase matrilysin, which could stimulate invasion at a later stage of tumourigenesis (He et al., 1998; Brabletz et al., 1999; Crawford et al., 1999).

The development of colorectal cancer is histologically defined by distinct steps that reflect tumour acquiring mutations. This is called the adenoma-to-carcinoma sequence. Four to five mutations in oncogenes and tumour suppressor genes are thought to be necessary for the development of a malignant tumour (Fearon and Vogelstein, 1990). The first mutation is usually in APC and induces the formation of an adenoma (Powell et al., 1992). A clear example of the early effects of APC mutations in colorectal cancer is the familial adenomatous polyposis syndrome, or FAP (Kinzler et al., 1991; Nishisho et al., 1991). This autosomal dominant disease is characterized by inherited mutations in APC and results in an early manifestation of hundreds of adenomatous lesions in the colon and rectum with an increased risk of progression of the benign polyps into adenocarcinomas.

When Wnt signaling is activated and target gene expression leads to clonal expansion, the environment is set for the acquisition of additional mutations. In the adenoma-to-carcinoma sequence, K-Ras is often mutated as a second hit and mutation of this oncogene acts synergistically with Wnt signaling (Janssen et al., 2006). This effect is explained by the ability of K-Ras to induce phosphorylation of ß-catenin on tyrosine, which increases nuclear ß-catenin levels due to decreased affinity of ß-catenin for E-cadherin (Kinch et al., 1995). Additional mutations in the colorectal sequence occur in members of the TGFß pathway and p53; these result in increased ß-catenin signaling as well as genomic instability (Vogelstein et al., 1988).

There are two types of genomic instability: micro satellite instability (MIN), which is characterized by a high mutation rate due to defects in mismatch repair genes, and chromosomal instability (CIN), characterized by chromosomal rearrangements due to mitotic defects (Rajagopalan and Lengauer, 2004). In colorectal cancer, CIN occurs in 85% of the tumours and MIN in 15% (Lengauer et al., 1997; Lindblom, 2001). MIN plays an important role in hereditary non-polyposis colon cancer (HNPCC) that is caused by germ line mutations in mismatch repair genes (Lynch and Lynch, 2000). Defects in chromosomal segregation and aneuploidy occur already early in tumourigenesis, before loss of p53 (Shih et al., 2001). In colorectal cancer the incidence of mutations in spindle checkpoint genes is low (Cahill et al., 1998). Some recent studies may provide an explanation, as they describe a direct role for APC in chromosome segregation and CIN. During mitosis, APC localizes to the kinetochores, while truncating mutations in APC have been shown to cause spindle aberrations, aneuploidy and structural abnormalities in chromosomes (Fodde et al., 2001; Kaplan et al., 2001; Dikovskaya et al., 2004; Green and Kaplan, 2004; Tighe et al., 2004). Moreover, Wnt signaling could be involved in tumour progression through CIN at the level of ß-catenin/TCF-mediated transcription. This effect may be indirect, through regulation of Cdc2

or direct through increased transcription of Conductin, which is thought to regulate the spindle checkpoint (Aoki et al., 2007; Hadjihannas et al., 2006).

Finally, the microenvironment plays an important role in tumourigenesis. In colon cancer, inflammation further increases Wnt signaling as, for example, macrophages can secrete Wnt3a (Smith et al., 1999). In addition, release of prostaglandin E2 during infection results in activation of prostaglandin E2 receptors that couple to G proteins. These G proteins bind Axin, which is sequestered to prevent downregulation of ß-catenin. (Castellone et al., 2005). These effects may explain the success of anti-inflammatory drugs like aspirin in the treatment of colon cancer as these drugs inhibit cyclo-oxygenase 2 (COX-2), the rate-limiting enzyme in prostaglandin synthesis (Brown and DuBois, 2005).

Our knowledge of the Wnt signaling pathway has increased dramatically in the past decades and has identified many targets for drug interference to treat cancer. Small molecule inhibitors and antagonists of the pathway are promising new drugs for the future. A better understanding of the Wnt pathway will help to explain why certain genetic profiles are linked to poor diagnostic outcome.

Aim and outline of this thesis

ß-Catenin is an important protein for cancer research as it influences numerous events in the cell that lead to the development of cancer when gone awry (reviewed in Giles, 2003). At the adherens junctions, ß-catenin functions in cell-cell adhesion to maintain epithelial organisation (McCrea et al., 1991; Kemler, 1993). As an effector of Wnt signaling, ß-catenin controls numerous developmental processes as well as homeostatic self-renewal (Nusse, 2005). The effector function of ß-catenin is to form a transcriptional complex in the nucleus with TCF/Lef transcription factors to regulate target gene expression (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). Due to the dual function of ß-catenin in cell adhesion and signaling, there are different pools of the protein. The research described in this thesis focuses on the role of ß-catenin in the Wnt signaling pathway. What is the pool of ß-catenin that is active in signaling? Where is active ß-catenin localized? Where and how is ß-catenin activated and how is its nuclear export regulated to terminate Wnt signaling.

Chapter 1 provides a general introduction about the different aspects of nuclear transport and the Wnt signaling cascade, putting it into the context of cancer development. **Chapter 2** describes the identification of Ran-binding protein 3 (RanBP3) as a novel regulator of the active signaling form of ß-catenin. We initiated this study to investigate the nuclear translocation of ß-catenin and found that RanBP3 directly inhibits ß-catenin signaling by stimulating nuclear export of the transcriptionally active form of ß-catenin. The active form of ß-catenin is unphosphorylated on its N-terminus, and covers only a small fraction of the total amount of ß-catenin in the cell. We therefore continued to study the localization of this pool of ß-catenin in **Chapter 3**. We describe that a relative large pool of unphosphorylated ß-catenin resides at the adherens junctions, where it most likely functions in cell-cell adhesion. As Wnt treatment induces recruitment of unphosphorylated ß-catenin to the plasma membrane, it is impossible to distinguish the resident junctional pool of unphosphorylated ß-catenin from the signaling pool. We emphasize the importance of an E-cadherin null background in studying signaling competent unphosphorylated ß-catenin. In **Chapter 4**, we study the unphosphorylated ß-catenin pool at the plasma membrane upon Wnt signal induction in E-cadherin knock out cells. Plasma membrane recruitment of ß-catenin in the early steps of the Wnt signaling cascade fits with recent new insights, which suggest recruitment of Axin and Dvl to the activated Wnt receptor LRP5/6. We expand these insights by showing that active ß-catenin, Axin, APC and activated LRP6 receptor all localize to the plasma membrane upon Wnt stimulation. Moreover, we find that Wnt induced ß-catenin is transcriptionally more active than overexpressed ß-catenin. We suggest a model in which plasma membrane recruitment of ß-catenin represents an important step in ß-catenin processing and Wnt signal transduction. In **Chapter 5**, we determine the nuclear export kinetics of ß-catenin in human cells and show that ß-catenin exits the nucleus very fast, independently of the CRM1 export pathway and that ß-catenin can enhance export of the small molecule GFP (green fluorescent protein). These observations fit into a model in which ß-catenin can translocate quickly into and out of the nucleus independently of nuclear transport receptors. Therefore, the activity and localization of ß-catenin are likely to be regulated by retention of the protein in the nucleus, cytoplasm and plasma membrane. Finally, in **Chapter 6** we reconcile our findings with current knowledge of the Wnt signaling cascade.

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CHAPTER 2

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

2

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

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

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 ß-catenin. Cytoplasmic ß-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 3ß (GSK3ß; He et al., 1995). In the absence of a Wnt signal, this complex rapidly phosphorylates ß-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–GSK3ß 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 ß-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 ß-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 ß-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 ß-catenin–TCF transcription complexes (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). ß-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 ß-catenin and TCF4) and Chibby are identified as nuclear proteins that repress Wnt signaling by compet-

ing with TCF for binding to ß-catenin (Tago et al., 2000; Takemaru et al., 2003).

In this study, we aimed to identify new modulators of ß-catenin in the nucleus. We used the nuclear marker RanGTP to select for nuclear factors that directly bind ß-catenin and identified Ran binding protein 3 (RanBP3). We show that RanBP3 inhibits ß-catenin–TCF4–mediated transactivation in human cell lines by relocalization of active ß-catenin from the nucleus to the cytoplasm. In addition, we show that RanBP3 causes ventralization and inhibits ß-catenin–induced double axis formation in *X. laevis* embryos. Loss of *D. 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 ß-catenin and identify its gene as a candidate human tumor suppressor in the commonly deleted chromosomal region 19p13.3.

Results

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

To study the interaction between ß-catenin and nuclear transport factors, we used GST-tagged ß-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 ß-catenin and by the central armadillo (ARM) repeat region (Chapter 5, Fig 5). Interestingly, we found a strong interaction between ß-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 ß-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 ß-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 ß-

catenin (Fig. 1 B). To mimic nuclear conditions, 2 µM of a nonhydrolysable mutant of the small GT-Pase Ran (RanQ69L-GTP) was added, resulting in increased interaction between ß-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 ß-catenin (Fig. 1 B, lane 2).

To investigate whether the binding between ß-catenin and RanBP3 was direct, we performed pull down assays with GST-tagged ß-catenin and recombinant RanBP3. Human RanBP3-b interacted directly with GST-ß-catenin with an optimum at 0.5 µM RanBP3 (Fig. 1C, lane 3). These binding characteristics resemble the interaction of RanBP3 with 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 due to a point mutation in its RanGTP binding domain (RanBP3 "wv" mutant (Englmeier et al., 2001). This mutant interacted only very weakly with ß-catenin and lost its ability to bind at an optimum concentration (Fig. 1C, lanes 5-7). These data suggest that RanGTP increases the affinity of RanBP3 for ß-catenin. To confirm the RanGTP dependency, RanBP3 was bound to ß-catenin columns at the optimal concentration of 0.5 µM in the presence of RanGTP and eluted either in the absence or presence of the recombinant Ran cofactors RanBP1 and RanGAP (lanes 8-11). While virtually no RanBP3 was eluted with buffer only, significant amounts were detected after elution in the presence of either 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 ß-catenin that forms active transcriptional regulation complexes with transcription factors of the TCF/Lef family. A well-established functional read-out of Wnt signaling makes use of TCF responsive luciferase reporter constructs (Korinek et al., 1997). To test the functional relevance of the interaction between ß-catenin and RanBP3, we transfected human embryonic kidney cells (HEK293) with reporter constructs that contain either three optimal TCF binding sites (TOP) or three mutated binding sites (FOP). Transfection of a Wnt1 plasmid resulted in a strong activation of the TOP reporter but not of the FOP control (Fig. 2B). Co-transfection of increasing amounts of RanBP3 repressed Wnt1/ß-catenin transactivation dose-dependently (Fig 2B). A mutant of RanBP3 that cannot interact with RanGTP and
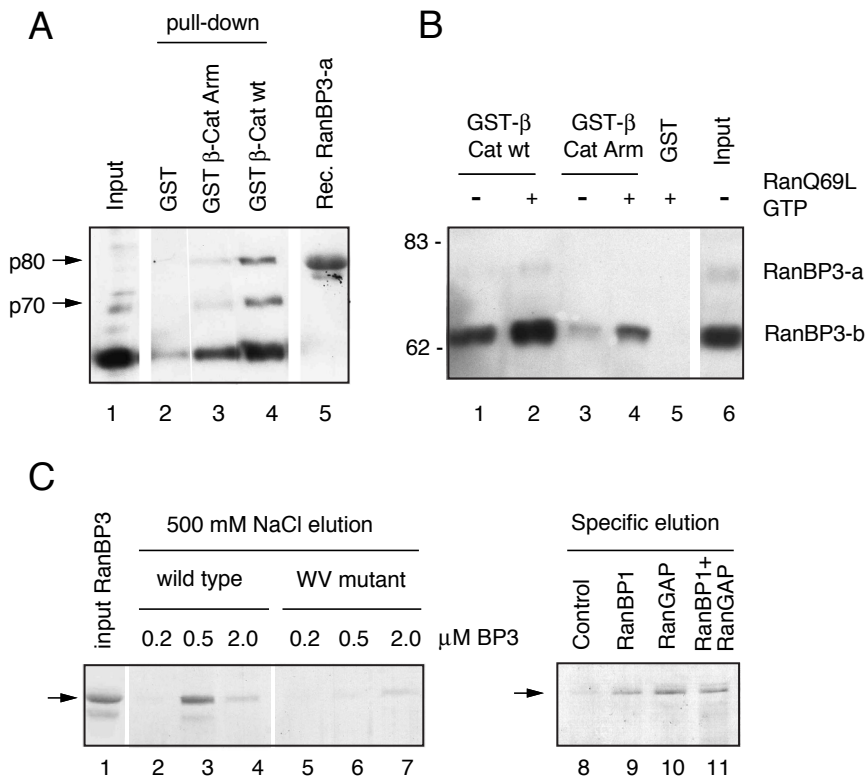
Figure 1. Identification of RanBP3 as an interaction partner of ß-Catenin. A. Pull down experiment using immobilized GST (lane 2), GST-tagged ß-Catenin ARM repeats 1-12 (lane 3) and full length ß-Catenin (lane 4) incubated with Xenopus egg extract (input, lane 1). Bound proteins were analyzed by Western blot using Mab 414 recognizing a subset of nucleoporins. Two unknown proteins p80 and p90 are marked with an arrow. 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 ß-Catenin. GST-tagged full length ß-Catenin (lanes 2-11) was incubated with 2 μM RanGTP and 0.2 μM (lanes 2 and 5), 0.5 μM (lanes 3, 6, 8-11) or 2.0 μM (lanes 4 and 7) wild type (lanes 2-4, 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.

binds ß-catenin with less affinity (Fig. 1C) was less active than wild type RanBP3 (Fig. 2B). To investigate whether RanBP3 inhibits Wnt signaling downstream or upstream of ß-catenin, we mimicked Wnt signaling in HEK293 cells by expressing ß-catenin. RanBP3 could still specifically inhibit activation of the TOP reporter (Fig. 2C), while the RanBP3 "wv" mutant was less effective. These experiments show that RanBP3 inhibits TCF-dependent transcription by acting either on ß-catenin itself, or on regulators downstream of ß-catenin. We confirmed that the expression levels of our wild type and "wv" mutant RanBP3 constructs were equal by analyzing cell lysates from transfected HEK293 cells on Western blot (Fig. 2A).

The interaction of recombinant ß-catenin with RanBP3 (Fig. 1C) implies that RanBP3 can bind N-terminally unphosphorylated ß-catenin, which is thought to be the signaling-competent form of the protein. To test whether this is the case *in vivo*, we used a ß-catenin mutant that contains alanines in all four N-terminal GSK3ß phosphorylation sites (ß-cateninΔGSK3ß, Barth et al., 1999), and therefore is constitutively active. This mutant stimulated expression of the TCF reporter to 2-3 fold higher levels than wild type ß-catenin (data not shown). Co-expression of wild-type RanBP3 lead to a significant reduction in transactivation by ß-cateninΔGSK3ß (Fig. 2D). Again, the RanBP3 RanGTP-binding mutant was less able to repress ß-cateninΔGSK3ß mediated
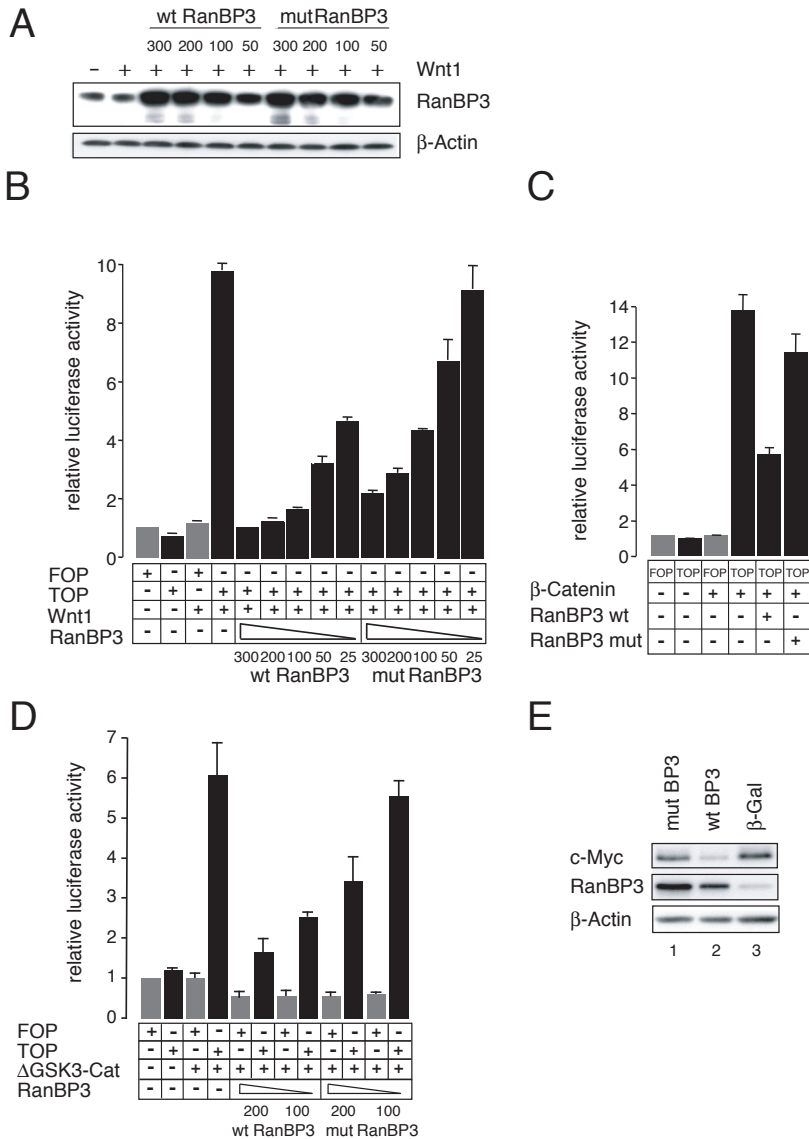
Figure 2. Expression of RanBP3 inhibits ß-Catenin/TCF mediated transcriptional activation. A. Wild type and "wv" mutant RanBP3 are expressed at equal levels. HEK293 cells were transfected with indicated constructs (ng) and lysates were analyzed 48 hrs post-transfection by Western blot with indicated antibodies. B. RanBP3 represses Wnt-1 induced ß-Catenin/TCF mediated transcriptional activation dose-dependently. HEK293 cells were transfected with TOP (black bars) or the control FOP (grey bars), Wnt1, and decreasing amounts of RanBP3 wt or "wv" mutant as indicated (ng) and luciferase activity was measured after 48 hours. C and D. RanBP3 represses transcriptional activation induced by wild type ß-Catenin (C) or ΔGSK3-ß-Catenin (D). HEK293 cells were transfected with indicated constructs and luciferase activity was measured 48 hrs after transfection. In all experiments, normalized relative luciferase values are shown as corrected with pRL-CMV Renilla. Bars represent standard errors of means of independent experiments E. RanBP3 inhibits the expression of the endogenous Wnt target c-Myc. HCT116 colon carcinoma cells expressing Δ45-ß-Catenin were transfected with GFP and ß-galactosidase, RanBP3 wt or mutant plasmids. 2 days after transfection, GFP-positive cells were sorted using flow cytometry, lysed in sample buffer and analyzed by Western blot using indicated antibodies.
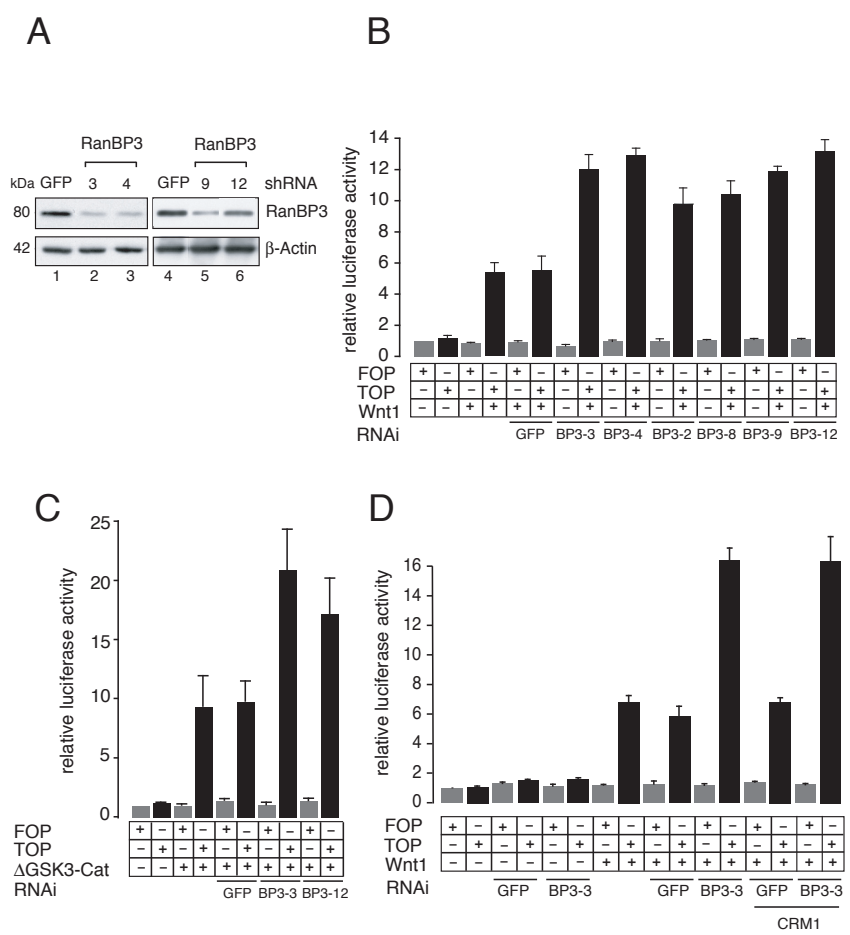
Figure 3. Reduction of RanBP3 by RNAi results in increased ß-Catenin/TCF mediated transcription activation. A. Western blot showing that different short hairpin RNAs (shRNA) against RanBP3 reduce RanBP3 protein levels in HEK293 cells. cells were transfected with shRNAs and pHA262-PUR was co-transfected to introduce puromycin resistence. 24 hrs after transfection, cells were grown on puromycin medium for 48 hrs and cell lysates were prepared and analyzed on Western blot with indicated antibodies B. RNAi against RanBP3 increases Wnt1-induced ß-Catenin/TCF-mediated transcription. HEK293 cells were transfected with indicated constructs and activity of TOP (black bars) and FOP (grey bars) were measured 72 hours after transfection C. RNAi against RanBP3 increases ß-Catenin/TCF driven transcription in HEK293 cells that transiently express an active form of ß-Catenin (ΔGSK3-ß-Catenin). Cells were transfected with indicated constructs and luciferase activity was measured after 72 hours. D. Co-expression of CRM1 with RanBP3 shRNA constructs does not affect ß-Catenin/TCF mediated transcription in Wnt1 transfected cells. HEK293 cells were transfected with indicated constructs and 72 hours after transfection luciferase activity was measured. For all experiments, relative luciferase levels are shown as corrected with CMV-Renilla-luc. Error bars in B represent standard deviations of technical replicates of a representative experiment. Bars in C and D represent standard errors of means of independent experiments.

transactivation.

To address whether RanBP3 could also affect expression of endogenous target genes of ß-catenin/TCF, we expressed RanBP3 in human colon carcinoma cell line HCT116. This cell line harbors an activating mutation in ß-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 proteins levels compared to control cells (Fig 2E, lanes 2 and 3). Although expressed in higher levels, the wv mutant RanBP3 was less capable of
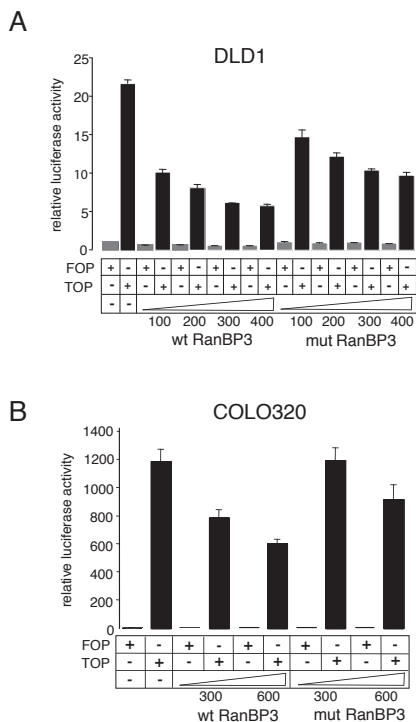
A

DLD1

relative luciferase activity

FOP	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
TOP	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+

100 200 300 400   100 200 300 400
wt RanBP3   mut RanBP3

B

COLO320

relative luciferase activity

FOP	+	-	+	-	+	-	+	-	+	-
TOP	-	+	-	+	-	+	-	+	-	+

300   600   300   600
wt RanBP3   mut RanBP3

Figure 4. RanBP3 antagonizes Wnt/ß-Catenin transactivation in APC mutated colon carcinoma cells. Luciferase assay showing that RanBP3 inhibits ß-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 C-terminal NESs. B. Luciferase reporter assay as in A, carried out 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 ß-Catenin binding and regulatory sites. Relative luciferase activity was measured 48 h post transfection. Bars represent standard deviations of a representative experiment.

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 different unique parts of RanBP3 that are present in all isoforms of RanBP3. We obtained several shRNA RanBP3 constructs that downregulate RanBP3 protein

levels in HEK293 cells (Fig. 3A).
When we co-expressed Wnt1 and RanBP3 shRNAs, we observed significant increases in TCF/LEF reporter activity compared to the GFP RNAi control (Fig. 3B). To test whether RanBP3 depletion also acts on N-terminally dephosphorylated ß-catenin, we cotransfected ß-cateninΔGSK3ß with anti-RanBP3 shRNA expression constructs (Fig. 3C). Reduction of RanBP3 increased reporter activity, confirming that RanBP3 can act on the N-terminally dephosphorylated or "activated" form of ß-catenin. In the absence of Wnt signaling, depletion of RanBP3 did not result in increased reporter activity (Fig. 3D), arguing for a specific effect on ß-catenin. The direct binding of RanBP3 to ß-catenin that we observed (Fig. 1C) indicated that RanBP3 may act on the Wnt signaling pathway independently of CRM1, that has been reported to play a role in ß-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. 3D, 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 ß-catenin.

RanBP3 downregulates ß-catenin-mediated transactivation independently of APC.
To further address the question whether RanBP3 represses ß-catenin transcriptional activation by stimulating export of ß-catenin via the APC/CRM1 pathway, we expressed RanBP3 in human colorectal cancer cell lines that express C-terminal truncations of APC. First, we tested DLD1 cells, which express APC1-1417 that retains some ß-catenin binding sites but lacks all C-terminal nuclear export signals (NES) which are the ones most highly conserved in evolution. As shown in Fig. 4A, ß-catenin/TCF activity is already high in these cells. Expression of RanBP3 wt or "wv" mutant could still dose dependently downregulate transcriptional activity, the mutant again being a less potent inhibitor (Fig. 4A). As APC in DLD1 cells can still bind to ß-catenin, and NESs have also been reported in the N-terminus of APC, we repeated the experiment in COLO320 cells. These cells express a very short APC truncation (1-811) that lacks all ß-catenin binding sites. ß-catenin/TCF activity was much higher in

these cells than in DLD1 cells correlating with the severity of the APC mutation (Fig. 4B, Rosin-Arbesfeld et al., 2003). Nevertheless, transfection of the RanBP3 expression constructs caused a significant downregulation of transcription (Fig. 4B). Therefore, the mechanism by which RanBP3 inhibits ß-catenin is independent of a nuclear export function of APC.

RanBP3 influences subcellular localization of active ß-catenin

A

Whole cell extracts

B

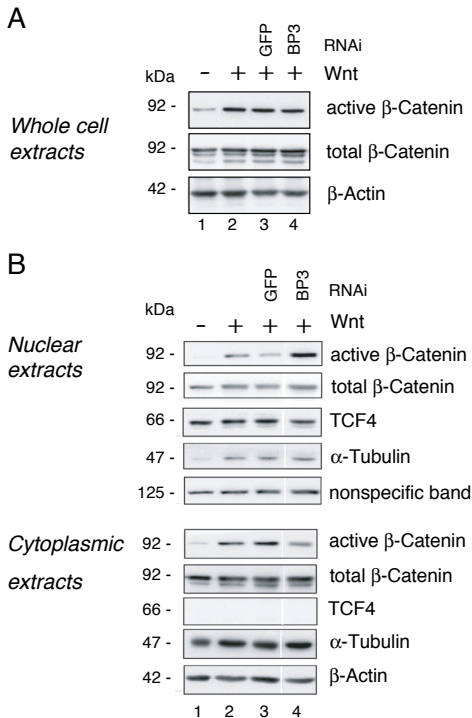*Nuclear extracts*

Cytoplasmic extracts

Figure 5. Depletion of RanBP3 results in nuclear accumulation of active ß-Catenin. A. Depletion of RanBP3 does not alter the levels of both total and active dephosphorylated ß-Catenin. HEK293 cells were transfected with or without Wnt1 and shRNA constructs against GFP or RanBP3. 72 hours after transfection, whole cell lysates were analyzed by Western blot with indicated antibodies. B. RNAi against RanBP3 results in increased levels of active ß-Catenin in the nucleus. HEK293 cells were transfected with indicated constructs and 72 hours post 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 non-specific reaction of the antibody recognizing active ß-Catenin are shown.

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

We next prepared nuclear and cytoplasmic extracts from HEK293 cells transfected with or without Wnt1 and RNAi against GFP or RanBP3. Total ß-catenin was mostly detected in the cytosol fraction (Fig. 5B). No change in total ß-catenin levels was observed in the nucleus or cytoplasm after transfection with Wnt1 (Fig 5B, lane 2), or treatment with RNAi (lanes 3 and 4). When we stained for active ß-catenin, a clear increase was evident after stimulation with Wnt1 (Fig. 5B, lane 2). Interestingly, when cells were transfected with RNAi against RanBP3, active ß-catenin significantly increased in the nuclear fraction and decreased in the cytosolic fraction (Fig. 5B, lane 4), suggesting that RanBP3 relocates active ß-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 allways reflect the subcellular localization in living cells, as pools of proteins that are not tightly bound to nuclear or cytoplasmic structures and are relatively small may leak through NPCs of permeabilized cells. We therefore assayed the effect of RanBP3 overexpression on active ß-catenin in situ using the anti-active ß-catenin antibody. In our hands, this antibody did not visualize endogenous dephosphorylated ß-catenin in Wnt1 transfected HEK293 cells (data not shown). We therefore tested two colon carcinoma cell lines, SW480 and DLD1 that have a constitutively activated ß-catenin due to a mutation in APC (Rosin-Arbesfeld et al., 2003). In SW480, but not in DLD1, the anti-dephospho-ß-catenin antibody recognizes a clear nuclear signal above background (Fig. 6A and C). The presence of this signal correlates with the exceptionally high ß-catenin activity as measured in luciferase assays (Fig. 6D), that is approximately 30 fold higher than in DLD1. Importantly, RanBP3 overexpres-
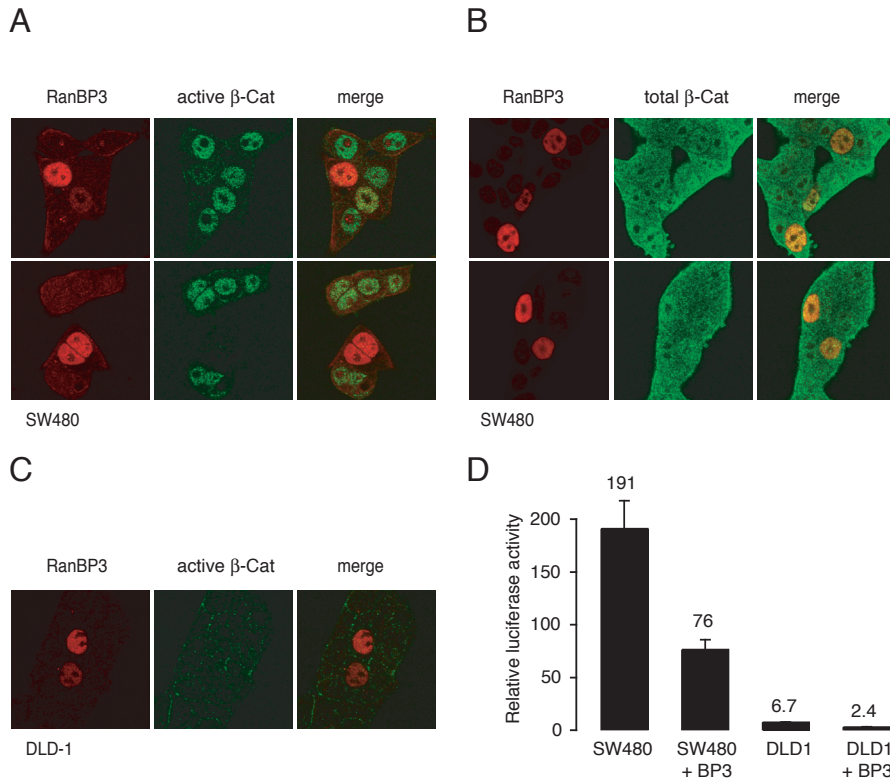
Figure 6. RanBP3 induces specific depletion of endogenous nuclear active ß-Catenin. SW480 (A, B) or DLD1 (C) colon carcinoma cells were transfected with RanBP3 expression plasmids and stained after 45h for dephospho-ß-Catenin (A and C) or total ß-Catenin (B). RanBP3 expression was visualized in the same cells using a RanBP3 polyclonal (A and C) or monoclonal antibody. D. Luciferase reporter assay as in Fig. 2-4 measuring relative ß-Catenin activity. Cells were transfected as in A and C; Error bars represent standard deviations of technical replicates.

sion leads to a clear reduction of active ß-catenin signal from the SW480 nuclei (Fig. 6A), but has no influence on total ß-catenin localization (Fig. 6B). This indicates that, even in the extremely active SW480 cell line, only a very small proportion of total ß-catenin is properly dephosphorylated and active, and that this is the pool RanBP3 acts upon.

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

Reduction of active nuclear ß-catenin by Ran-BP3 in SW480 cells was not accompanied by an increase in cytoplasmic signal, raising the question whether RanBP3 induces enhanced nuclear export of active ß-catenin or its increased phospohorylation. However, enhanced nuclear export would result in dilution in a cytoplasmic volume that is roughly ten fold larger than that of the nucleus, precluding detection by the anti-dephos-

pho-ß-catenin antibody. To discriminate between the two possibilities, we mimicked the active state of ß-catenin using a monomeric RFP (mRFP) tagged, constitutively active form of ß-catenin, the previously employed ß-cateninΔGSK3ß. To test 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 ß-catenin gene (Calvo et al., 2000). This prevented possible activating effects of this mutant on endogenous ß-catenin. mRFP-ß-cateninΔGSK3ß activated the very low endogenous TCF activity of these cells to a great extent (Figure 7B). We next compared the subcellular localization of this protein in the presence or absence of exogenous RanBP3 (Fig. 7A). Care was taken to record cells of similar low expression levels (Fig. 7C). In control cells, more mRFP-ß-cateninΔGSK3ß was present in the nuclei compared to the cytoplasm (median nuclear
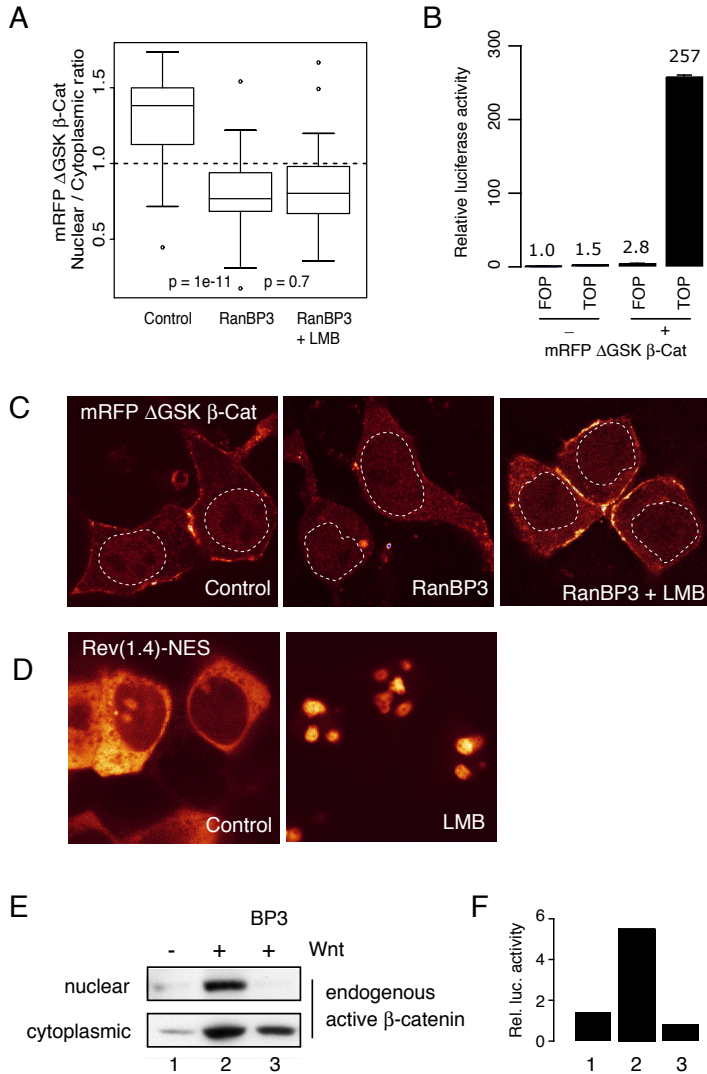
A

mRFP ΔGSK β-Cat
Nuclear / Cytoplasmic ratio

p = 1e-11   p = 0.7

Control   RanBP3   RanBP3
                        + LMB

B

Relative luciferase activity

257

1.0   1.5   2.8

FOP   TOP   FOP   TOP

−   +

mRFP ΔGSK β-Cat

C

mRFP ΔGSK β-Cat

Control   RanBP3   RanBP3 + LMB

D

Rev(1.4)-NES

Control   LMB

E

BP3

−   +   +   Wnt

nuclear   endogenous
                           active β-catenin
cytoplasmic

1   2   3

F

Rel. luc. activity

1   2   3

Figure 7. RanBP3 enhances nuclear export of active ß-Catenin independently of CRM1. A and C. Effect of RanBP3 on mRFP-ΔGSK ß-Catenin nucleocytoplasmic distribution in HEK293 cells in presence or absence of 50 nM LMB for 3h. A. Box plot showing the distribution of nuclear-cytoplasmic ratios of mRFP-ΔGSK ß-Catenin of two independent experiments. P values are according to Mann-Whitney tests. Representive mRFP fluorescence images are shown in C. Highlighted nuclear borders are drawn on the basis of accompanying phase contast images. B. Functionality of mRFP-ΔGSK3-ß-Catenin. NCI-H28 cells (lacking endogenous ß-Catenin) were transfected with indicated constructs and 48 hours after transfection luciferase activity was measured. Shown are relative luciferase levels as corrected for transfection efficiency (Renilla luciferase activity). Bars represent standard deviations. D. Representive fluorescence images of HEK293 cells expressing GFP-Rev(1.4)-NES in the presence or absence of 50 nM LMB for 3h. E and F. Endogenous activated ß-Catenin relocalizes from the nucleus to the cytoplasm upon over-expression 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 48h as in Fig. 5. Localization of active ß-Catenin was monitored using anti-active ß-Catenin antibody. Amounts of protein loaded were normalized on transfection efficiency (Renilla luciferase activity). Normalized ß-Catenin/TCF dependent luciferase activity is depicted in F.

to cytoplasmic ratio of 1.38, n=37).

In contrast, cells expressing exogenous Ran-BP3 showed a higher cytoplasmic than nuclear mRFP-ß-cateninΔGSK3ß levels (median nuclear to cytoplasmic ratio of 0.77, n = 41). Importantly, addition of 50 mM of the CRM1 inhibitor leptomycin B (Wolff et al., 1997) did not significantly change the effect of RanBP3 (median nuclear to cytoplasmic ratio of 0.80, n=52), eventhough photobleaching experiments show that mRFP-ß-cateninΔGSK3ß rapidly shuttles between the nucleus and cytoplasm (data not shown). Identical LMB treatment dramatically relocalized the NES-containing reporter protein Rev(1.4)-NES-GFP (Henderson and Eleftheriou, 2000) to the nucleus (Fig. 6D). We conclude that RanBP3 enhances nuclear export of active ß-catenin, and that this export is independent of CRM1. To confirm that endogenous activated ß-catenin relocalises 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 ß-catenin levels in both nuclear and cytoplasmic fractions, of which the nuclear pool was more sensitive to RanBP3 overexpression than the cytoplasmic pool (Fig 7E). The decrease in cytoplasmic active ß-catenin is consistent with increased nuclear export of ß-catenin and subsequent degradation in the cytoplasm.

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

To study the role of RanBP3 in Wnt signaling in a physiological context, we used a *X. laevis* axis duplication assay. During *X. laevis* embryonic development, Wnt signaling determines patterning along the dorsal ventral axis. Ectopic ventral injection of ß-catenin mRNA in 4-cell embryos resulted in clear axis duplication (Fig. 8A,B). The majority of the embryos (75%) showed a complete duplication of the dorsoventral axis. 22% of the embryos showed a partial duplication i.e. secondary axis without duplicated cement gland. However, co-injection of ß-catenin mRNA with RanBP3 mRNA resulted in a strong suppression of the double axis phenotype in the majority of the embryos (63%). Only few partial or very partial secondary axis (24% and 13% respectively) were observed in these embryos (Fig. 6B). We also co-injected ß-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 such a potent inhibitor as the wild type RanBP3 (Fig. 8A, B; p=4e-8). This data correlates with our findings

that this RanBP3 mutant binds ß-catenin with less affinity (Fig. 1) and that it is less active in repressing transcriptional activity of a TCF reporter gene in human cell lines (Fig. 2 and 4). If RanBP3 is an inhibitor of nuclear ß-catenin function, dorsal injection of RanBP3 mRNA is expected to result in ventralization of the embryo. We therefore injected 4-cell embryos dorsally with either RanBP3 or control mRNA and scored ventralization after three days of development by dorso-anterio index (DAI). Mild to severe ventrilization was observed (DIA1-4) in 80% of RanBP3 injected embryos (Fig 8C), while only less then 10% of control injected embryos showed these phenotypes. Complete ventrilization (DIA0) was not observed. An important direct downstream target of dorsal nuclear ß-catenin acitvity is the early Wnt-inducible homeobox gene Siamois (Brannon et al., 1997). We therefore tested whether expression levels of this gene were reduced in the RanBP3 injected embryos by RT-PCR. In four independent experiments, we detected a ~2 fold decrease in Siamois levels in late stage 9 embryos (Figure 8D and E). This decrease is rather mild, consistent with the incomplete ventralization phenotypes observed. Together, we conclude that RanBP3 is not only a repressor of Wnt signaling in human cell lines but it 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 Drosophila RanBP3 homologue and used RNAi to study its role in Drosophila 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. 9A). Wingless (Wg, Drosophila Wnt) signaling increases levels of Armadillo (ß-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 e.g. RNAi against Daxin (Willert et al., 1999). As a control, we injected embryos with ß-galactosidase double stranded RNA (dsRNA) and observed that the majority (97%) developed into larvae that were indistinguishable from non-injected wild-type larvae (Fig. 9A). 3% of these control embryos showed some very weak effects
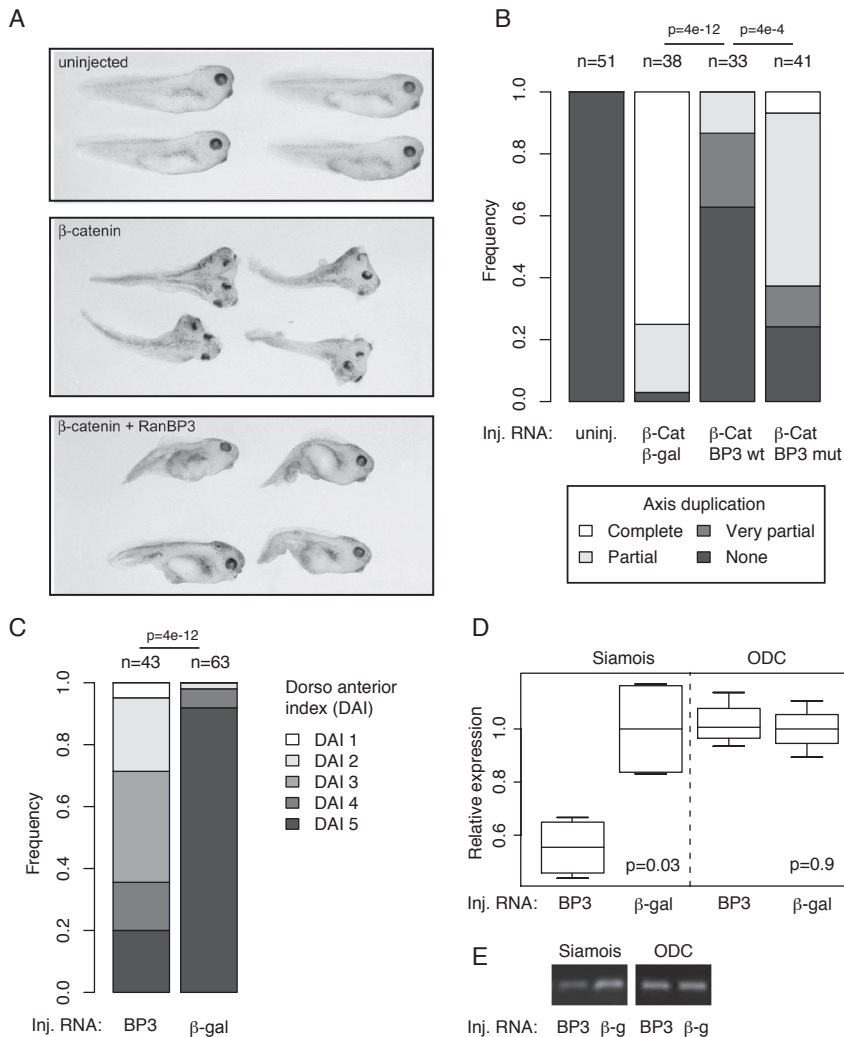
A

uninjected

β-catenin

β-catenin + RanBP3

B

p=4e-12   p=4e-4

n=51   n=38   n=33   n=41

Frequency

1.0
0.8
0.6
0.4
0.2
0.0

Inj. RNA: uninj.   β-Cat   β-Cat   β-Cat
                        β-gal   BP3 wt   BP3 mut

Axis duplication
☐ Complete   ▨ Very partial
☐ Partial   ▪ None

C

p=4e-12

n=43   n=63

Frequency

1.0
0.8
0.6
0.4
0.2
0.0

Inj. RNA: BP3   β-gal

Dorso anterior index (DAI)

☐ DAI 1
☐ DAI 2
▨ DAI 3
▨ DAI 4
▪ DAI 5

D

Siamois   ODC

Relative expression

1.0
0.8
0.6

p=0.03   p=0.9

Inj. RNA: BP3   β-gal   BP3   β-gal

E

Siamois   ODC

Inj. RNA: BP3   β-g   BP3   β-g

Figure 8. RanBP3 rescues ß-Catenin-induced double axis formation in *X. laevis* embryos. A. *X. laevis* embryos were injected ventrally at the 4-cell stage with ß-Catenin mRNA, in the presence or absence of control ß-galacto-sidase or Xenopus RanBP3-b mRNA. In the upper panel wild type non-injected embryos are shown. Middle panel shows double axis phenotype as induced by the injection of ß-Catenin mRNA. Lower panel shows embryos that are rescued from the double axis phenotype by co-expression of RanBP3 and ß-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 Chi-squared test for count data. C. Dorsal injection of RanBP3 results in ventralization of Xenopus embryos. 4-cell stage embryos were injected dorsally with RanBP3 or control (ß-galactosidase) mRNA and analyzed three days later for ventralization using the standarized dorso-anterior index (DAI)(Kao and Elinson, 1988). This scale runs from 0 (complete ventraliza-tion) to 5 (normal development). Frequencies are derived from three independent experiments. P-values as in B. D. The ß-Catenin downstream target siamois is significantly downregulated 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 ß-galactosidase in-jected embryos and represented in a boxplot. P-values are according to Mann-Whitney tests. E. Representive signals from RT-PCR reactions visualized by ethidium bromide staining.
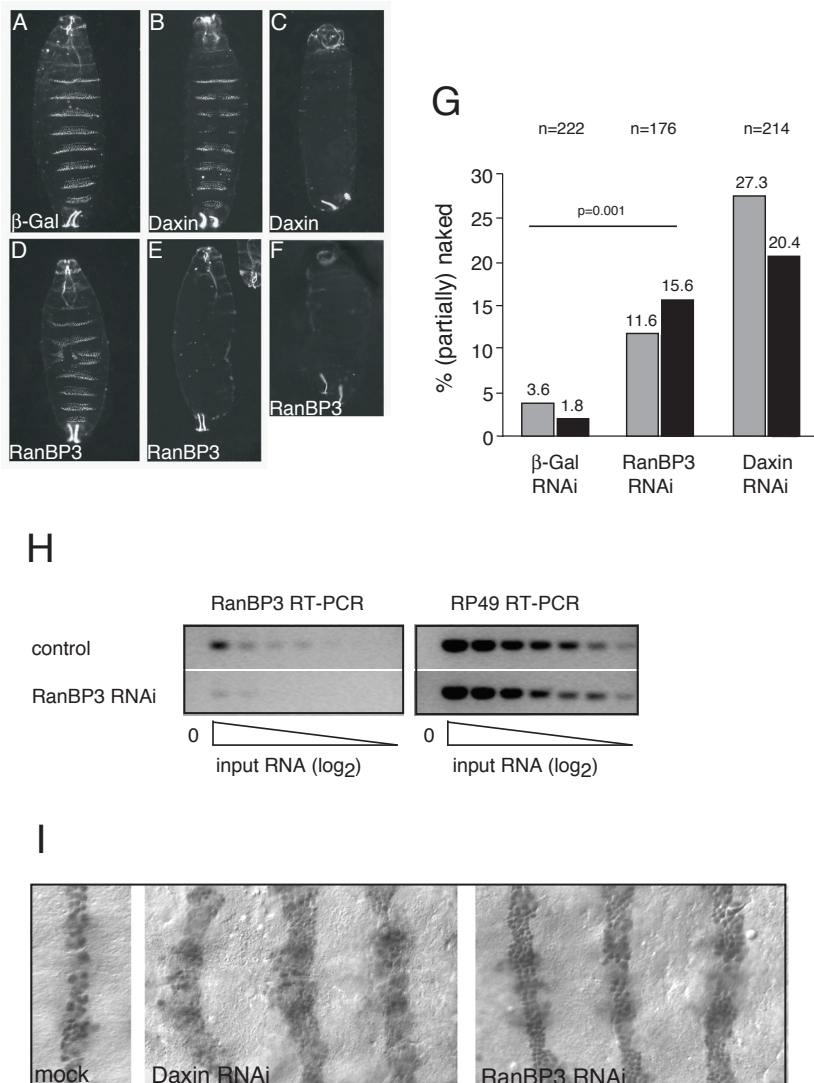
Figure 9. Loss of RanBP3 by RNAi results in a naked cuticle phenotype in Drosophila. Shown are dark field images of cuticle preparations of control (ß-galatosidase) (A), Drosophila Daxin (B and C) and Drosophila RanBP3 dsRNA injected embryos (D, E, F). Loss of Daxin and RanBP3 results in increased Wnt signaling and replacement of denticles by naked cuticle. Partially naked cuticles (B and D), nearly naked (E) cuticles 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 Figure 7B. Note that the contribution of the completely naked phenotype in the RanBP3 RNAi embryos is relatively high (results not shown). 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 15h of development. RT-PCRs specific for RanBP3 or control (ribosomal protein RP49) were performed using nothing (0) or a series of 2 fold dilutions of extracted RNA. I. Loss of RanBP3 function by dsRNA injection results in increased expression of the wg target gene engrailed. Shown are Engrailed antibody staining of buffer injected embryo (left), Daxin dsRNA injected embryo (middle) and 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, explaining the larger cells in the former embryo. The number of Engrailed positive cell rows between stage 10 and 11 is identical. Ventral-lateral view is shown, posterior is left.

on denticle belt formation (Fig. 9G).

RNAi against the (Daxin) resulted in a significant increase in naked cuticle phenotype in 24% of the Daxin dsRNA injected embryos (Fig. 9G) with phenotypes varying from partial loss of denticles to completely naked embryos (Fig. 9B 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. 9D-F). The most severe phenotypes of the RanBP3 RNAi embryos showed deformation of both the head and spiracles (Fig. 9F), resembling Daxin RNAi (Fig 9C). 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-PCRs on buffer and RanBP3 dsRNA injected embryos. Fig. 9H shows that RanBP3 mRNA levels were indeed decreased in RanBP3 dsRNA injected embryos while 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. 9I, left). Removal of the Wnt signaling inhibitor Daxin by dsRNA injection resulted in a broader Engrailed expression pattern that extended from 2 to 4 rows of cells (Fig. 9I; middle). In RanBP3 dsRNA injected embryos, Engrailed expression expanded by one row of cells (Fig. 9I, 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 ß-catenin directly by enhancing nuclear export of its active form. We show that RanBP3 binds directly to ß-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 over-activation of Wnt signaling in tissue culture cells and in *D. melanogaster* embryos. In addition, expression of RanBP3 in human cells specifically reduces active ß-catenin levels in the nucleus and relocates ∆GSK3-ß-catenin from the nucleus to the cyto-

plasm, 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 ß-catenin activity would therefore be increased nuclear export via the CRM1 pathway. Although the nuclear export mechanisms of ß-catenin are not fully understood, two pathways have been proposed (Henderson and Fagotto, 2002). In the first, ß-catenin exits the nucleus independently of nuclear export receptors by interacting directly with proteins of the nuclear pore complex (Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). In the second pathway, ß-catenin exits the nucleus via the CRM1 pathway, but as ß-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 ß-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 ß-catenin. Second, ß-catenin activity is RanBP3-sensitive in colon carcinoma cell line COLO320 (Quinn et al., 1979) that expresses a short type I APC truncation lacking all ß-catenin interaction sites (Rosin-Arbesfeld et al., 2003). We cannot formally exclude that the neuronal APC-like protein APC2 (van Es et al., 1999), which is expressed in certain colon carcinoma cell lines compensates for loss of APC. However, in luciferase reporter assays, CRM1 overexpression does not reverse stimulation of ß-catenin activity caused by depletion of RanBP3. Finally, RanBP3 mediated relocalization of active ß-catenin is insensitive to leptomycin B, a potent CRM1 inhibitor (Wolff et al., 1997). Therefore, we conclude that the mechanism by which RanBP3 inhibits ß-catenin is independent of CRM1 and APC.

Recently, it has been suggested that nuclear ß-catenin signaling is carried out mainly by ß-catenin dephosphorylated at serine 37 and threonine 41, which are main target sites of GSK3ß (Staal et al., 2002; van Noort et al., 2002). Depletion of RanBP3 by RNAi specifically increases the amount of dephosphorylated ß-catenin in

nuclear fractions, while RanBP3 overexpression has the opposite effect. No concomitant increase of cytoplasmic endogenous active ß-catenin was observed by overexpression of RanBP3, rather a small decrease. We attribute this to cytoplasmic phosphorylation and subsequent degradation of wild-type ß-catenin.

Endogenous active ß-catenin was visualized in situ, using the anti-active ß-catenin antibody recognizing desphosphorylated ß-catenin. This was only possible in SW480 colon carcinoma cells that contain a high level of active ß-catenin, due to severely defective APC function (Korinek et al., 1997). RanBP3 overexpression reduced active ß-catenin levels in the nucleus, but had no effect on total ß-catenin. This suggests that only a small proportion of total ß-catenin is active in SW480 cells and confirms the specificity of RanBP3 for active ß-catenin. Apparently, absence of proper ß-catenin phosphorylation and degradation is not sufficient for ß-catenin to be in an active, dephosphorylated state. Also, we infer that the modulation by RanBP3 of ß-catenin activity as measured in our luciferase reporter assays acts on a small dephosphorylated pool, explaining why RanBP3 modulates wild-type and ΔGSK3 ß-catenin to a similar extend (Fig. 2 and 4).

To discriminate whether RanBP3 enhances ß-catenin N-terminal phosphorylation or nuclear export, we have visualized both nuclear and cytoplasmic distribution of active ß-catenin. For this, we used a fluorescently tagged ß-cateninΔGSK3 that is resistant to N-terminal phosphorylation and degradation. As shown in Figure 7, RanBP3 causes a clear and highly significant shift of ß-cateninΔGSK3 from the nucleus to the cytoplasm. We therefore conclude that RanBP3 directly enhances nuclear export of active ß-catenin. How does RanBP3 perform this task? Recent studies have indicated that the interactions of nuclear factors with chromatin or with each other are highly dynamic (Dundr et al., 2002; Phair et al., 2004). This suggests that RanBP3 does not need to actively remove ß-catenin from the TCF/LEF-chromatin complexes. We therefore favor the possibility that association with RanBP3 prevents association of active ß-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 ß-catenin to the cytoplasm and acts as a true nuclear export factor. The stimulatory effect of RanGTP on the ß-catenin/RanBP3 interaction, and the consistently weaker inhibitory effects on

ß-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 ß-catenin for subsequent interactions with the cytoplasmic interacting proteins, such as E-cadherin or the APC/Axin/GSK3ß 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 ß-catenin-dependent dorsoventral axis formation. RNA interference of the *D. melanogaster* homologue of RanBP3 causes naked cuticle phenotypes and a broader Engrailed expression domain due to 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 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); (Miyai et al., 2004); (Kato et al., 2004); (Yang et al., 2004). Further work is required to determine if 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 ß-catenin. This function is separate from its role in CRM1-mediated nuclear export. The structural similarities between CRM1 and ß-catenin suggest that RanBP3 may be a more general cofactor for nuclear export of Armadillo 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 ß-catenin (C19220) (Trans-

duction Lab.) and H-102 (Santa Cruz), RanBP3 (R33620) (Transduction Lab.) and PA1-084 (Affinity Bioreagens), active ß-catenin (ABC 8E7), TCF4 (6H5-3) (Upstate), 414 (Eurogentec/Babco), α-Tubulin (YL1/2, ECACC), Actin (Ab-1, Oncogene) and c-Myc (9E10, Santa Cruz). The 4D9 anti-Engrailed/invected mAb was a gift from Corey Goodman (Patel et al., 1989).

Plasmids

The following plasmids were used: GST-ß-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), pRev(1.4)-RevNES-GFP (Henderson and Eleftheriou, 2000). pQE32-Ran and pQE32-RanQ69L were kind gifts from D. Görlich (ZMBH Heidelberg, Germany). TOP/FOP-Tk and Wnt1, GFP-ß-catenin and pSUPER plasmid were kind gifts from H. Clevers (Hubrecht Laboratory, Utrecht, The Netherlands), R. Kypta (UCSF, San Fransisco, USA) and R. Agami (NKI, Amsterdam, The Netherlands). pcDNA3-RanBP3-b wt and pcDNA3-RanBP3-b "wv" mutant were constructed by generating a blunt NdeI/EcoRV fragment from pET14b-h-RanBP3-b wt and "wv" mutant 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: RanBP3 2: AAGGCGGAGAAGATTCT-GACA 3: AAAGAGCCCCAGAAAAATGAG, 4: AAGAGCCCCAGAAAAATGAGT, 8: AAGCCGA-CATGGAGAATG-CTG, 9: AACCGCAACGAAC-TATTTCCT, 12: AAGGACACAGGTCAG-TTGTAT. pSUPER-GFP was a gift from S. Nijman (NKI) and pBS(SK-)-Daxin-myc from R. Nusse (Stanford). For *X. laevis* injection studies we used HA-ß-catenin (Funayama et al., 1995), ß-galactosidase in pCS2+ (gift from R. Rupp, Munich). pCS2+MT-RanBP3 t and "wv" mutant were constructed by inserting PCR fragments into the EcoRI and XbaI sites of pCS+Myc. mRFP-ΔGSK3 ß-catenin was constructed by inserting a BamHI/SacII digested PCR fragment spanning the ORF derived from pRK5–SK-catenin-GSK (R. Nusse) into the BglII and SacII sites of mRFP (Campbell et al., 2002).

Cell culture, transfection and reporter assays

Cells were cultured in DMEM or in RPMI (NCI-H28), supplemented with 10% fetal calf serum, penicillin/streptomycin (Gibco-BRL) and were transfected using Fugene-6 (Roche) as instructed by the supplier. For reporter assays, cells were cultured in 12 wells plates and transfected with 100 ng TOP/FOP-Tk-luc, 0.5 ng pRL-CMV, 10 ng Wnt1, 30 ng GFP-ß-catenin, 20 (HEK293) or 100 (NCI-H28) ng ΔGSK3-ß-catenin, 100 ng GFP-CRM1 and 100 ng RanBP3 wt/mutant or as indicated. Luciferase activity was measured 48 hrs post transfection using the Dual-Luciferase Reporter Assay System (Promega). Reporter assays using shRNAs were performed as above using 200 ng shRNA constructs and luciferase activity was measured 72 hrs after transfection. HCT116 cells were grown to 50% confluency in 10 cm dishes and transfected with 5 µg of either ß-Galactosidase, RanBP3 wt or wv mutant expression constructs and 0.5 µg EGFP-N3 plasmid to select for transfected cells. 40 hrs 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-ß-catenin (Wiechens and Fagotto, 2001) were expressed in E. coli strain BL21-pLysS and lysed by sonication in 500 mM NaCl; 20 mM Hepes-KOH pH 7.9; 8.7% glycerol; 2.5 mM 2-Mercaptoethanol supplemented with protease inhibitors (Complete protease inhibitor cocktail tablets, Roche). GST-ß-catenin fusion proteins were purified from post ribosomal supernatants using Protein G Sepharose (Amersham Biosciences). His-tagged Ran RanQ69L, RanBP1 and RanGAP were expressed as previously described (Englmeier et al., 2001; Izaurralde et al., 1997). 6x His-tagged RanBP3a/b wt and "wv" mutant proteins were gifts from Drs. L. Englmeier and I. Mattaj (EMBL, Heidelberg, Germany).

Western blotting

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (25 µg per lane) and Western blotting using Immobilon-P transfer membrane (Millipore). Aspecific sites were blocked with 5% non-fat milk at RT for one hour. Primary antibodies were incubated in 1% non-fat milk overnight at 4°C or 1-3 hours at RT in the following dilutions: ß-catenin C19220 1:5000, ABC 1:500; RanBP3 1:5000, TCF4 1:500; 414 1:1:1000; Tubulin 1:20; Actin 1:5000; C-Myc 1:1000. Blots were washed with phosphate buffered saline (PBS)/0.05% Tween 20. Enhanced chemiluminescence (Amersham) was used for detection of proteins.

Immunofluorescence and confocal microscopy

SW480 and DLD1 cells were transfected with 600 ng RanBP3 per 6 well using Fugene 6 (Roche). 45 hrs after transfection cells were either fixed for 10 min in 3.7% formaldehyde in PBS, permeabilized for 5 min in 0.2% Triton/PBS and incubated for one hour at room temperature with primary antibodies diluted in 0.05% BSA/PBS. Cells were washed in PBS and incubated in fluorescently conjugated secondary antibody (Molecular Probes) and mounted in Vectashield (Vector Laboratories). Images were recorded using a Leica NT confocal microscope. Hek 293 cells were transfected with 40 ng mRFP-GSK3-ß-catenin, 200 ng RanBP3 and/or 200 ng GFP-Rev-NES per 6 well using Fugene 6. After 40 hrs cells were either treated or not treated with 50 nM LMB for 1 hour. 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 Leica SP2 TCS AOBS confocal microscope. Nuclear and cytoplasmic regions of confocal images were quantified, background subtracted and nuclear-cytoplasmic ratios were calculated using Image J software.

In vitro binding studies
In pull down assays 750 pmol GST, GST-ß-catenin or GST-ARM were incubated for 1 hr at 4°C with *X. laevis* extracts (Hetzer et al., 2000) 1:1 diluted in 200 mM NaCl, 20 mM Hepes-KOH pH 7.9, 8.7% glycerol, 2.5 mM 2-Mercaptoethanol (Buffer A). RanQ69L was added at 2 µM. In binding assays using HeLa nuclear extracts (obtained from 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 hr at 4°C; 1.5 µM GST-ß-catenin beads with 0.2, 0.5 or 2 µM wt or "wv" mutant RanBP3 and 2 µM Ran-GTP in PBS, 8.7% glycerol, 2 mM $MgCl_2$. Proteins were eluted with 500 mM NaCl, 8.7% glycerol, 2 mM $MgCl_2$, 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 $NaVO_3$ and protease inhibitors (Complete protease inhibitor cocktail tablets minus EDTA, Roche) were added to the lysis buffers. After incubation in hypotonic buffer, NP-40 was added to a concentration of 10%, samples were vortexed shortly and passed through a 25G needle. Whole cell extracts were reconstituted by mixing nuclear and cytosol 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 4-cell stage as described previously (Fagotto et al., 1996; Fagotto et al., 1997). Embryos were raised in 0.1x MBSH (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM HEPES, pH 7.4, 10 mg/ml benzylpenicillin, 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*
ß-galactosidase, Daxin and RanBP3 dsRNAs were synthesized according to Kennerdel and Carthew (1998) and purified using S400 Spin Columns (Pharmacia). 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 open reading frame (ORF) from position 341-1104, fragment 2 from position 683 to 3'UTR position 1423. The following primers were used: BP3 sense primer-1: AGAA-CAACATGCCAAATGTTCAG; BP3 anti sense primer-1: GACGCCG-TTTTTCGCTTCCTCT; BP3 sense primer-2: AGAAACGCAAATACGAGGAG-GT; BP3 anti sense primer-2: GGCGCGCTT-TATTAATTAGTGT. pBS(SK-)-Daxin-myc (Willert et al., 1999) was used as a template to generate a 750 bps dsRNA Daxin fragment spanning nucleotides 1462-2210. Daxin sense primer: GAGAAGTTTGCACTGGACGAAGA and Daxin antisense primer: GGCTTGACAAGACCCATC-GCTT. For ß-gal dsRNA, nucleotides spanning 1296 to 1921 of the lac operon (NCBI accession # J01636.) were sub-cloned into pGEMT-easy and T7 RNA polymerase promoters were added by PCR of the linearized plasmid.

Cuticle analysis and immuno histochemistry
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, an 1:1 mixture of two dsRNA fragments was used. After

injection, the embryos were covered with oil and incubated for 48 hrs at 18°C in a humified 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 days at 55°C and visualized by dark field microscopy. For anti-Engrailed antibody staining, embryos were incubated for 15 hrs, fixed, manually devitalinized and processed for antibody staining according to standard procedures (Patel, 1994).

D. melanogaster RT-PCR

Dechorionated wild type embryos were injected with buffer or RanBP3 dsRNA and then aged at 16 degrees for 15 hours. 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 manufacturers protocol. Samples for the RP49-specific control PCRs were initially diluted 80-fold to compensate for higher expression levels. Subsequently, a series of 2-fold dilutions was performed for each sample; one 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 (AGTGACAGCGATAACACAGCGATAA), RanBP3 Reverse (GCAGAAACG-GATTATTCAGCAGG), RP49 Forward (ATGACCATCCGCCCAGCA) and RP49 Reverse (TTGGGGTTGGTGAG-GCGGAC). 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.

Acknowledgements

Abbreviations list

Abbreviations used in this paper: APC, adenomatous polyposis coli; GSK3ß, glycogen synthase kinase 3ß; RNAi, RNA interference; shRNA, short hairpin RNA; dsRNA, double stranded RNA, NES, nuclear export signal

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CHAPTER 3

Wnt stimulation-independent plasma membrane localization of dephospho-ß-catenin

Manuscript in preparation

3

Wnt stimulation-independent plasma membrane localization of dephospho-ß-catenin

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ß-Catenin is the nuclear effector of the Wnt signaling pathway. Recently, a small pool of N-terminally dephosphorylated ß-catenin was shown to transduce transcriptional activation of Wnt target genes. We show that in a panel of colon carcinoma cell lines, dephospho-ß-catenin localizes to the plasma membrane and/or nucleoplasm. Plasma membrane localization of dephospho-ß-catenin correlates with expression of E-cadherin. Dephospho-ß-catenin localizes specifically to adherens junctions while total ß-catenin staining labels along the baso-lateral membrane. Upon cellular polarization, dephospho-ß-catenin is recruited to the apical actin-based adherens junctions and colocalizes with the adenomatous polyposis coli protein. Immunohistochemistry on tissue sections shows that dephospho-ß-catenin is also enriched at apico-lateral cell-cell borders in the intestinal crypt. In fractionation experiments, neither E-cadherin-bound nor free dephospho-ß-catenin is predictive of Wnt signaling output in our panel of colon carcinoma cell lines. Our data suggest multiple levels of regulation of signaling output and emphasize the need for an E-cadherin negative background in studying the Wnt-responsive dephosphorylated pool of ß-catenin.

Wnts are secreted signaling molecules that regulate embryonic development and adult tissue homeostasis. Deregulation of the Wnt signaling pathway is implicated in tumourigenesis (Nusse, 2005). Compared to other signaling pathways, the Wnt cascade is complex as it contains numerous players (for a complete overview of the pathway see the Wnt homepage on http://www.stanford.edu/~rnusse/wntwindow.html). The output of the cascade is determined by nuclear ß-catenin levels, which regulate transcription of target genes in complex with TCF/Lef transcription factors (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). To further increase our knowledge of the Wnt signaling pathway, it is important to understand the regulatory mechanisms that control the levels and activity of nuclear ß-catenin.

The current canonical model of ß-catenin-dependent Wnt signaling holds that an important regulatory step in the pathway is the constant and rapid degradation of free ß-catenin in the cytoplasm. This mechanism is active in the absence of Wnt signaling and ensures that free ß-catenin molecules are bound and phosphorylated by a complex containing APC/Axin/GSK3/CK1 (Hart et al., 1998; Liu et al., 2002; Amit et al., 2002; Yanagawa et al., 2002). N-terminal phosphorylation marks ß-catenin for degradation by the proteasome (Hart et al., 1999; Aberle et al., 1997). Due to this constant degradation of ß-catenin in the cytoplasm, ß-catenin localization is restricted to the plasma membrane in non-stimulated epithelial cells. This pool of ß-catenin at the plasma membrane functions in cell-cell adhesion as a structural component of Ca^{2+}-dependent adherens junctions.

Another mechanism to control ß-catenin activity is retention mediated by ß-catenin binding proteins. Due to overlapping binding proteins, there is competition between binding of ß-catenin at the plasma membrane by E-cadherin, Axin, APC and ICAT in the cytoplasm/nucleus, and by TCF in the nucleus. In addition, Gottardi and Gumbiner (2004) have suggested that there are molecular forms of ß-catenin that show differential binding to E-cadherin and TCF. They showed that Wnt signaling generates a monomeric form that preferentially binds TCF over E-cadherin. This could be accomplished by a fold-back mechanism in which the C-terminus of ß-catenin binds to its final armadillo repeats, masking part of the E-cadherin binding domain (Gottardi and Gumbiner, 2004).

Staal et al. (2002) were able to show that immunoreactivity for an antibody recognizing N-terminally non-phosphorylated ß-catenin (ABC) correlates much better with Wnt activity than im-

munoreactivity for total ß-catenin. This form of ß-catenin accumulates in Wnt-activated cells and is localized only in the nucleus (Staal et al., 2002). In earlier studies, we confirmed these findings by Staal et al. and identified RanBP3 as a specific nuclear export factor for dephospho-ß-catenin (Hendriksen et al., 2005).

Based on our work examining the transcriptionally active pool of ß-catenin, we determined the intracellular localization of dephospho-ß-catenin in a panel of colon carcinoma cell lines. Surprisingly, nuclear dephospho-ß-catenin is observed in only 3 out of 8 cell lines, whereas the majority of cell lines in our panel show plasma membrane localization. Plasma membrane localization of dephosphorylated ß-catenin correlates with E-cadherin expression. Upon close inspection of HCT15 cells, we find that total ß-catenin antibodies label along the baso-lateral membrane, while the ABC antibody specifically labels the apical region of the baso-lateral membrane. We confirmed the apical localisation of dephospho-ß-catenin using single polarized cells. In this system, dephospho-ß-catenin localizes to the apical actin cap along with APC. Next, we determined the localization of dephospho-ß-catenin in normal adult small intestine and found that in the crypt area where Wnt signaling is active, dephospho-ß-catenin was enriched at the apico-lateral cell border. Total ß-catenin did not show a preferential accumulation. Finally, by focusing on the free pool of dephosphorylated ß-catenin we demonstrate that the correlation between this pool and Wnt signaling activity in colon carcinoma cell lines is poor, which suggests multiple levels of regulation of signaling output. We stress that an E-cadherin null background is required in studying the dephosphorylated pool of ß-catenin in Wnt signal transduction.

Results and Discussion

Nuclear localization of ß-catenin has long been a surrogate marker for Wnt signaling activity, even though it correlates poorly with TCF reporter activity in *in vitro* assays. The ABC antibody, which specifically recognizes N-terminally unphosphorylated ß-catenin, was shown to correlate much better with Wnt signaling activity when compared to antibodies recognizing total ß-catenin (Staal et al., 2002). To gain more insight into the behaviour of unphosphorylated (and therefore possibly signaling competent) ß-catenin, we have investigated the intracellular localization of the dephosphorylated form of ß-catenin in various colon cancer cell lines. In these Wnt-activated cells, we detected dephospho-ß-catenin in the nucleus of SW480, LS174T and Colo320 cells (Fig 1A) as was suggested by the work of (Staal et al., 2002). Interestingly, several cell lines showed prominent dephospho-ß-catenin staining at the plasma membrane, including HCT15, Colo205, SW48, DLD1 and Caco2. Low amounts of dephospho-ß-catenin were detected at the cell-cell contacts of Lovo cells, whereas dephospho-ß-catenin was not detected in HT29 cells (Fig 1A, data not shown for DLD1 and Caco2). Plasma membrane localization of dephospho-ß-catenin in these cell lines correlates with total ß-catenin staining. This localization of the dephosphorylated form of ß-catenin is somewhat surprising. A previous study has claimed that N-terminally dephosphorylated ß-catenin can localize to the plasma membrane in epithelial cells (Gottardi and Gumbiner, 2004). However, the anti-dephospho-ß-catenin antibody used in the this study has recently been shown to be aspecific (van Noort et al., 2007). To our knowledge, we are the first to describe that a large pool of dephospho-ß-catenin resides at the plasma membrane.

As the dephosphorylated pool of ß-catenin has been equated with the signaling competent pool of ß-catenin (Staal et al., 2002), we focused on this membrane-associated pool more closely. Close inspection of our colon carcinoma cell lines expressing plasma membrane dephospho-ß-catenin revealed that localization patterns of dephospho-ß-catenin and total ß-catenin do not overlap. Confocal scanning showed that, whereas total ß-catenin labels along the lateral plasma membrane, dephospho-ß-catenin accumulates at the apico-lateral cell-cell border (Fig 1B and C). The observed difference in localization is most apparent in cell lines, such as HCT15 cells, that retain the ability to grow in monolayer and therefore show proper polarization. We sought to investigate differences in plasma membrane accumulation of dephospho-ß-catenin between colon cancer cell lines in further detail. The known mutation status of ß-catenin and APC did not reveal any associations with nuclear or plasma membrane dephospho-ß-catenin levels (Table 1). The localization of dephospho-ß-catenin was investigated in relation to the expression of E-cadherin in our panel of cell lines. Using quantitative western blot analysis, we find a relationship between plasma membrane localization of dephospho-ß-catenin and E-cadherin protein levels. Cell lines with little membrane-associated dephospho-ß-catenin express low (SW480) to undetectable (Colo320, LS174T) levels of E-cadherin (Figure 1A and D), whereas cell lines showing prominent plasma membrane staining of de-
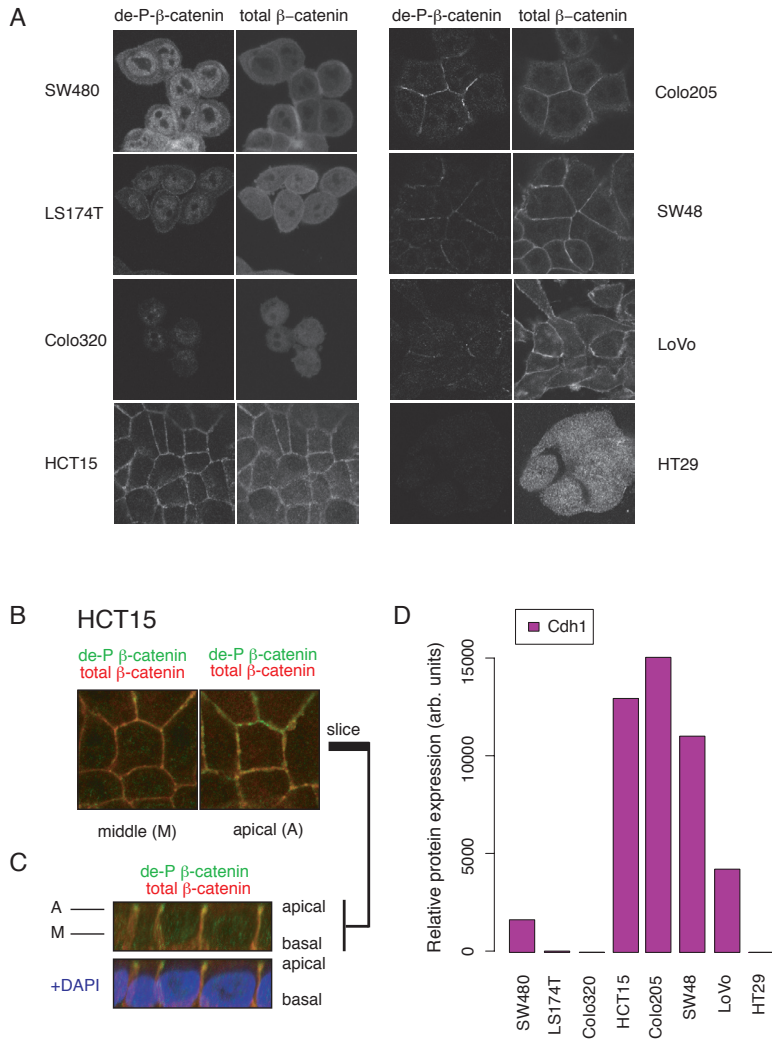
Figure 1. Plasma membrane localization of dephospho-ß-catenin correlates with E-cadherin expression. A. Subcellular localization of total and dephospho-ß-catenin in colon carcinoma cell lines. B. Dephospho-ß-catenin is concentrated at adherens junctions. Confocal sections taken from a z-series through the mid (left) and apical (right) planes of confluent HCT15 cells stained for total (red) or dephospho-ß-catenin (green). C. Orthogonal slice of z-series, labelled as in B. The lower panel includes the DAPI channel to visualize the positions of the nuclei (blue). D. Relative Cdh1 protein levels in cell lines shown in Figure 1A. 20 μg of total cellular protein was separated on SDS-PAGE, blotted and probed with an anti-Cdh1 antibody. Western blot signals were quantified using a luminoscan analyzer. Equal loading was confirmed using ß-actin detection, levels of which varied less than 25%.

phospho-ß-catenin (HCT15, Colo205, SW48 and Lovo) all express high levels of E-cadherin (Figure 1A and D). Low E-cadherin levels in SW480 and LS174T have been described in earlier reports (Gottardi et al., 2001; Elefstathiou et al., 1999; Muller et al., 2002). This shows that the amount of membrane-associated dephospho-ß-catenin correlates with E-cadherin expression, and suggests that the pool of dephospho-ß-catenin likely resides in a junctional complex. These results are consistent with previous findings showing that exogenously expressed N-terminal truncation mutants of ß-catenin colocalize with E-cadherin at cell-cell contacts in MDCK epithelial cells

mutation status (grey is LOH)

	SW480	LS174T	Colo320	HCT15	Colo205	SW48	LoVo	HT29	HCT116	CaCo2
β-cat	wt	S45F	wt	wt	N287S	S33Y	wt	wt	Δ45	G245A
APC	1338	wt	811	1416	1554	wt	1114	853 / 1555	wt	1367

Table 1. ß-Catenin and/or APC mutation status of colon carcinoma cell lines used in this study.
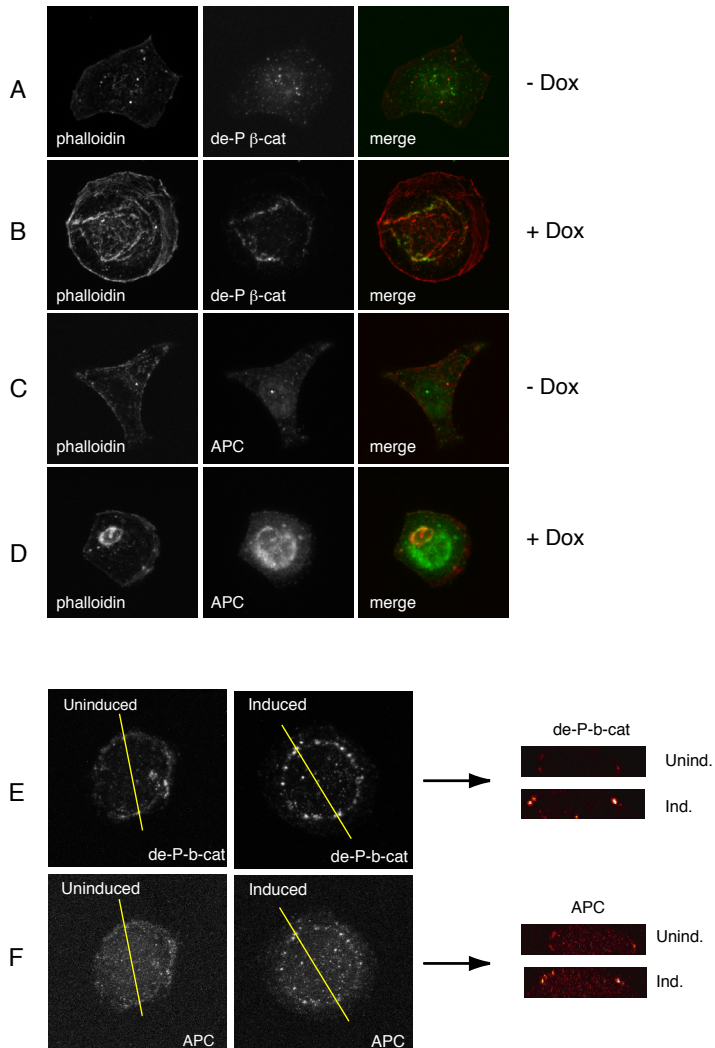

Figure 2. Dephospho-ß-catenin and APC localize to the apical membrane in polarized cells A-D. Immunofluorescence images of DLD1-W5 cells before and after polarization induced with doxycycline, stained with indicated antibodies. Dephospho-ß-catenin and APC colocalize with actin in the apical brush border after polarization. E and F. Z-stack projection of confocal images of DLD1-W5 cells before and after induction with doxycycline, showing apical localization of dephopho-ß-catenin (E) and APC (F) after polarization.

(Barth et al., 1997). Their data suggest that cellular dephospho-ß-catenin levels per se are not to be equated with ongoing Wnt signaling activity.

We continued to study the localization of dephospho-ß-catenin in a model of cellular polarization. For this, we used the human colon cancer cell line DLD1-W5 that can be induced to polarize at the single cell level (Baas et al., 2004). Upon doxycycline-induced expression of STRAD, isolated DLD-1-W5 cells show several hallmarks of polarization, such as organization of the actin cytoskeleton including a prominent apical ring-like actin cap (Fig. 2B and D). While there was no co-localization between actin (phalloidin) and dephospho-ß-catenin before polarization, in the polarized HCT15 monolayer, dephospho-ß-catenin localized to this apical actin structure (Fig. 2A and B). Dephospho-ß-catenin co-localized specifically with apical actin, whereas it did not co-localize with a well-known tight-junction marker ZO-1 (data not shown). Data from *in vivo* systems both in the *D. melanogaster* embryonic epidermis (Yu et al., 1999; McCartney et al., 1999; Cliffe et al., 2004) and in the human adult gastrointestinal epithelium (Anderson et al., 2002) have revealed that APC localizes to adherens junc-

tions along with ß-catenin. However, the exact location of APC in cultured mammalian cell lines has remained unclear (Brocardo et al., 2005). We therefore stained polarized and unpolarized DLD-1-W5 cells with the N-APC monoclonal antibody (Midgley et al., 1997) that is a specific probe for endogenous APC in cultured cells (Kita et al., 2006). Like dephospho-ß-catenin, APC localizes to the ring-like apical actin cap in polarized epithelial cells (Fig. 2D), whereas no co-localization is apparent before polarization (Fig. 2C). We conclude that upon cellular polarization both dephospho-ß-catenin and APC are recruited to the presumptive apical adherens junction in this model system. Our results confirm studies in *D. melanogaster* showing that E-APC localizes to adherens junctions, where it co-localizes with ß-catenin and E-cadherin (Yu et al., 1999).

In order to determine the localization of dephospho-ß-catenin in the adult human system *in vivo*, we stained paraffin-embedded consecutive sections of normal adult small intestinal epithelium with antibodies recognizing either the total or dephosphorylated pool of ß-catenin. We find that both antibodies reveal an increased labelling on the plasma membrane at the level of the
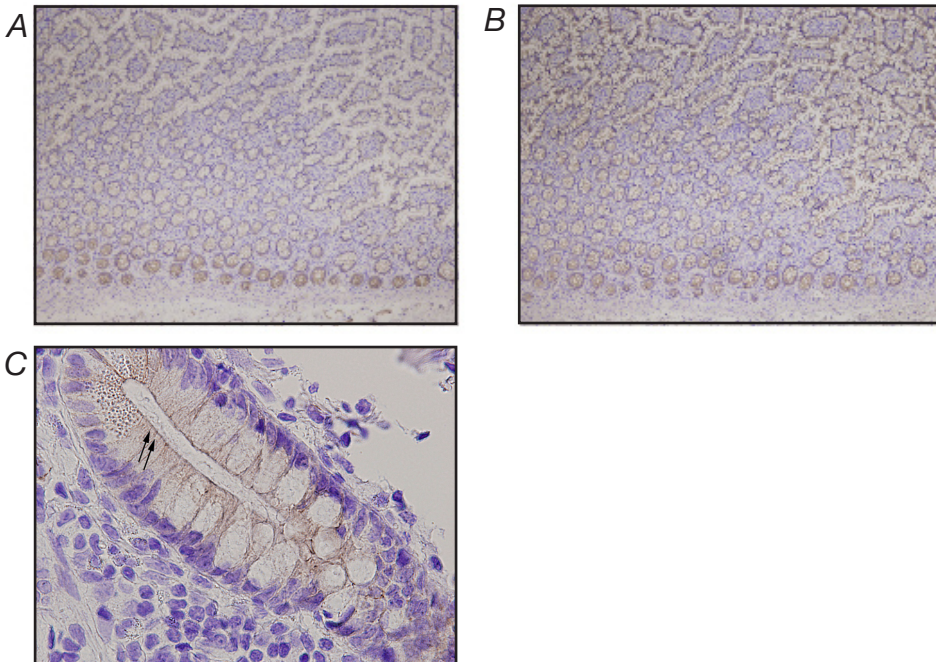
3

Figure 3. Immunohistochemistry of total (A) and dephospho-ß-catenin (B) in normal human small intestine. Both antibodies reveal increased membrane labeling at the level of the intestinal crypt. C. Zoom-in Fig 3B. Dephospho-ß-catenin shows a punctuate staining at the apico-lateral cell-cell border.

crypt over the villous epithelial cells (Fig 3A and B). By performing a dilution series we find that at limiting dilution, dephospho-ß-catenin accumulates on the apico-lateral membrane at the presumptive adherens junctions (Fig 3C), whereas dilution series for total ß-catenin did not reveal a similar preferential accumulation, in line with our data obtained in the polarized cell lines HCT15 and DLD-1-W5. Importantly, this apico-lateral accumulation is specific for crypt compared to villous epithelial cells, suggesting that the apical accumulation of dephospho-ß-catenin might be linked to active Wnt signal transduction.

Our data show that total cellular levels of dephospho-β-catenin are not predictive of Wnt signaling activity due to cadherin-mediated dephospho-ß-catenin membrane sequestration. However, we were interested to analyze whether subpools of dephospho-ß-catenin might correlate better with Wnt signaling activity. The lectin protein Concavalin A (Con A) binds with high affinity to glycosylated proteins and has been used by several laboratories to distinguish between E-cadherin-bound and free ß-catenin (Aghib and McCrea, 1995; Funayama et al., 1995). We used Con A to precipitate E-cadherin and associated proteins, including pools of ß-catenin from cell lysates, and analyzed fractions on semi-quantitative western blot. In our panel of colon cancer cell lines, 4 cell lines showed a dispersed growth pattern, absence of ß-catenin in cell-cell contacts and low (SW480) to undetectable E-cadherin levels (HT29, LS174T, Colo320) (Fig 1A and D). Analysis of total ß-catenin protein levels before and after Con A binding did not reveal a correlation between E-cadherin expression and Con A-bound ß-catenin (Fig 4A). A possible explanation could be expression of other cadherins in these cell lines. We next determined dephospho-ß-catenin levels and found that 3 out of 4 cell lines with low E-cadherin levels show high levels of non-Con A-bound or free dephospho-ß-catenin (SW480, Colo320 and LS174T, Fig 4B). Out of these 3 cell lines, only SW480 and Colo320 show high Wnt signaling activity in the TOP/FOP assay as a readout for TCF-dependent transcriptional activation (Fig 4C). We conclude that high levels of free dephospho-ß-catenin still correlate poorly with Wnt signaling activity as only two out of three cell lines match high levels of free dephospho-ß-catenin to robust TCF reporter output.

It remains to be established whether quantitative analyses of the pool of dephospho-β-catenin at the plasma membrane correlates with Wnt signaling activity. Our analyses in an E-cadherin null background provide evidence for plasma membrane recruitment of dephospho-ß-catenin upon Wnt treatment (Chapter 4). From the results of the Con A-bound pool of dephospho-ß-catenin in our panel of cell lines, it is clear that there is no strict correlation between this pool and Wnt signaling activity, much like the situation for free dephospho-ß-catenin. Therefore, our data of Con A-bound versus free dephospho-ß-catenin provide no evidence for a correlation between either of these pools and Wnt signaling activity. This underscores the importance of an E-cadherin null background in studying signaling competent dephospho-ß-catenin. Moreover, levels of free dephospho-ß-catenin still correlate poorly with Wnt signaling output, even if cell lines expressing low levels of E-cadherin are scored separately, which suggests multiple levels of regulation of signaling output. This is in accordance with data obtained in an E-cadherin null background (Chapter 4). To further investigate the impact of E-cadherin expression on dephospho-ß-catenin localization, we compared dephospho-ß-catenin localization in HCT15 cells grown at different densities. Nuclear levels of dephospho-ß-catenin were found to be higher in HCT15 cells grown in low density compared to confluent cells (Fig 4D). This suggests that increased cell-cell contacts can downregulate nuclear dephospho-ß-catenin levels. Indeed, overexpression of E-cadherin in these cells reduced TCF-dependent transcription (Fig 4E). Earlier studies have also shown that modulation of E-cadherin levels can affect Wnt signaling output. In particular, overexpression of E-cadherin antagonizes Wnt signaling by sequestering ß-catenin at the plasma membrane (Heasman et al., 1994; Fagotto et al., 1996; Sanson et al., 1996; Orsulic et al., 1999). Likewise, reduction in E-cadherin increased armadillo signaling in Drosophila (Cox et al., 1996). However, E-cadherin does not appear to regulate the Wnt pathway *in vivo* as loss of cadherin function did not enhance Wnt signaling in either human tumors or murine cancer models (Caca et al., 1999; Smits et al., 2000; Vasioukhin et al., 2001; van de Wetering et al., 2001; Derksen et al., 2006).

In this study, we have shown that a pool of dephospho-ß-catenin resides at the apico-lateral cell-cell border of the plasma membrane. Plasma membrane localization of dephospho-ß-catenin correlates with E-cadherin expression, which suggests that at least part of this pool is involved in cell-cell adhesion. Therefore, the mere presence of dephospho-ß-catenin is not predictive of Wnt signaling activity. However, since Wnt treatment induces plasma membrane recruitment of
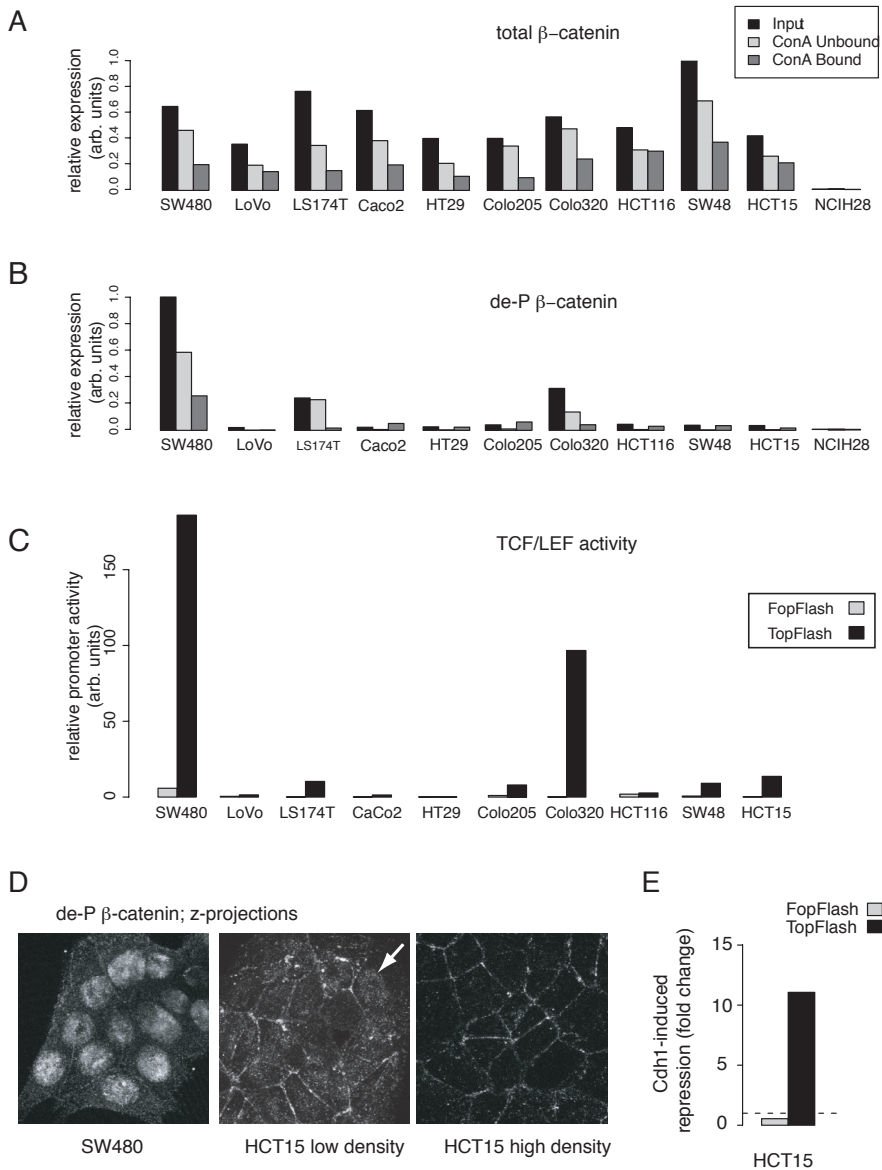
Figure 4. Relationship between ß-catenin levels, E-cadherin binding and Wnt signalling activity. A and B. Cell lysates from indicated colon carcinoma cell lines were subjected to binding to Con A to pull down E-cadherin-binding proteins. Input, Con A-bound (E-cadherin-bound) and Con A-unbound fractions were analyzed by semi-quantitative western blot and analyzed with an antibody recognizing total (A) or dephospho-ß-catenin (B). C. ß-Catenin/TCF-mediated transcriptional activity in colon carcinoma cell lines. Cells were transfected with TOP or the control FOP luciferase reporter to measure Wnt signalling activity 24 hours after transfection. Co-transfection of the pRL-CMV Renilla construct was used to correct for transfection efficiency. D. Detection of dephospho-ß-catenin in low density (middle) and high density (right) HCT15 cells. SW480 cells stained in parallel and imaged with the same settings are shown for comparison (left). Images represent projections of top-to-bottom confocal z-series. An arrow marks nuclear staining in low-density HCT15 cells. E. HCT15 cells were assayed for TCF/LEF-dependent transcriptional activity using TOP and FOP-TK-luciferase reporters in the presence or absence of Cdh1 expression. Ratios with/without Cdh1 are plotted as fold change. Dashed line: fold change equals 1.

dephospho-ß-catenin (Chapter 4), a fraction of this membrane-associated pool might in fact be involved in Wnt signal transduction. The mechanistic details of dephospho-ß-catenin routing in response to Wnt stimulation are unclear. Therefore, it is impossible to discern a resident junctional pool from a recruited signaling competent pool of dephospho-ß-catenin residing at the plasma membrane. It is imperative that future work addresses these issues to develop adequate immunological tools. In this respect, it is interesting to note that dephospho-ß-catenin accumulates at the apico-lateral cell-cell border to a greater degree in crypt epithelial cells, which are thought to be Wnt responsive. Whether this reflects ongoing Wnt signal transduction requires further study. Lastly, we show by fractionation experiments that neither the Con A-bound pool of dephospho-ß-catenin nor the free fraction of dephospho-ß-catenin correlates with Wnt signaling output. In addition to underscoring the importance of an E-cadherin null background, our results suggest multiple levels of regulation of Wnt signaling output.

Materials and methods

Cell culture and luciferase reporter assay
All cell lines were cultured in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco-BRL) and were transfected using Fugene-6 (Roche) as instructed by the supplier. For reporter assays, cells were cultured in 12-wells plates and transfected with 200 ng TOP-Tk-lluc or the control FOP-Tk-luc together with 1 ng pRL-CMV Renilla to control for transfection efficiency. Cells were lysed after 48 hours and luciferase activity was measured using the Dual-luciferase reporter assay system (Promega).

Western blotting
Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (25 μg per lane) and western blotting using Immobilon-P transfer membrane (Millipore). Aspecific sites were blocked with 5% skim milk (Oxio, Hampshire, England) at room temperature for one hour. Note that detection of dephospho-ß-catenin with the ABC antibody was inhibited by certain brands/lots of skim milk. Primary antibodies were incubated in 1% skim milk for 2 hours at room temperature in the following dilutions: E-cadherin 1:1500; ß-catenin mAb C19220 1:5000, ABC 1:500; actin 1:5000. Blots were washed with phosphate buffered saline (PBS)/0.05% Tween 20. Enhanced chemiluminescence (Amersham) was used for detection of proteins.

Immunofluorescence and confocal microscopy
For immunofluorescence, cells were grown on glass coverslips coated with fibronectin (Sigma) and fixed in 3.7% formalin in PBS for 10 min and permeabilized for 5 min in 0.2% Triton/PBS. Primary antibodies were incubated for 2 hours in 1% purified BSA/PBS using the following dilutions; ABC 1:200; total ß-catenin C19220 1:250. Cells were washed shortly in PBS and incubated in conjugated fluorescent secondary antibodies (Molecular Probes) and DAPI in 1% BSA/PBS for 30 min, washed shortly in PBS and mounted in Mowiol. Images were recorded using a Leica NT, SP2 or SP2 AOBS confocal microscope. Antibodies used were against ß-catenin (C19220) (Transduction Labs), active ß-catenin (ABC 8E7), E-cadherin (C20820, Transduction Labs).

Concavalin A purification
For Concavalin A (Con A) purification, cells were lysed in 0.1% NP-40, 20 mM HEPES-KOH (pH 7.9), 200 mM NaCl 1 mM 2-mercaptoethanol and protease inhibitors (Complete-EDTA; 0.5 tablet per 10 ml), cleared by centrifugation and bound to 10 microliter Con A Sepharose 4B (Pharmacia) for 2.5 h at 4°C. Beads were washed 3 times in lysis buffer and eluted using SDS-PAGE sample buffer.

Immunohistochemistry
Sections (4 μm) were deparaffinized and antigen retrieval was carried out by boiling 10 min in 10 mM Tris/1 mM EDTA (pH 9). Subsequently, slides were immersed in 0.3% hydrogen peroxide in methanol for 30 min and nonspecific binding was blocked with 5% normal goat serum for 1 hr at room temperature. The sections were incubated for 1 hr at room temperature in primary antibodies against total ß-catenin (C19220 Transduction Labs) and active ß-catenin (ABC 8E7 Upstate Biotechnology). The Ultravision antipolyvalent HRP detection system (Lab Vision Corp., Fremont, CA, USA) was used to visualize antibody binding sites with 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin.

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Chapter 4

Plasma membrane recruitment of signaling-competent ß-catenin upon activation of the Wnt pathway

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Plasma membrane recruitment of signaling competent ß-catenin upon activation of the Wnt pathway

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The standard model of Wnt signaling specifies that after receipt of a Wnt ligand at the membrane-associated receptor complex, downstream mediators inhibit a cytoplasmic destruction complex, allowing ß-catenin to accumulate in the cytosol and nucleus and co-activate Wnt target genes. Unexpectedly, upon Wnt treatment, we detected the dephosphorylated form of ß-catenin at the plasma membrane, displaying a discontinuous punctate labeling. This pool of ß-catenin could only be detected in E-cadherin (-/-) cells, because in E-cadherin (+/+) cells Wnt-induced, membrane-associated ß-catenin was concealed by a constitutive junctional pool. Wnt signaling-dependent dephosphorylated ß-catenin co-localized at the plasma membrane with members of the destruction complex APC and Axin and the activated Wnt co-receptor LRP6. ß-Catenin induced through the Wnt receptor complex was transcriptionally significantly more competent than overexpressed ß-catenin, both in cultured cells and in early Xenopus embryos. Our data reveal an unappreciated step in processing of the Wnt signal and suggest multiple levels of regulation of signaling output beyond the level of protein accumulation.

The Wnt pathway is a critical determinant of cell proliferation during development and regenerative processes such as stem cell proliferation in the adult (Clevers, 2006). Aberrant activation of the pathway has been linked to oncogenesis in multiple systems. A central player in the Wnt pathway is ß-catenin. Most of the cellular pool of ß-catenin is tethered to E-cadherin (encoded by the Cdh1 gene) as an adherens junction component mediating cell-cell adhesion (McCrea et al., 1991; Peifer et al., 1994a). A less abundant pool of ß-catenin, often referred to as the 'free' pool of ß-catenin, functions in complex with TCF/Lef transcription factors as a transcriptional co-activator of Wnt signaling in the nucleus (Cadigan and Nusse, 1997). In the absence of a Wnt signal, the free pool of ß-catenin is tightly regulated through phosphorylation at specific N-terminal residues by a so-called 'destruction complex' consisting of the serine kinases CK1α and GSK3 and the tumor suppressors Adenomatous Polyposis Coli (APC) and Axin (Logan and Nusse, 2004). Phosphorylated ß-catenin is marked for rapid ubiquitinization and degradation by the proteasome. Receipt of a Wnt ligand at the membrane-associated receptor complex results in inhibition of ß-catenin breakdown, allowing ß-catenin to accumulate, enter the nucleus and activate a Wnt target gene program (Logan and Nusse, 2004).

However, our current understanding of Wnt signal transduction and ß-catenin processing suffers from significant gaps. In particular, the make-up and subcellular localization of the mature destruction complex is unclear at present. For this reason, the mechanism through which the destruction complex senses ligand engagement at the Frizzled/LRP receptor complex remains unidentified. Recently, increasing evidence suggests important regulatory steps in the turn-over of the destruction complex may take place at the plasma membrane (for a review see Cadigan and Liu, 2006). Engagement of the Frizzled and LRP5/6 co-receptors on the cell surface by Wnt ligands, results in the phosphorylation of the intracellular domain of LRP5/6 by CK1γ and/or GSK3ß. Phosphorylated LRP5/6 presents a docking site for Axin that is recruited to the plasma membrane in response to Wnt stimulation (Cliffe et al., 2003; Davidson et al., 2005; Zeng et al., 2005) along with other canonical Wnt pathway components including Axin, GSK3ß and Fz8 (Bilic et al., 2007). The scaffold protein dishevelled (Dvl) appears to be required for this trans-

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location (Schwarz-Romond et al., 2007). Although it remains unproven, it has been hypothesized that cytoplasmic destruction of ß-catenin is halted as a result of Axin relocation, allowing ß-catenin to redistribute to the nucleus.

Nuclear localization of ß-catenin is considered a hallmark of Wnt activation, yet in many systems it is only incidentally detected in the nucleus (Anderson et al., 2002; Kobayashi et al., 2000). The nuclear level of the N-terminally dephosphorylated (or 'dephospho') form of ß-catenin has been shown to correlate much better with Wnt activity (Staal et al., 2002). Dephospho-ß-catenin has been suggested to reflect the *de novo* translated form of ß-catenin, which is involved in signal transduction (Willert et al., 2002). We set out to optimize experimental conditions for the detection of dephospho-ß-catenin in cultured mammalian cell lines. In a series of colon carcinoma cell lines, dephospho-ß-catenin often localizes to the plasma membrane. Although we find no correlation with either APC or ß-catenin mutation status, the plasma membrane localization of dephospho-ß-catenin does correlate with E-cadherin expression. Surprisingly, stimulation of E-cadherin -/- cells with Wnt3A resulted in the appearance of dephospho-ß-catenin at the plasma membrane, where it co-localizes with the activated form of LRP6, APC and Axin. By unmasking the transcriptionally competent pool of ß-catenin, we provide evidence for a key step in ß-catenin processing and Wnt signal transduction at the plasma membrane.

Results

Dephospho-ß-catenin is present in cadherin complexes

Because N-terminally dephosphorylated ß-catenin represents a better marker for Wnt signaling activity than total ß-catenin, we optimized experimental conditions allowing detection of dephospho-ß-catenin by the α-ABC (8E7) antibody (van Noort et al., 2002) in cultured mammalian cell lines. This antibody specifically reacts with an N-terminally unphosphorylated peptide (amino acids 36-44) that contains the GSK3ß target residues S37 and T41 (van Noort et al., 2007). Reproducible detection of dephospho-ß-catenin with the α-ABC antibody was highly dependent on fixation and blocking conditions, and further improved after antigen retrieval (see Materials and Methods). Screening of a set of colon carcinoma cell lines using these conditions showed both nuclear and plasma membrane localization of dephospho-ß-catenin, depending on the cell line (Fig 1A, Chapter 3, this thesis). Although we found no correlation with either APC or ß-catenin mutation status, the plasma membrane localization of dephospho-ß-catenin does correlate positively with E-cadherin expression (Fig 1, Chapter 3, this thesis).

Cadherin-independent plasma membrane localization of dephospho-ß-catenin upon Wnt3A stimulation

In order to eliminate E-cadherin expression as a confounding factor in our interpretation of endogenous Wnt-induced dephospho-ß-catenin accumulation, we turned to the murine mammary epithelial cell line Kep1, which does not express E-cadherin due to Cre-mediated recombination of both E-cadherin alleles, and compared it with its E-cadherin (+/+) isogenic control counterpart Kp6 (Derksen et al., 2006). In the absence of Wnt stimulation, these cell lines line do not activate a Wnt-responsive luciferase reportergene, indicating that these cell lines do not carry Wnt pathway activating mutations (Fig 1A). Earlier studies have demonstrated that in the presence of an intact destruction complex the loss of E-cadherin is neutral with respect to Wnt stimulation (van de Wetering et al., 2001). No or very little ß-catenin could be detected in unstimulated Kep1 cells. After 2.5 h stimulation with Wnt3A protein, however, a clear accumulation of the dephospho-ß-catenin form was seen (Fig 1B). In contrast, in the E-cadherin +/+ Kp6 cells dephospho-ß-catenin was clearly detected before Wnt stimulation (Fig1B).

In view of the transcriptional activation, we anticipated that in Wnt-stimulated Kep1 cells, dephospho-ß-catenin would be mainly nuclear. Surprisingly, stimulation of E-cadherin -/- Kep1 cells with Wnt3A resulted in the appearance of dephospho-ß-catenin at the plasma membrane (Fig. 1C). Nuclear staining of the ABC antibody is also observed. Note that this is partly aspecific, as some nuclear staining is also observed in unstimulated Kep1 cells and in NCI-H28 ß-catenin knock-out cells (data not shown), which is likely caused by a cross-reacting protein (in Fig. 1B marked by an asterisk). As expected, a similar plasma membrane accumulation in response to Wnt stimulation in E-cadherin -/- Kep1 cells is confirmed with antibodies to total ß-catenin (Fig. 2C). The discontinuous punctate plasma membrane labeling of dephospho-ß-catenin is strikingly similar to the plasma membrane-associated puncta described for LRP6-Axin (Bilic et al., 2007) and dishevelled (Dvl) (Schwarz-Romond et al., 2007) appearing upon Wnt treatment (see below). In unstimulated isogenic E-cadherin +/+ Kp6 cells dephospho-ß-catenin is prominent at the plasma membrane (Fig. 1C, right panel). This pool reflects transcriptionally
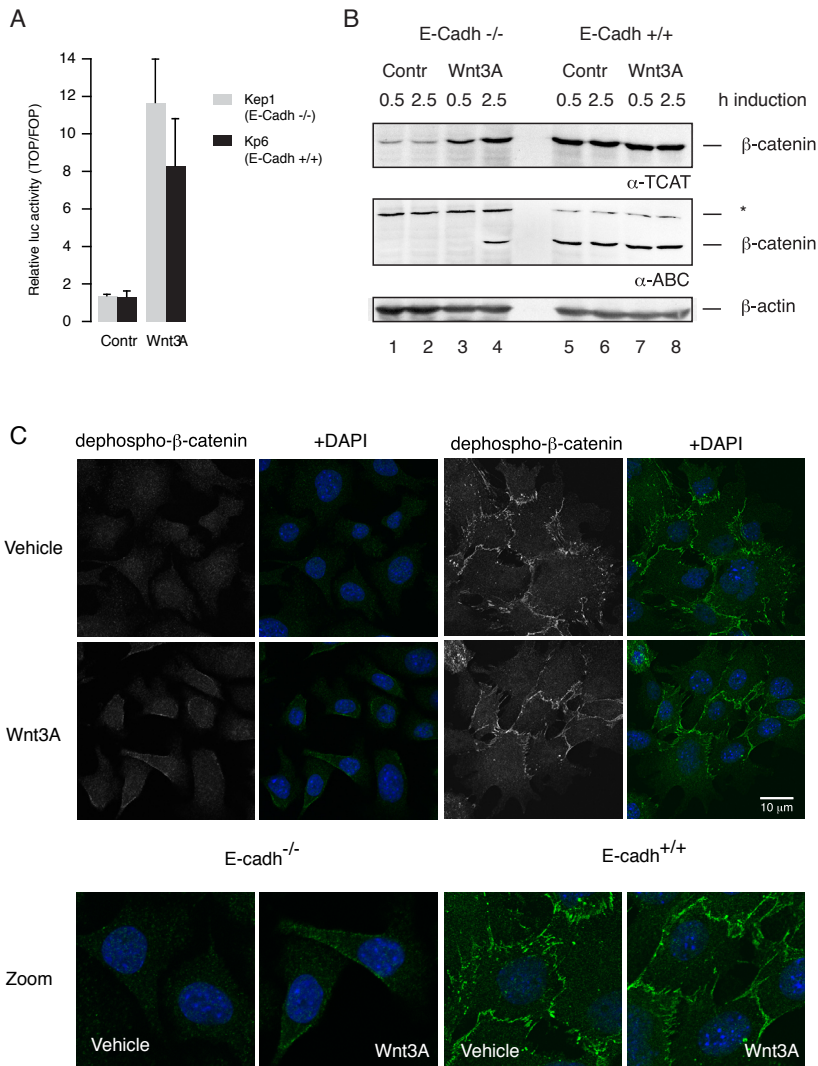
Figure 1. Cadherin-independent plasma membrane localization of dephospho-ß-catenin upon Wnt3A stimulation. A. E-cadherin negative cells respond normally to Wnt3a. Luciferase reporter assay in Kep1 (E-cadherin -/-) and Kp6 (E-cadherin +/+) cells using the TCF reporter TOP-TK and the control FOP-TK, normalized for transfection efficiency using pRL-CMV-Renilla. 24 Hours after transfection cells were stimulated overnight with Wnt3a conditioned or control medium and luciferase activity was measured. B. (Dephospho) ß-catenin levels in Kep1 or Kp6 cells. Cells were induced with Wnt3A protein or control and analyzed 0.5 or 2.5 h after induction by Western blotting using an antibody recognizing all forms of ß-catenin (α-TCAT) or an antibody specific for the N-terminal dephospho form (α-ABC). *, cross-reacting epitope. C. Subcellular localization of dephospho-ß-catenin in E-Cadherin positive or negative cells upon Wnt stimulation. Kep1 (E-cadherin -/-) or Kp6 (E-cadherin +/+) were induced with Wnt3A protein or control and analyzed 2.5 h after induction by immunolocalization with an antibody specific for the N-terminal dephospho form (α-ABC). DAPI was used as a nuclear marker. Note that dephospho-ß-catenin levels in E-cad -/- cells are much lower than in E-cad +/+ cells, requiring unequal confocal settings to be used.

inactive ß-catenin, as no reporter activity is detected (Fig. 1A). Wnt3A induces reporter activity in Kp6 (Fig. 1A), and a minor increase in signal is indeed detected on Western blot (Fig. 1B), but this increase does not translate in any noticeable increase in dephospho-ß-catenin staining in situ

(Figure 1C, right panel). This indicates that the signaling-competent dephospho-ß-catenin induced in response to Wnt stimulation is relatively minor in comparison to the steady-state junctional pool of dephospho-ß-catenin.

The absence of classical cadherins from Kep1 cells was confirmed using a 'pan-cadherin' antibody, which recognizes E, N and P cadherin. This antibody failed to show any membrane staining in Kep1 cells, neither before nor after Wnt3A stimulation (Supplementary Figure S1A). We wanted to further rule out the possibility that the recruitment of dephospho-ß-catenin to the plasma membrane could be the result of a Wnt-induced upregulation of a cadherin or another membrane protein acting as a cryptic docking site. Therefore, we studied the localization of dephospho-ß-catenin in Kep1 cells stimulated with Wnt3A protein, in the presence or absence of the transcription inhibitor actinomycin D. Under these conditions, ß-catenin was still stabilized and recruited to the plasma membrane (Supplementary Figure S1B), indicating that this process is independent of the induction of Wnt target genes.

Dephospho-ß-catenin colocalizes with APC, Axin and LRP6 at the plasma membrane after Wnt3A stimulation

Earlier studies have suggested translocation of members of the destruction complex to the LRP6 co-receptor upon Wnt signaling (Cliffe et al., 2003; Tolwinski et al., 2003). Phosphorylation on threonine 1479 of LRP6 is required for the recruitment of Axin and is carried out by CK1γ in response to Wnt signaling (Davidson et al., 2005). We therefore used a phospho-specific antibody recognizing this residue of LRP6 in immunofluorescence in Kep1 cells. We found that after stimulation with Wnt3A, phospho-LRP6 co-localized with dephospho-ß-catenin on the plasma membrane (Fig 2A). Likewise, we found that Axin colocalized with dephospho-ß-catenin (Fig 2B). These results suggest that the N-terminally dephosphorylated, or signaling-competent form, of ß-catenin is translocated to the receptor in a Wnt signaling-associated complex. Another component of the destruction complex, APC, has been reported to localize to the plasma membrane in different epithelial cell lines, including colon carcinoma cell lines (Miyashiro et al., 1995). As many available antibodies against APC are not reliable for immunofluorescence (Brocardo et al., 2005), we developed a monoclonal rat antibody, 3E7, that detects endogenous APC in immunofluorescence studies and on Western blot (Supplementary Figure S2). APC detected

with this antibody also clearly colocalized with ß-catenin on the membrane of Wnt3A stimulated Kep1 cells (Fig. 2C).

To further characterize the involvement of the Wnt receptor complex in recruitment of ß-catenin, we expressed ΔN-LRP6, a dominant active LRP6 receptor that mimics Wnt ligand engagement at the receptor complex. We could express this protein in SK-BR-3 cells, a breast cancer cell line with a homozygous deletion of E-cadherin (van de Wetering et al., 2001). Expression of this construct in Kep1 cells failed, since it was not properly presented at the plasma membrane in these cells (data not shown). As shown in Figure 2D, expression of ΔN-LRP6 resulted in a prominent plasma membrane localization of dephospho-ß-catenin, resembling the appearance of dephospho-ß-catenin in Kep1 cells at the plasma membrane after Wnt stimulation (Fig. 2A, B). This indicates that activation of LRP6 is involved in the E-cadherin-independent plasma membrane recruitment of dephospho-ß-catenin in response to Wnt signaling. Expression of ΔN-LRP6 in SK-BR-3 also resulted in colocalization of dephospho-ß-catenin with APC (Fig. 2E). We were unable to detect Axin in SK-BR-3 cells, possibly due to very low expression levels (data not shown). SK-BR-3 cells were found to be unresponsive to Wnt3A stimulation (data not shown) possibly due to the fact that these cells lack the appropriate Frizzled receptor for this ligand. We conclude that activation of the Wnt pathway by either Wnt3A or dominant active LRP6 leads to recruitment of Axin and/or APC and signaling competent ß-catenin to the plasma membrane.

LRP6-initiated dephospho-ß-catenin is transcriptionally significantly more competent than 'downstream-initiated' dephospho-ß-catenin

Our data so far are consistent with a model of Wnt signal transduction where, upon Wnt stimulation, *de novo* synthesized ß-catenin is attracted to the Wnt receptor complex together with members of the destruction complex. As Wnt activation results in co-activation of genes by ß-catenin in the nucleus, ß-catenin likely is released from the membrane complex and is routed to the nucleus. In order to test the relevance of membrane association of ß-catenin, we compared the activity of ß-catenin, either routed or not routed through the Wnt receptor complex. To mimic ß-catenin accumulation due to receptor activation we expressed ΔN-LRP6 in SK-BR-3 cells. To produce ß-catenin accumulation without receptor activation, we over-expressed wild-type ß-catenin. If the transactivating potential is similar regardless
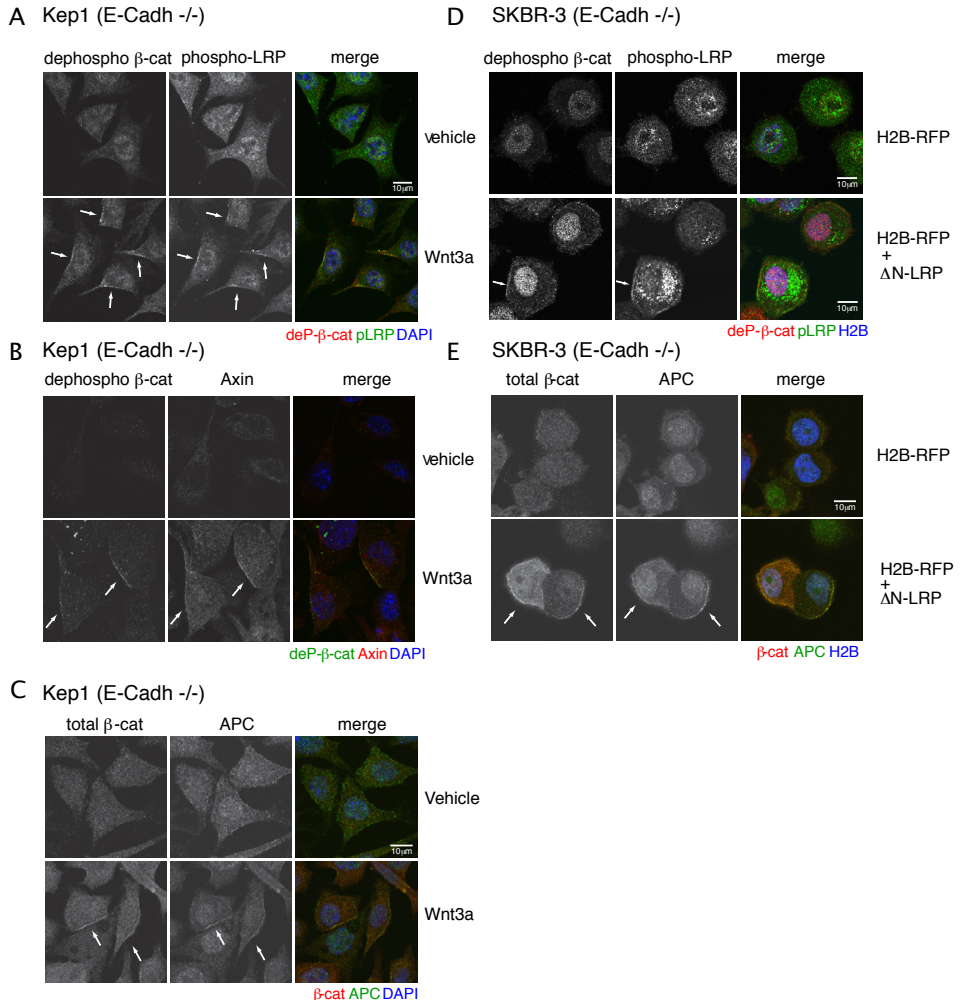
Figure 2. Dephospho-ß-catenin colocalizes with p-LRP6, Axin and APC at the plasma membrane in a Wnt-dependent (Kep1) or ∆N-LRP6 dependent (SK-BR-3) manner. Kep1 cells were induced with purified Wnt3A protein or vehicle for 2.5 h (Kep1), or transfected with ∆N-LRP6-encoding plasmid (SK-BR-3) and analyzed for subcellular localization of dephospho-ß-catenin and phospho-LRP6 (A and D), dephospho-ß-catenin and Axin (B), total ß-catenin and APC (C), and dephospho-ß-catenin and APC (E). Arrows denote plasma membrane localizations. Merged images are also shown that include DAPI as a nuclear marker (A-C) or H2B-mRFP as a transfection marker (D and E).

the source of dephospho-ß-catenin, the amount of luciferase output is expected to closely parallel the amount of dephospho-ß-catenin generated. As shown in Fig. 3, expression of ∆N-LRP6 resulted in significant upregulation of a lucifease reporter gene (Fig. 3C). A similar degree of TCF-reporter activation could be produced by transfection of wild-type ß-catenin, but this was accompanied by much higher cellular levels of dephospho-ß-catenin (Fig. 3D and E). Also, there was no enrichment of dephospho-ß-catenin on the plasma membrane under these conditions (Fig. 3B), while a prominent plasma membrane localization of the comparatively minor pool of dephospho-ß-catenin was induced by ∆N-LRP6 (Fig. 3A). Thus, in spite of significantly lower cellular levels of dephospho-ß-catenin, a similar degree of TCF reporter output can be achieved through LRP6 co-receptor activation.

Supraphysiological levels of exogenous ß-catenin are required to mimic Wnt activity in *Xenopus* embryos

We sought to support these observations in a second model. Wnt signaling activity in the early *X. laevis* embryo can be readily monitored by the formation of an ectopic body axis. We thus compared the levels of exogenously expressed ß-catenin required to induce secondary axes, to the levels produced by the endogenous dorsalizing center, or by a much stronger activation of the pathway by ectopic Wnt expression (Fig. 4G).

The endogenous Wnt pathway is active in the blastula (stage 8.5-9.5) and can be detected as
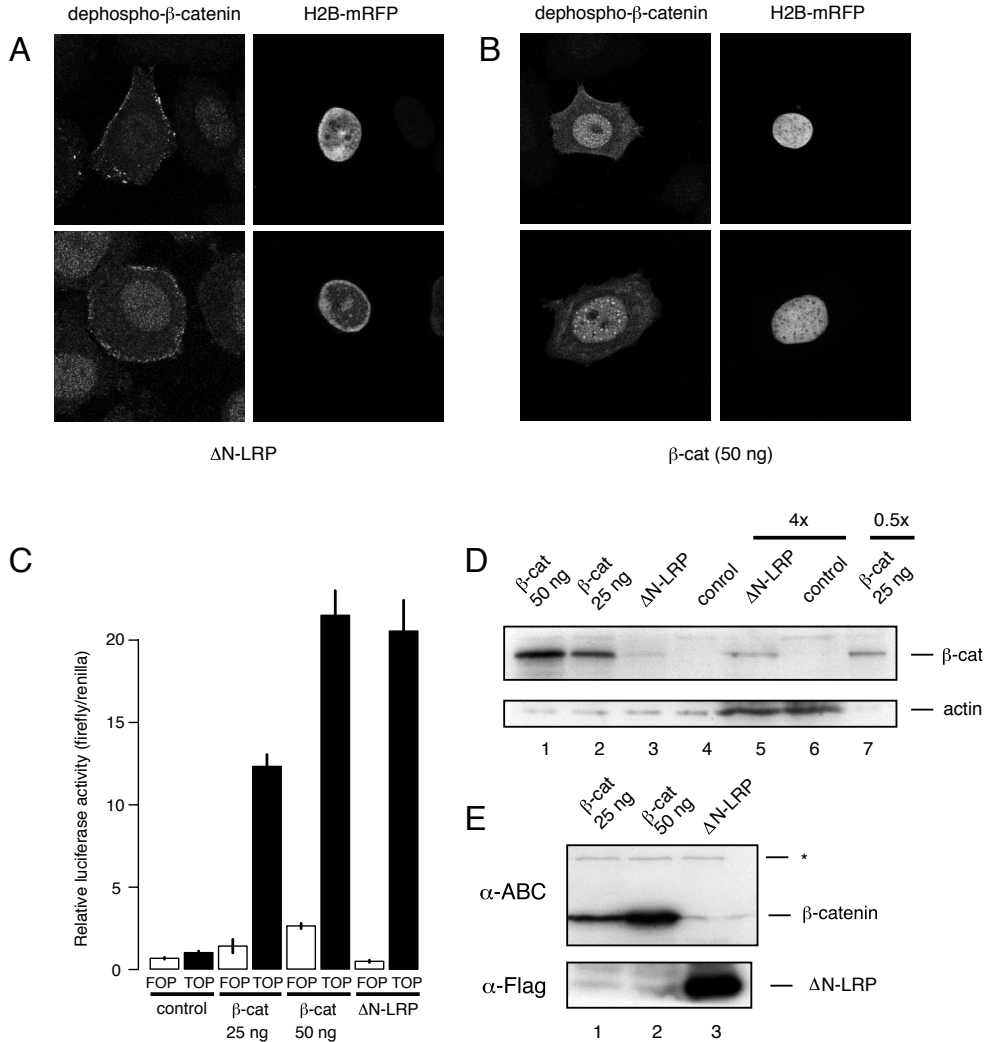
Figure 3. LRP6-initiated dephospho-ß-catenin is transcriptionally more active than downstream-initiated dephospho-ß-catenin. Cadherin-deficient SK-BR-3 breast carcinoma cells were transiently transfected with 25 or 50 ng plasmid encoding wild-type ß-catenin or 160 ng of a plasmid encoding ΔN-LRP6 and in parallel analyzed by immunolocalization (A and B), TCF transcriptional activity (C) and Western blotting (D and E). A-B. Immunolocalization of dephospho-ß-catenin in cells exogenously expressing ß-catenin or ΔN-LRP6, identified by co-expression of mRFP-tagged histone H2B. C. TCF-dependent transcriptional activity in SK-BR3 cells transfected with indicated plasmids 24 hours after transfection. TOP, TCF-reporter luciferase activity; FOP, mutated TCF-reporter activity. Values were normalized to a transfection control (constitutive Renilla luciferase reporter). D-E, Western blot analysis of cells shown in A-C, detecting total (D) or dephospho-ß-catenin (E) levels. 4x, four fold loaded; 0.5x, one half loaded.
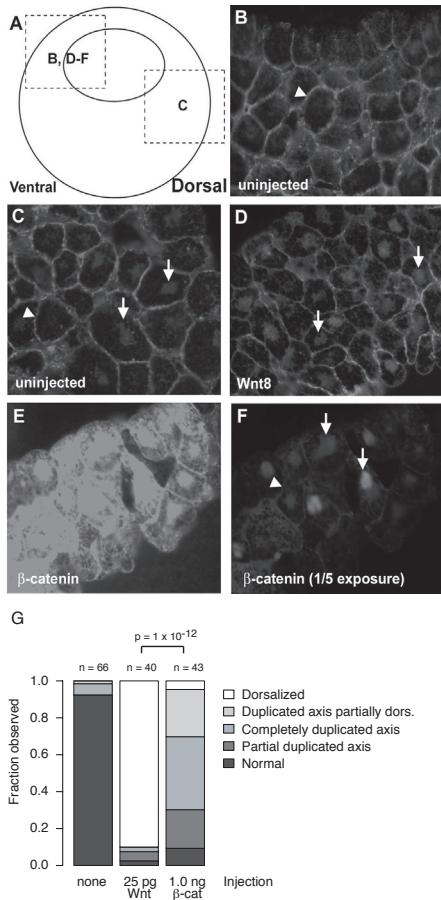
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Figure 4. Supraphysiological levels of exogenous ß-catenin are required to mimic Wnt activity in *Xenopus* embryos. ß-Catenin (1.0 ng) or Wnt8 (50 pg) mRNA was injected into the ventral side of 4-cell stage embryos and total ß-catenin levels achieved at blastula stage were estimated by immunofluorescence on cryosections. A. Diagram of crossection of a blastula embryo indicating the areas used to compare ß-catenin staining. B, C. Ventral-animal and dorsal regions of uninjected embryos. Arrows: nuclear ß-catenin. Arrowheads: plasma membranes. D. Ventral region from Wnt8-injected embryo. Note the increased nuclear signal. E. Ventral region from ß-catenin-injected embryo. Note the very strong signal throughout the cells. F. Same field recorded with a 5 times shorter exposure time. G. Comparison of dorsalizing activity of Wnt and ß-catenin in early Xenopus embryos. The degree of dorsalization obtained was scored at tailbud stage. The phenotypes were classified in five categories of increasing dorsalization: normal, partial duplicated axis, complete duplicated axis, complete duplicated axis with shorten axis indicating partial global dorsalization and completely dorsalized, i.e. global dorsalization with reduced or no axis and radial head structures. p value according to χ2 test.

nuclear accumulation of ß-catenin strongest in the dorsal side, but also spread throughout the prospective mesoderm, while the signal remains lower in the ectoderm (Schohl and Fagotto, 2002; Schohl and Fagotto, 2003). With 50 pg Wnt8 mRNA, which is in excess of the amount required to induce complete dorsalization, ß-catenin nuclear levels were found to be only slightly higher than levels induced by the endogenous pathway (Fig. 4). However, the levels of exogenous ß-catenin corresponding to induction of a secondary axis were well beyond physiological levels, both in the cytoplasm and in the nucleus. These observations indicate that exogenous ß-catenin is less effective at activating the pathway than endogenous ß-catenin regulated by Wnt signals, consistent with the hypothesis that Wnt-induced ß-catenin is qualitatively different.

Quantitative differences in dephospho-ß-catenin plasma membrane labeling in Wnt responding tissues *in vivo*

Our data in the isogenic Kep1 and Kp6 cell lines demonstrate that dephospho-ß-catenin is recruited to the plasma membrane in response to Wnt stimulation in an E-cadherin independent fashion. Such an unbiased analysis would not be feasible in other *in vitro* model systems such as the commonly used colon cancer cell lines, where the Wnt pathway is constitutively active and E-cadherin expression varies even amongst subclones of low-expressing cell lines such as LS174T (unpublished data). We find that in our model system the increase in dephospho-ß-catenin is subtle and not detectable *in situ* in an E-cadherin +/+ background. However, based on earlier data in the *Drosophila* system from the Wieschaus lab (see below) we optimized our staining protocol for the detection of dephospho-ß-catenin in other systems as well. As a first approach we double-stained *Xenopus* embryo sections for total and dephospho-ß-catenin (Fig. 5). We detected a clear signal for dephospho-ß-catenin at the plasma membrane of mesodermal cells, but less in ectodermal cells (Fig 5B). Wnt over-expression led to membrane recruitment of dephospho-ß-catenin in ectodermal cells (Fig 5A) and increased membrane staining of total ß-catenin. Despite the fact that this model system lacks the advantage of an E-cadherin negative background, the observations in a physiological setting of a quantitative difference in (dephospho-)ß-catenin accumulation at the plasma membrane in Wnt responding versus Wnt non-responding cells are in agreement with the observations in our initial model system not suffering from this drawback. In order to unam-

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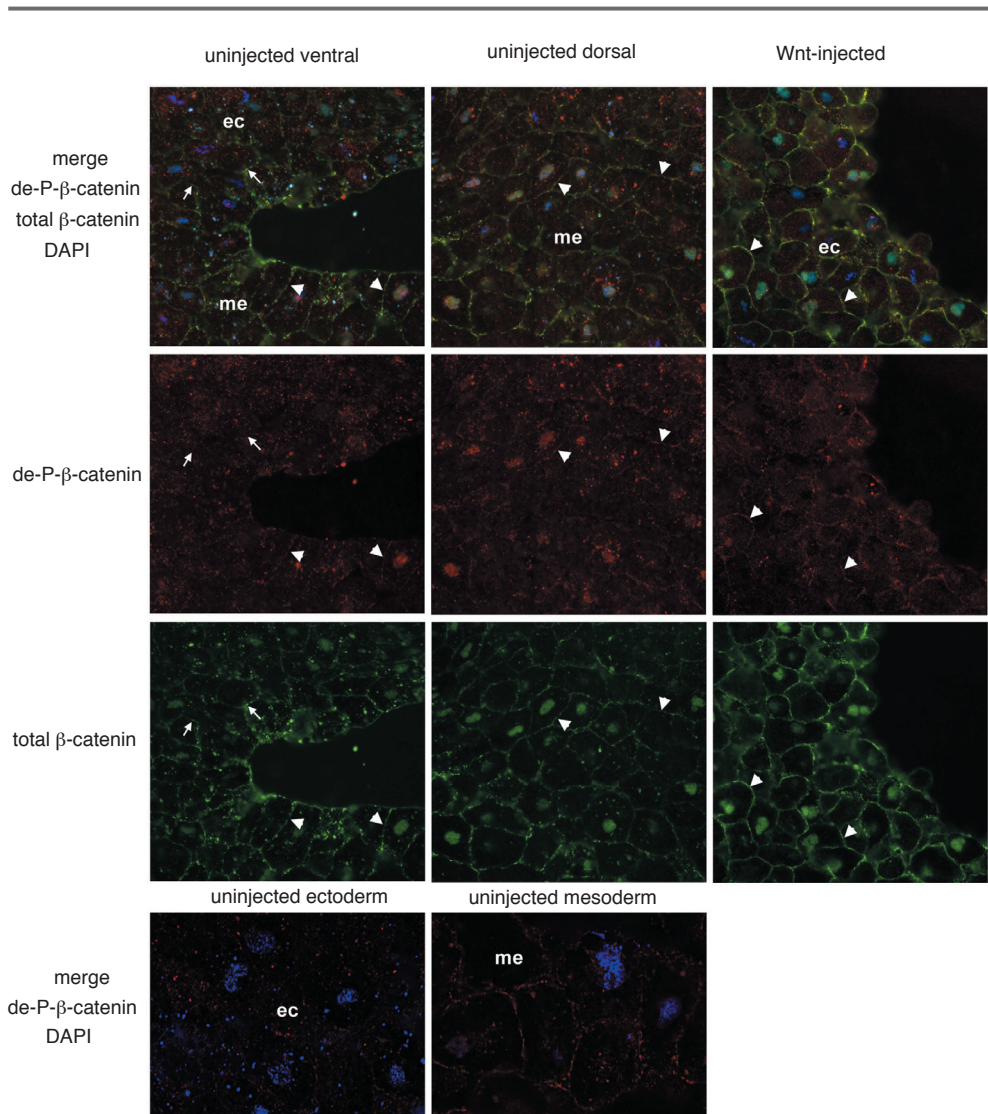**Figure 5. Membrane localization of dephospho-ß-catenin in the early *Xenopus* embryo.** Cryosections of stage 9 embryos were stained for dephospho-ß-catenin (ABC) and total ß–catenin (H102) and nuclei were counterstained with DAPI. A. Selected fields of the ventral presumptive ectoderm and mesoderm (uninjected ventral), dorsal mesoderm (uninjected dorsal), and ventral ectoderm of an embryo injected with 50 pg Wnt8 mRNA (Wnt-injected). In uninjected embryos, ABC stains the outlines of mesodermal cells (me) (arrowheads) but not ectodermal cells (ec) (arrows). Membrane ABC is detected in ectodermal cells of Wnt-injected embryos (arrowheads). Small bright cytoplasmic spots seen all three color channels correspond to autofluorescent pigment granules. B. Higher magnification view of ABC staining of ectodermal (ec) and mesodermal (me) cells of uninjected cells. These two images were obtained by collection of z-stacks followed by 2D-nearest neighbors diconvolution and merge of 10 images from each stack.

biguously assign translocation of Wnt-induced dephospho-ß-catenin as E-cadherin-independent however, an E-cadherin negative background is required. We point out that the results

on dephospho-ß-catenin plasma membrane accumulation in response to Wnt stimulation in the *Xenopus* model system parallel earlier data obtained in *Drosophila* where Wg signaling similarly
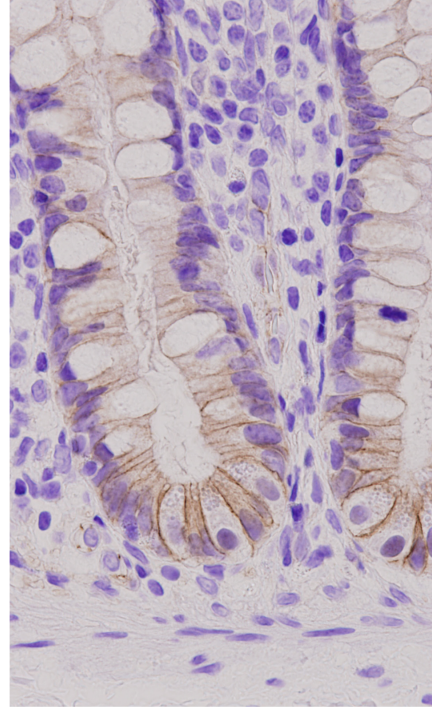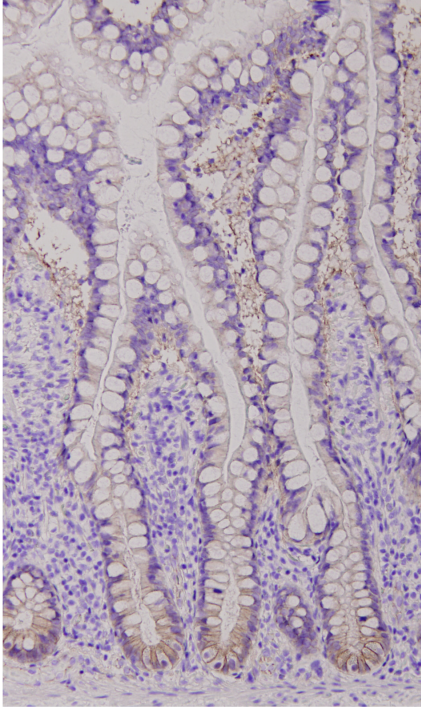
Figure 6. Dephospho-ß-catenin is enriched on the plasma membrane in human intestinal crypts. A. Low-power photomicrograph of a normal human small intestinal crypt-villus axis. Dephospho-ß-catenin is enriched on the crypt epithelial plasma membrane when compared to the plasma membrane labeling on differentiated villus cells. B. High-power photomicrograph of crypt compartment shown in A. Crypt epithelial cells including the presumptive intestinal epithelial stem cells or crypt base columnar (CBC) cells (Barker et al., 2007) show robust plasma membrane labelling.

increases plasma membrane levels of Armadillo (the fly ß-catenin homologue) in Wg-responding stripe regions, in addition to elevating cytosolic ,levels of the protein (Peifer et al., 1994b). This suggests that an increase in Armadillo at the plasma membrane is important for endogenous Wg signaling in the fruit fly as well.

We also tested Wnt-induced dephospho-ß-catenin plasma membrane enrichment in a second *in vivo* model of Wnt signaling. The intestinal epithelium of the human small intestine is organized into flask-shaped invaginations called crypts and finger-like projections termed villi. Wnt signaling has been shown to be essential for maintaining stem cell turn-over in the intestinal crypt (Korinek et al., 1998). We stained normal human small intestinal epithelium for dephospho-ß-catenin and found that similar to the situation in *Xenopus*, dephospho-ß-catenin is enriched at the plasma membrane of Wnt-responsive crypt epithelial cells when compared to differentiated cells on the villus epithelium (Fig. 6, Chapter 3,

Fig 3A and B).

We emphasize that it remains to be tested whether in these two *in vivo* examples of Wnt signaling, the plasma membrane localization of (dephospho-)β-catenin, which coincides with regions of known Wnt activity, represents the E-cadherin-independent signaling competent form, as this form could still potentially be masked.

Discussion

The currently prevailing model of Wnt signal transduction specifies that upon receipt of a Wnt ligand at the membrane-associated receptor complex, ß-catenin proteolysis is prevented and the protein accumulates in the cytosol. It has remained unclear how cytosolic destruction complexes sense ligand engagement at the plasma membrane. Recent studies have shown that Wnt treatment induces the formation of LRP6 co-receptor aggregates at the plasma membrane

(Bilic et al., 2007). These receptor aggregates in turn promote the recruitment of canonical Wnt pathway components including dishevelled, Axin and GSK3ß (Bilic et al., 2007; Schwarz-Romond et al., 2007). These observations parallel earlier data obtained in the *Drosophila* system where similarly recruitment of pathway components to the plasma membrane has been recorded upon activation of the pathway (Cliffe et al., 2003). How the inhibition of ß-catenin proteolysis ties up with the formation of these LRP6 signalosomes and whether ß-catenin itself may translocate to the plasma membrane along with its canonical destruction complex members is unknown. Here we show using an *in vitro* model system, that endogenous dephosphorylated ß-catenin indeed appears on the plasma membrane upon Wnt3A treatment. This translocation occurs independent of E-cadherin and dephosphorylated ß-catenin co-localizes at the plasma membrane with phospho-LRP6, Axin and APC. Together, our results suggests that Wnt signal transduction may be regulated at multiple levels other than, or in addition to, the inhibition of breakdown and that routing of *de novo* synthesized ß-catenin through the Wnt receptor complex is required for optimal transcriptional activity of the protein. This step in the processing of signaling-competent ß-catenin may have remained difficult to detect so far due to plasma membrane masking by the junctional pool of ß-catenin. We note that the punctate plasma membrane labeling observed in our model system bears a striking resemblance to the plasma membrane labeling described in the former studies (Bilic et al., 2007; Schwarz-Romond et al., 2007). In contrast to what has been described in these studies however, our plasma membrane enrichment appeared to occur at a later time-point. With regards this temporal difference, we stress that in contrast to these studies using overexpression assays to achieve stoichiometric amounts of destruction complex members, we have focused on the endogenous fraction of Wnt-responsive dephosphorylated ß-catenin only which, as we show, is relatively minor.

We propose that under normal physiological conditions, ß-catenin is activated at the plasma membrane upon Wnt stimulation, generating a signaling competent form. In line with this, Gottardi and Gumbiner have shown that Wnt stimulation generates a monomeric form of ß-catenin that selectively binds TCF and not E-cadherin (Gottardi and Gumbiner, 2004). Therefore, the activation step may constitute this transition. Plasma membrane activation is not absolutely required for signal transduction, as increasing ß-catenin to supraphysiological levels by interfering with its degradation will lead to transactivation as well. It will be important to test this hypothesis by studying the routing of Wnt-induced ß-catenin at endogenous levels.

Previous work in *X. laevis* has generated evidence for the notion that ß-catenin stability alone may not explain Wnt signaling outcome (Guger and Gumbiner, 2000; Nelson and Gumbiner, 1999). Later studies from the Wieschaus lab in the fly embryo using hypomorphic Armadillo alleles show that modulation of Wg signaling can occur in the presence of uniformly high levels of Armadillo (Tolwinski et al., 2003; Tolwinski and Wieschaus, 2001; Tolwinski and Wieschaus, 2004). Moreover, studies in cultured mammalian cell lines show that receptor-mediated signal transduction events such as Wnt stimulation or secreted Frizzled related protein (sFRP) inhibition can impinge on Wnt signaling output even in the presence of downstream mutations preventing ß-catenin breakdown (He et al., 2005; Suzuki et al., 2004). We are currently in the process of studying potential post-translational modifications on ß-catenin employing the E-cadherin -/- Kep1 cell line.

A model of ß-catenin activation at the receptor complex would allow ß-catenin output to be regulated on a direct stoichiometic 'per molecule' basis, in theory allowing one Wnt molecule to liberate a predetermined quanta of signaling-competent ß-catenin molecules. If correct, this regulation would be considerably more efficient than the currently proposed models in which Wnt signaling input is titrated against the activity of cytoplasmic degradation complexes to regulate gene expression in the nucleus. Regulation at multiple levels is similarly observed in the Hedgehog signal transduction pathway where stabilization of the transcriptional co-activator does not suffice for full activation (Methot and Basler, 1999). It is currently not known what mediates this activation step in Hedgehog signal transduction at the plasma membrane (Hooper and Scott, 2005). Regulation at multiple levels beyond the mere inhibition of proteolysis would allow the Wnt pathway to join other developmental pathways such as the Hedgehog and Notch signaling pathways, where the transcriptional co-activator is licensed for signaling at the plasma membrane.

Acknowledgements

Materials and Methods

Data analysis

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

Cell culture, transfection and reporter assays

SK-BR-3, Kep1 (E-cadherin-/-, p53 -/-), Kp6 (E-cadherin +/+, p53 -/-), SW480, LS174T, Colo320, HCT15, Colo205 and SW48 were cultured in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco-BRL) and were transfected using Fugene-6 (Roche) as instructed by the supplier. 1 x 105 cells were transfected with 300 ng TOP/FOP-TK-luc, 1.5 ng pRL-CMV, 325 ng ΔNLRP6, 50 or 100 ng ß-Catenin, 10 ng Wnt1 and 50 ng H2B-mRFP. Luciferase reporter activity was measured 24 hours after transfection in SK-BR-3. 24 hours after transfection with Top/Fop-TK-luc, Kep1 cells were stimulated with Wnt3A for 7 hours, after which luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

APC antibodies

Domains of mouse APC, termed APC-A (amino acids 788-1038), APC-B (amino acids 2170-2394), and APC-C (amino acids 2644-2845), were fused to GST and purified in bacteria. Rabbit polyclonal antisera were prepared as described (Hoogenraad et al., 2000). Rat monoclonal antibodies against APC were generated by Absea (China) using the same GST fusion proteins. Hybridomas were first tested for specific recognition of the respective GST fusion proteins. Positive clones (51 hybridomas for APC-A, 44 hybridomas for APC-B, and 58 hybridomas for APC-C) were subsequently tested on Western blot for recognition of eCFP-tagged APC domains and by immunofluorescence for detection of endogenous APC. We screened 9 rabbit polyclonals against the A, B and C domains, but none of the rabbit polyclonal antibodies were monospecific (data not shown). We subsequently screened 51 antibody-producing rat hybridomas for APC-A (amino acids 788-1038), 44 hybridomas for APC-B (amino acids 2170-2394) and 58 hybridomas for APC-C (amino acids 2644-2845). Two rat monoclonal antibodies (13F7, APC-A-derived, and 3E7, APC-B-derived) detected both overexpressed, eCFP-tagged APC (Supplementary Figure S2 C), as well as endogenous APC

on Western blots (Supplementary Figure S2 D). These antibodies also recognized GFP-tagged full length APC in transfected COS-1 cells (data not shown) and endogenous APC in different cell lines (Figure S2 D and data not shown). As 3E7 recognized clusters of full length APC in MDCK cells, and not in SW480 cells that contain truncated APC (Supplementary Figure S2 E, F), we conclude that 3E7 detects endogenous APC in immunofluorescence studies.

Other antibodies and reagents

Further antibodies used were against ß-Catenin (C19220) (Transduction Labs) and H-102 (Santa Cruz), active ß-Catenin (ABC 8E7), Actin (Ab-1, Oncogene), N-Axin (Fagotto), E-cadherin (C20820, Transduction Labs), pan-cadherin (C3678, Sigma), Tp1479 LRP6 (Niehrs), M2 mAb FLAG (Sigma). Purified recombinant Wnt3A was a kind gift from R. Nusse (Stanford, CA) or obtained from R&D Systems. 4',6-diamidino-2-phenylindole (DAPI) and Actinomycin D were obtained from Sigma.

Plasmids

Top/Fop-TK, pRL-CMV and pRK5SK-ß-catenin were described before (Hendriksen et al., 2005) and ΔN-LRP6 was a kind gift from H. Clevers (Hubrecht laboratory, Utrecht, The Netherlands).

Western blotting

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (25 µg per lane) and Western blotting using Immobilon-P transfer membrane (Millipore). Aspecific sites were blocked with 5% skim milk (Oxio, Hampshire, England) at room temperature for one hour. Note that detection of dephospho-ß-catenin with the αABC antibody was inhibited by certain brands/lots of skim milk. Primary antibodies were incubated in 1% skim milk for 2 hours at room temperature in the following dilutions: E-cadherin 1:1500; ß-Catenin mAb C19220 1:5000, ABC 1:500; Actin 1:5000; mFLAG M2 1:500. Blots were washed with phosphate buffered saline (PBS)/0.05% Tween 20. Enhanced chemiluminescence (Amersham) was used for detection of proteins.

Immunofluorescence and confocal microscopy

For immunofluorescence, cells were grown on glass coverslips coated with fibronectin (Sigma) and fixed in 3.7% formalin in PBS for 10 min and permeabilized for 5 min in 0.2% Triton/PBS. For antigen retrieval, cells were incubated in 10 mM citric acid buffer pH 6 at 95°C for 20 min and blocked in 5% BSA/PBS at room temperature

for 10 min. Primary antibodies were incubated for 2 hours in 1% purified BSA/PBS using the following dilutions; ABC 1:200; total ß-Catenin C19220 1:250; total ß-Catenin H102 1:65; N-Axin 1:50; N-APC 1:100; APC 3E7 1:100; p-LRP6 1:250; LRP6 1:300; pan-cadherin 1:5000. Cells were shortly washed in PBS and incubated in fluorescently conjugated secondary antibodies (Molecular Probes) and DAPI in 1% BSA/PBS for 30 min, washed shortly in PBS and mounted in Mowiol. Images were recorded using a Leica NT, SP2 or SP2 AOBS confocal microscope.

Embryo injections and immunofluorescence
4-cell stage embryos were injected in one ventral blastomere with 25 or 50 pg Wnt8 mRNA or 1000 pg myc-tagged ß-catenin mRNA as previously described (Fagotto et al., 1996). Stage 9 embryos were fixed in 3-4% paraformaldeyde and sections were prepared and stained as previously described (Schohl and Fagotto, 2002). Antibodies used were total anti-ß-catenin H102 diluted 1:50, ABC 1:250, and secondary goat Alexa546/Alexa488 anti-rabbit/anti-mouse (Molecular Probes). Images were recorded with a Leica microscope using a narrow Cy3 filter and a 20x oil immersion objective.

Immunohistochemistry
Sections (4 μm) were deparaffinized and antigen retrieval was carried out by 10 min of boiling in 10 mM Tris/1 mM EDTA (pH 9). Subsequently slides were immersed in 0.3% hydrogen peroxide in methanol for 30 min and nonspecific binding was blocked with 5% normal goat serum for 1 hr at room temperature. The sections were incubated for 1 hr at room temperature in primary antibodies against total ß-Catenin (C19220 Transduction Labs) and active ß-Catenin (ABC 8E7). The Ultravision antipolyvalent HRP detection system (Lab Vision Corp., Fremont, CA, USA) was used to visualize antibody binding sites with 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin.

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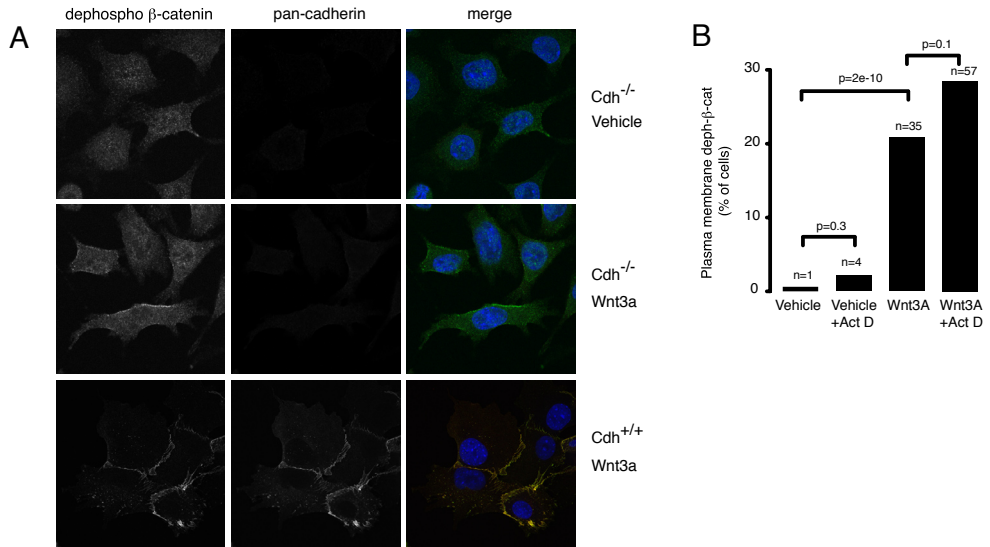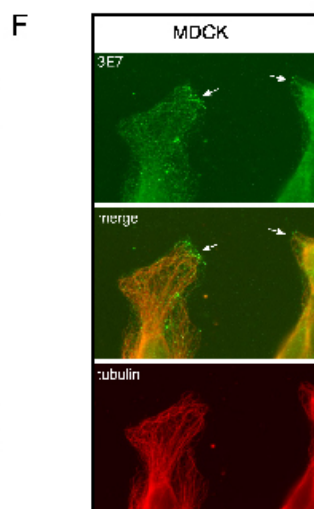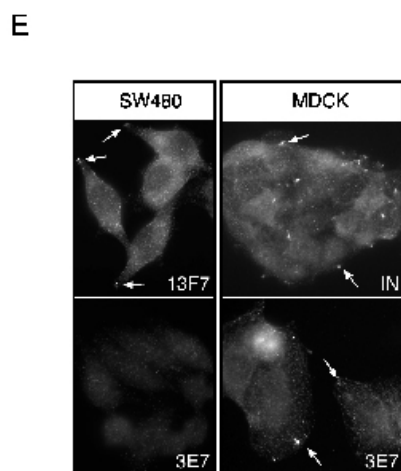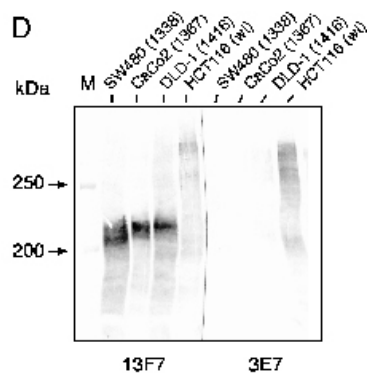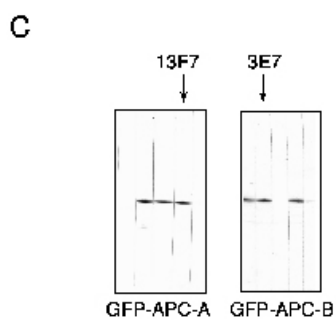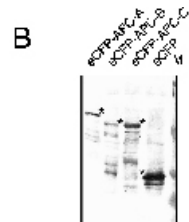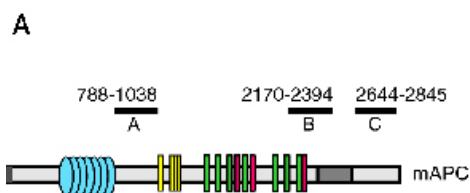
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Figure S1 A. Membrane localization of dephospho-ß-catenin does not coincide with membrane localization of cadherins. Cells were stimulated as in A and stained for dephospho-ß-catenin and cadherins using a pan-cadherin antiserum. As a reference, E-cad +/+ cells are used. B. Plasma membrane recruitment of dephospho-ß-catenin is independent of ongoing transcription. E-cadherin -/- cells were analyzed as in A in the presence or absence of the transcription inhibitor Actinomycin D at 4 μg/ml. No significant difference in plasma membrane localization of dephospho-ß-catenin was observed. P values according to Fisher's exact tests.

Figure S2 Generation of anti-APC antisera. A. Domain structure of APC. Coiled coil and basic regions are shown as dark grey boxes at the N- and C-terminal ends of APC, respectively. Armadillo repeats (light blue), and ß-catenin (yellow and green) and axin (red) binding regions are also depicted. The domains (amino acids indicated) used to make GST fusion proteins are shown above the sequence. B - D. Western blot analysis. In (B) a blot is shown of overexpressed eCFP, and eCFP-tagged APC-A, -B, and -C in COS-1 cells. Proteins were detected with anti-GFP antibodies. In (C) a blot is shown of eCFP-APC-A (left panel) or eCFP-APC-B (right panel), overexpressed in COS-1 cells, and detected with 5 rat monoclonals against APC-A or with 5 monoclonals against APC-B. Note that only some antibodies recognize the eCFP-tagged proteins. Strips incubated with 13F7 and 3E7 are indicated. In (D) blots are shown with indicated cell lysates. Of the 4 cell lines used only HCT116 expresses full length APC (indicated as wt). The amino acids at which APC is truncated in the other cell lines are indicated. Note that 3E7 only recognizes full length APC (and degradation products), while 13F7 recognizes both full length and truncated forms of APC (E, F) on immunofluorescence. In (E) SW480 and MDCK cells were fixed and stained with the indicated antibodies (IN: polyclonal rabbit antibody donated by Inke Nathke). APC is found clustered in peripheral domains (indicated by arows). Note that 3E7 fails to stain such clusters in SW480 cells. In (F) a double labelling of 3E7 with anti-tubulin antibodies was performed. APC is concentrated at the ends of a subset of microtubules (arrows). The staining patterns obtained with 3E7 and 13F7 are comparable to that obtained with the IN antibody and resemble published patterns.

A

788-1038   2170-2394   2644-2845
     A   B   C

mAPC

B

eGFP-APC-A
eGFP-APC-B
eGFP-APC-C
eGFP
fl

C

13F7   3E7

GFP-APC-A   GFP-APC-B

D

kDa M

SW480 (1338)
CrCo2 (1367)
DLD-1 (1416)
HCT116 (wt)
SW480 (1338)
CrCo2 (1367)
DLD-1 (1416)
HCT116 (wt)

250 →

200 →

13F7   3E7

E

SW480   MDCK

13F7   IN

3E7   3E7

F

MDCK

3E7

merge

tubulin

Chapter 5

Rapid nuclear export of GFP-ß-catenin via a facilitated mechanism independent of CRM1

Manuscript in preparation

5

Rapid nuclear export of GFP-ß-catenin via a facilitated mechanism independent of CRM1

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Stimulation of cells with Wnt results in nuclear translocation of ß-catenin and transcriptional activation of target genes. Export of ß-catenin out of the nucleus could be an important mechanism to end transcriptional activation. Currently, there are two models for ß-catenin nuclear export. The first suggests that nuclear export of ß-catenin is mediated by co-transport of APC (or Axin) via the CRM1 nuclear export pathway. The second model predicts that ß-catenin mediates its own nucleocytoplasmic transport. We analyzed the in vivo nuclear export kinetics of GFP-ß-catenin in Hek293 cells using Fluorescence Loss In Photo bleaching (FLIP). We show that GFP-ß-catenin nuclear export is very fast and exceeds the nuclear exit by diffusion of the small GFP molecule, suggesting that GFP-ß-catenin uses a fascilitated nuclear export mechanism. Furthermore, we find that nuclear export of GFP-ß-catenin is not influenced by leptomycin B (LMB) treatment that inhibits CRM1-mediated export. We find that ß-catenin interacts with FG repeat nucleoporins Nup62, Nup153, Nup214 and Nup358. We conclude that ß-catenin mediates its own nuclear export, supporting the idea that its localisation is regulated by retention.

The Wnt family of secreted signaling molecules regulate numerous processes during animal development and tissue homeostasis. Deregulation of the Wnt pathway is linked to many diseases including cancer (Nusse, 2005; Clevers, 2006). In canonical Wnt signaling, Wnt signals via ß-catenin to transduce the signal from the plasma membrane to the nucleus. In the nucleus, ß-catenin interacts with transcription factors of the TCF/Lef family and the co-activators Pygopus and Legless to regulate the expression of Wnt target genes (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997; Takemaru and Moon, 2000; Townsley et al., 2004).

Nuclear import and export may control the availability and thereby the activity of ß-catenin. ß-Catenin has a molecular weight of 92 kDa and is therefore expected to depend on the importin/exportin system for nucleo-cytoplasmic transport. However, ß-catenin contains no recognizable nuclear localization signal (NLS), or nuclear export signal (NES), which are required for receptor-mediated nucleo-cytoplasmic transport (Mattaj and Englmeier, 1998; Gorlich and Kutay). ß-Catenin can shuttle in and out of the nucleus (Fagotto et al., 1998; Yokoya et al., 1999; Prieve and Waterman, 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001; Suh and Gumbiner, 2003; Koike et al., 2004). Micro-injection and semi-permeabilized cell assays have shown that ß-catenin does not need Importin-ß, CRM1 or RanGTP to exit the nucleus (Prieve and Waterman, 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). Structural similarities between the ß-catenin armadillo repeats and the HEAT repeats of importins, have led to the hypothesis that ß-catenin mediates its own transport by interacting directly to the NPC proteins (Cingolani et al., 1999; Fagotto et al., 1998; Yokoya et al., 1999; Wiechens and Fagotto, 2001). Nucleocytoplasmic transport of ß-catenin is, however, still under debate. An alternative model specifies, that the APC tumour suppressor exports ß-catenin out of the nucleus, resulting in the degradation of ß-catenin in the cytoplasm. This model is based upon the observation that APC shuttles between the nucleus and cytoplasm, and that ß-catenin mimics the localization of APC containing either active or inactive NESs (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). Similar to APC, Axin and LZTS2 shuttle in and out of the nucleus, and a role for these proteins in ß-catenin nuclear transport has been suggested (Cong and Varmus, 2004; Thyssen et al., 2006).

In this study, we examined the nuclear export kinetics of GFP-ß-catenin in living cells, by performing *in vivo* nuclear transport assays in

Hek293 cells. We expressed GFP-tagged ß-catenin and performed Fluorescence Loss In Photo bleaching experiments (FLIP). We found that nuclear export of GFP-ß-catenin is fast, as full recovery of steady state levels is observed within 3 minutes. Furthermore, inhibition of the CRM1 pathway does not affect GFP-ß-catenin nuclear export. Furthermore, we show that ß-catenin interacts with FG repeat nucleoporins and that coupling of GFP to ß-catenin increases the nuclear export rate of GFP. We conclude that ß-catenin exits the nucleus on its own by inter-acting with FG repeat nucleoporins, and may act as a transport receptor itself.

Results and discussion

GFP-ß-catenin is active in Wnt signaling
To analyze the kinetics of ß-catenin in living cells, we expressed a carboxy-terminal fusion of GFP to ß-catenin in Hek293 cells. In a previous study, this GFP-ß-catenin fusion protein formed rod-like aggregates in the nuclei of COS and MDCK cells (Giannini et al., 2000). We observed these aggregates as well in the nuclei of Hek293 cells but only when high levels of the plasmid were transfected and/or after long expression times (data not shown). It is conceivable that these ß-catenin aggregates are formed to protect the cell from toxic levels of GFP-ß-catenin (Giannini et al., 2000). To prevent the formation of these ag-gregates in the nucleus, we expressed GFP-ß-catenin at very low levels for a short period (i.e. 100 ng per 3.10^5 cells for 20 hours). Under these conditions, the intracellular distribution of GFP-ß-catenin is uniformly distributed between the nucleus and cytoplasm, yet the nucleoli were ex-cluded (Fig 2A left image and data not shown).
To determine the expression of our GFP-ß-catenin fusion protein, we transfected Hek293 cells and analyzed cell lysates 24 hours after transfection by western blot. An anti-GFP mono-clonal antibody detected only full length GFP-ß-catenin (~115 kDa) and no free GFP (~25 kDa), showing proper expression of the GFP-ß-catenin fusion protein (Fig 1A). We next tested whether the GFP-ß-catenin fusion protein is functional in Wnt signaling by performing luciferase assays using the TCF-responsive TOP reporter (Korinek et al., 1997). In the ß-catenin deficient cell line NCI-H28, GFP-ß-catenin activates the TOP re-porter but not the control FOP (Fig 1B). These results show that the GFP-ß-catenin fusion pro-tein is properly expressed and active in transac-tivation.
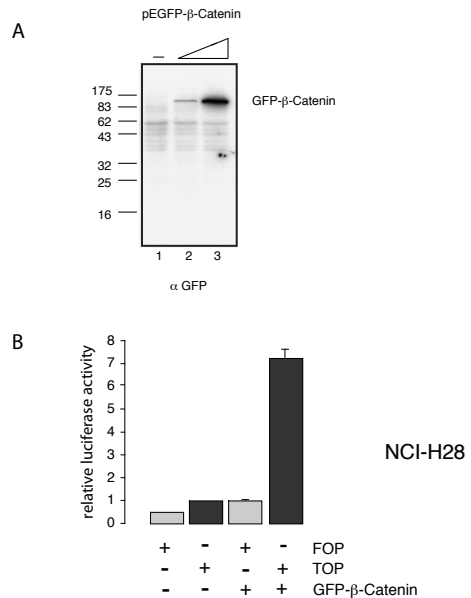
ß-Catenin exists in pools of different mobility

Figure 1. GFP-ß-catenin is functional. A. Hek293 cells were transfected with GFP-ß-catenin in increas-ing amounts. After 24 hours, cell lysates were ana-lyzed by western blot using a monoclonal antibody recognizing GFP. B. NCI-H28 cells were transfected with the TOP (dark bars) or the control FOP (light bars) luciferase reporter together with pRL-CMV, in pres-ence or absence of GFP-ß-catenin. Cell lysates were analyzed 24 hours after transfection. Shown are nor-malized relative luciferase values corrected with the pRL-CMV Renilla luciferase reporter. Bars represent SEMs of independent experiments.

To analyze the kinetics of ß-catenin nuclear ex-port in living cells, we expressed GFP-ß-catenin at low levels in Hek293 cells and performed Fluorescence Loss In Photo bleaching (FLIP) experiments. In these experiments, the entire nucleus or cytosol is bleached after which time laps images are taken to monitor the recovery rate. To control the precision of the laser beam, we performed similar bleach experiments on formalin fixed cells expressing GFP-ß-catenin. A well-defined spot was permanently bleached showing that there was no leakage of the laser beam to regions outside our indicated region of interest (data not shown). Many nuclear proteins, including transcription factors are highly mobile as measured by FRAP analysis (Fluorescence Recovery After Photo bleaching). Although these proteins move rapidly, their movements are not as fast as a free diffusing molecule such as GFP (Misteli, 2001).

We noticed that the mobility of GFP-ß-catenin in both the nucleus and cytoplasm was very high, as we could bleach either compartment effectively and uniformly by using 4 point bleaches of 3 seconds, suggesting that during this short bleaching time, all GFP-ß-catenin molecules in the bleached compartment had passed the laser beam.

Typically, after bleaching, fluorescence dropped in the bleached compartment to ~80%, whereas the compartment that was not bleached lost only ~20% (data not shown). To measure nuclear export, we bleached the whole cytoplasm and used time-lapse microscopy to monitor the recovery. The subsequent increase in cytoplasmic fluorescence of GFP-ß-catenin was equal to the decrease in nuclear fluorescence, suggesting that the cytoplasmic increase was the result of nuclear transport events. From the recovery curves, we calculated the tau value, i.e. the time needed for the fluorescence to recover to 63% between its level after bleach and the plateau
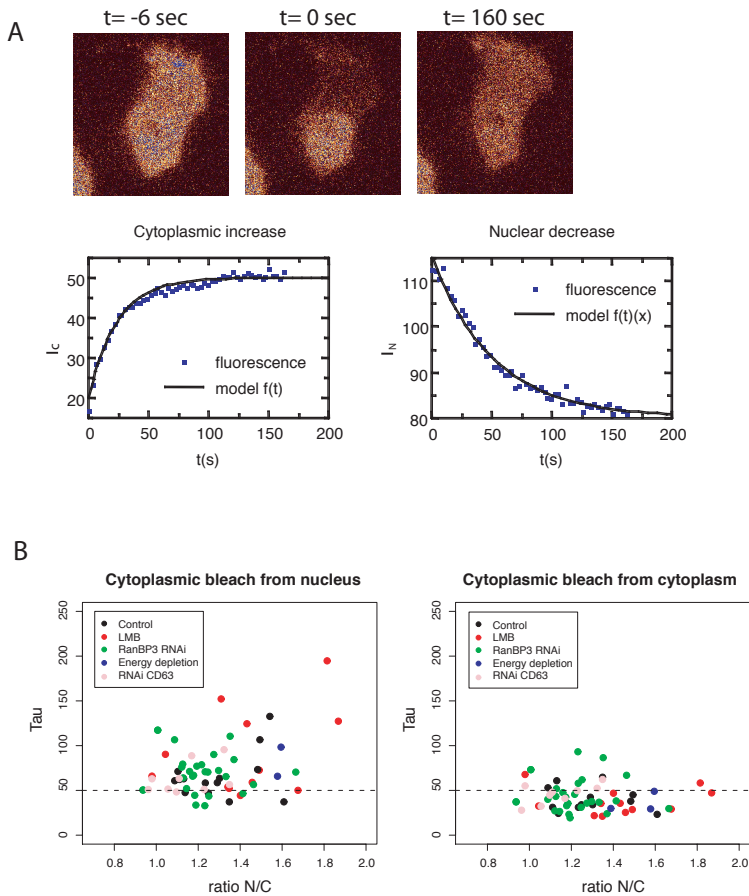

Figure 2. Time-lapse confocal images and recovery curves of a representative FLIP experiment. A. Hek293 cells were transfected with GFP-ß-catenin for 24 hours, after which FLIP experiments were carried out in imaging medium at 37°C. Two initial images (T = -6 sec) were taken after which the cytoplasm was bleached using 4 x 3 sec point bleaches. T = 0 is the first image of the time lapse that was used to monitor the fluorescent recovery. Steady state levels were reached within 160 seconds. Recovery curves were corrected for bleach depth, normalized and fitted to a single exponential function. B. Time constants (tau) of multiple experiments and experimental conditions were plotted against the nuclear/cytoplasmic (N/C) ratio of GFP-ß-catenin before bleaching. Observed from the cytoplasm, taus from a cytoplasmic bleach are close to 40 seconds independent of the N/C ratio (right). From a nuclear view, taus are higher and increase as the N/C ratio rises (left).

fluorescence (Fig 2A).

To measure export, we bleached GFP in the cytoplasm and measured the recovery time in both the nucleus and cytoplasm. The recovery curves were different depending on whether measurements were taken from the nucleus or the cytoplasm. Plotting the tau values of the recovery curves from the nucleus against the nuclear/cytoplasmic ratio, reveals that there are two pools of GFP-ß-catenin; a slow and a fast pool. The slow pool dominates export measured from a nuclear view and likely represents retention of GFP-ß-catenin in the nucleus, whereas the fast pool represents free GFP-ß-catenin. Increasing nuclear/cytoplasmic ratio's correlate with increasing tau values, suggesting that the slow pool of GFP-ß-catenin that is seen from the nucleus, is dependent on expression levels. However, when export was measured from the cytoplasm, we mostly detected the fast pool with no effects on expression levels (Fig 2B). We therefore studied GFP-ß-catenin nuclear export by measuring the fluorescent recovery in the cytoplasm.

The slow pool of GFP-ß-catenin that we observe in the nucleus may reflect nuclear retention by the formation of rod-like aggregate formation as described in Gianni et al. (Giannini et al., 2000). Nuclear levels of GFP-ß-catenin indeed rise upon higher expression levels and increased expression time. Alternatively, the slow and fast pool of GFP-ß-catenin in the nucleus may represent different molecular forms of ß-catenin that have been described earlier (Gottardi and Gumbiner, 2004). Higher levels of GFP-ß-catenin may shift the equilibrium to a molecular form of ß-catenin that is more likely to interact with nuclear proteins, such as TCF/Lef and BCL9 resulting in increased nuclear retention. Although retention is a likely mechanism to regulate ß-catenin, the different molecular conformations of ß-catenin remain speculative as no evidence for this theory was found in thermodynamic experiments on ß-catenin and its interacting partners (Choi et al., 2006).

Nuclear export of GFP-ß-catenin is fast and mediated by a facilitated transport pathway

To measure the export rate of GFP-ß-catenin in living cells, we bleached the cytoplasm of low expressing Hek293 cells and monitored the recovery time. Full recovery of fluorescence was observed within 160 seconds (2 min 40 sec) after bleaching, after which steady state levels were reached. We measured an averaged tau of 45 seconds, indicating that nuclear export of GFP-ß-catenin in Hek cells is fast (Fig 3). The nuclear

export rate that we measure for GFP-ß-catenin in Hek293 cells is more than twice as fast as described for YFP-ß-catenin in COS cells (Townsley et al., 2004). Differences in expression levels or cell types may account for this discrepancy. To compare the nuclear export kinetics of GFP-ß-catenin to those of other export substrates, we compared export of GFP-ß-catenin to the nuclear exit of free GFP, that can freely pass through the NPC by diffusion. Interestingly, the nuclear exit of the small GFP molecule was 1.5 times slower than that of GFP-ß-catenin, indicating that GFP-ß-catenin export is very efficient and mediated by a facilitated process. To confirm these findings in another cell line, we used the human colon cancer cell line SW480, which shows constitutively active Wnt signaling due to a truncation in APC. Nuclear export of GFP-ß-catenin in SW480 was as fast as in Hek293 cells and, likewise, the nuclear exit of free GFP was much slower than that of GFP-ß-catenin (Fig 3). These results indicate that differences in tissue origin and Wnt activation between these two cell types do not result in different nucleocytoplasmic transport kinetics of GFP-ß-catenin. Furthermore, the nuclear export rate of GFP-ß-catenin is faster than the nuclear exit of free GFP, indicating that ß-catenin export is mediated by an active transport mechanism (Ben-Efraim and Gerace, 2001).

Nuclear export of ß-catenin does not dependent of the CRM1 export pathway

Proteins of up to 30 kDa can diffuse through the NPC, yet larger proteins generally pass by a facilitated transport process that requires Ran and recognition by transport receptors (Mattaj and Englmeier, 1998; Gorlich and Kutay, 1999; Frey and Gorlich, 2007). The CRM1 nuclear export receptor is responsible for a considerable fraction of protein export out of the nucleus and recognizes its cargo by the NES. CRM1 binds directly to the NES in a RanGTP-dependent manner (Fornerod et al., 1997; Kudo et al., 1997; Stade et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997).

To test the involvement of the CRM1 pathway in ß-catenin nuclear export, we measured the nuclear export of GFP-ß-catenin in living cells in the presence and absence of the CRM1 inhibitor LMB. The earlier described shuttling substrate NLS-Rev-NES-GFP was used to control for the activity of LMB (Henderson and Eleftheriou, 2000). This substrate localizes to the cytosol in the absence of LMB due to its NES, but it accumulates in nucleoli when LMB is added. Hek293 cells were transfected with GFP-ß-catenin or
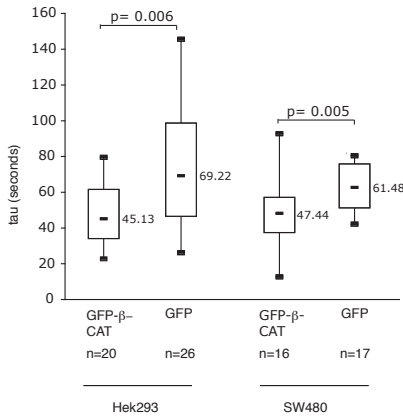
Figure 3. Nuclear export of GFP-ß-catenin is faster than that of GFP alone. Hek293 (left) and SW480 (right) cells were transfected with either GFP alone or with GFP-ß-catenin. After 24 hours, FLIP experiments were carried out to measure nuclear export using cytoplasmic bleaching. Kinetics of the cytoplasmic fluorescence recovery were analyzed by timelapse microscopy, corrected for bleach depth, normalized and fitted to a single exponential function. Taus are represented in a box plot. Indicated values are medians. P values are according to Mann-Whitney tests.

NLS-Rev-NES-GFP and either mock treated or incubated in imaging medium containing 50 nM LMB. Within 30 min, the NLS-Rev-NES-GFP reporter shifted from the cytoplasm to the nucleus, indicating that LMB was fully active (Fig 4A). We performed FLIP experiments on Hek293 cells expressing GFP-ß-catenin, and cultured the cells for at least 30 minutes to a maximum of 1.5 hours under LMB conditions. As shown in Figure 4B, the median tau value for GFP-ß-catenin decreased from 45 seconds without LMB to 34 seconds with LMB, but this drop was not significant (Fig 4B). This indicates that blocking the CRM1 pathway does not affect the nuclear export rate of GFP-ß-catenin. The steady state localization of GFP-ß-catenin was neither affected by LMB treatment, not even after treatment with LMB for more than 3 hours (data not shown). Our results show that in living cells, GFP-ß-catenin can exit the nucleus independently of the CRM1 nuclear export pathway. These results are in line with previous studies (Prieve and Waterman, 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001) and are not consistent with a role of APC, Axin or LTZS2 in ß-catenin nuclear export as these proteins all rely on the CRM1 nuclear export receptor to exit the nucleus.

ß-Catenin associates with FG repeat nucleoporins

As GFP-ß-catenin can exit the nucleus independently of the CRM1 export pathway, we sought for evidence that ß-catenin can translocate by itself through the nuclear pore. We therefore tested whether ß-catenin can interact with FG repeat nucleoporins using immobilized GST-tagged *X. laevis* ß-catenin to pull down interacting proteins from *X. leavis* egg extracts, which are highly concentrated in nucleoporins. We analyzed interacting proteins by western blot using monoclonal antibody 414 that recognizes a subset of FG repeat containing nucleoporins. Importantly, both full length and the armadillo repeat region of ß-catenin specifically interacted with FG repeat nucleoporins Nup62, Nup153, Nup214 and Nup358. Furthermore, a small amount of Nup214 also bound full length GST-ß-catenin (Figure 5). The interaction was not stimulated or weakened by non-hydrolysable forms of RanGTP (data not shown), indicating that the ß-catenin interaction was not mediated by nuclear trans-
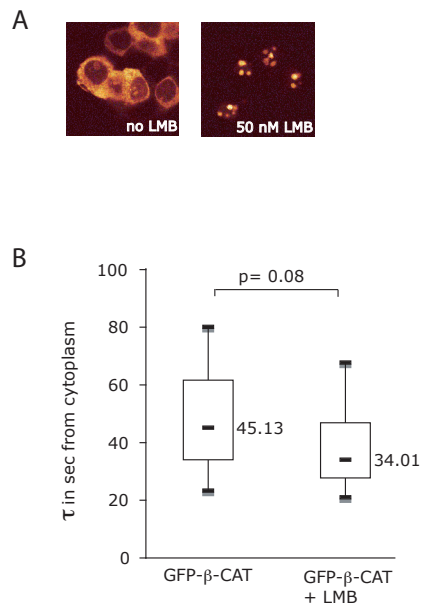
A

B

Figure 4. A. The CRM1 nuclear export pathway is efficiently blocked by addition of 50 nM LMB for 30 minutes. Hek293 cells were transfected with the shuttling substrate NLS-Rev-NES-GFP (Henderson and Eleftheriou, 2000). At 24 hours after transfection, cells were exposed to either normal imaging medium or imaging medium containing 50 nM LMB for 30 min. Thereafter, the cells were imaged to record the localization of the reporter. B. Blockade of the CRM1 pathway does not influence the nuclear export kinetics of GFP-ß-catenin. Hek293 cells were transfected with GFP-ß-catenin and export kinetics were determined as described in Figure 3.

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port receptors. These FG repeat nucleoporins are commonly known to bind nuclear transport receptors including those of the Importin-ß and NTF2 family (Moroianu et al., 1995; Bayliss et al., 2000; Fribourg et al., 2001; Conti and Izaurral-de, 2001; Vasu and Forbes, 2001; Bayliss et al., 2002). As ß-catenin shares structural homology with Importin-ß, it is interesting that it interacts with these common nucleoporins. Our results are in line with a previous study in which recom-binant *X. laevis* ß-catenin was shown to bind to the yeast Nup1 (Fagotto et al., 1998). Our data contradict a study (Stuh and Gumbiner, 2003) in which ß-catenin was tested for its ability to bind to the FG nups recognized by mAb 414, i.e. the same nups as tested here. In that study, the rela-tive binding of ß-catenin to FG repeat nups was compared to that of Importin-ß, but no interaction could be detected (Suh and Gumbiner, 2003). The affinity of Importin-ß for FG repeat nups is very high compared to other transport receptors (Ben-Efraim and Gerace, 2001). Therefore, it is imaginable that the relative weak interaction of ß-catenin with FG nups is below detection limits
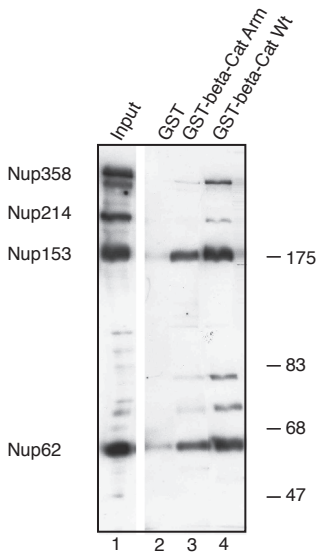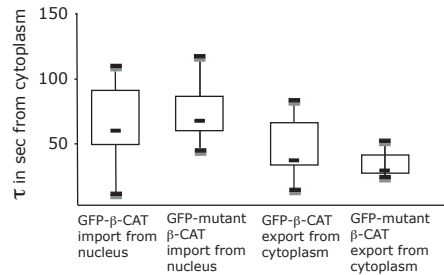

Figure 6. **Nuclear import and export kinetics of GFP-tagged ∆GSK3 mutant ß-catenin are not dif-ferent from wild type GFP-ß-catenin.** Hek293 cells were transfected with either GFP-ß-catenin or GFP-∆GSK3 ß-catenin. After 24 hours, import and export kinetics were measured. To measure import, the nucleus was bleached followed by monitoring the recovery of fluorescence in the nucleus. Transport ki-netics were measured, analyzed and represented as in Figure 3.

when compared to the strong affinity of Impor-tin-ß for FG nups. Moreover, weak interactions of transport receptors with FG repeats have been suggested to play an important role for efficient translocation through the inner channel of the NPC (Frey et al., 2006; Frey and Gorlich, 2007). In line with our observation that ß-catenin can exit the nucleus independent of CRM1, we find that ß-catenin interacts with FG repeat nups, suggesting that ß-catenin mediates its own translocation through the NPC.

ß-Catenin nuclear export is insensitive to GSK3ß phosphorylation

Our data so far have shown that GFP-ß-catenin can exit the nucleus by itself, most likely by me-diating its own nuclear export by interacting with the FG repeat nucleoporins. In recent years, it has been shown that not all ß-catenin molecules are equally active in transcriptional activation. Inhibition of ß-catenin degradation by abolish-ing proteosomal degradation results in increased ß-catenin levels, but not in increased transcrip-tion. However, blocking the phosphorylation of ß-catenin on its N-terminal GSK3/CK1 phos-phorylation sites does increase transcription. The use of the ABC antibody, which specifically recognizes ß-catenin that is not phosphorylated on its N-terminus, has been shown to report Wnt signaling activity more faithfully than antibodies directed against an epitope elsewhere in the pro-tein (Staal et al., 2002; Chan et al., 2002; Hen-driksen et al., 2005).

To test whether N-terminally dephosphorylated

Figure 5. **ß-Catenin interacts with FG- rich nu-cleoporins Nup62, Nup153, Nup214 and Nup 358** *in vitro*. GST (lane 2), GST-ß-catenin Arm (lane 3) and GST-ß-catenin (lane 4) were used to pull down inter-acting nucleoporins from *X. laevis* egg extracts in the presence of 2 µM RanQ69L. Bound proteins were analyzed by western blot using monoclonal antibody 414, recognizing FG repeat nucleoporins.

ß-catenin exhibits distinct nucleocytoplasmic shuttling behavior, we mimicked dephospho-ß-catenin by using the ΔGSK3 mutant ß-catenin in which all 4 GSK3 phosphorylation sites on its N-terminus are mutated to alanine. We tagged this protein to GFP and performed FLIP experiments to study its nuclear transport kinetics. In addition to measuring export, we also measured nuclear import of this substrate and compared it to the import of wild type GFP-ß-catenin. To measure import, we bleached the nucleus and analyzed the subsequent nuclear increase in fluorescence. We did not measure any significant differences in tau values of both the nuclear import and export between ΔGSK3 mutant and wild type GFP-ß-catenin (Fig 6). These results suggest that our ΔGSK3 mutant GFP-ß-catenin, which is more active in Wnt signaling, enters the nucleus as fast as GFP-ß-catenin. Furthermore, the results imply that N-terminal phosphorylations on positions 33, 37, 41 and 45 do not affect the ability of ß-catenin to interact with FG repeats to mediate its nuclear transport. However, as alanine mutations are not the same as natural non-phosphorylated residues, it is not clear whether this mutant is a good representative of active or dephospho-ß-catenin. As long as the exact nature of transcriptionally active or dephospho-ß-catenin remains elusive we cannot be totally sure whether the nuclear import of this protein is enhanced or its export decreased. Preliminary experiments using LiCl to block ß-catenin phosphorylation, however, did not show any differences in nuclear export kinetics either (data not shown).

In this study, we have confirmed that GFP-ß-catenin can exit the nucleus independently of CRM1 and hence, independently of APC and Axin (Prieve and Waterman, 1999; Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). We suggest that ß-catenin interacts with FG repeat nucleoporins to mediate its own nuclear export, which explains why GFP-ß-catenin nuclear export is very efficient even during LMB treatment. Moreover, coupling GFP to ß-catenin increases the nuclear export rate of GFP, suggesting that ß-catenin not only exits the nucleus similar to the transport receptors, but may also carry along substrates. Indeed, it has recently been shown that Lef1 can function as a natural nuclear import substrate for ß-catenin (Asally and Yoneda, 2005). It is fascinating that not only the mobility of GFP-ß-catenin within the cytoplasm and nucleus is very high, but also that GFP-ß-catenin is capable of shuttling quickly between these two compartments. Therefore, ß-catenin seems capable of relocalizing quickly in the cell to meet its

binding partners. A previously proposed model of retention seems applicable here (Tolwinski and Wieschaus, 2001). In this model, the binding partners of ß-catenin regulate its subcellular localization and therefore its activity. E-cadherin binds ß-catenin at the plasma membrane, APC, Axin and Dvl in the cytoplasm, and BCL9 and TCF in the nucleus. Therefore, Wnt signaling could regulate the availability of these pools of ß-catenin allowing the cell to respond quickly to the extracellular Wnt signal.

Materials and Methods

Plasmids
GST-ß-catenin and GST-ARM ((Wiechens and Fagotto, 2001)), GFP-ß-catenin (Giannini et al., 2000), pSUPER, pSUPER-RanBP3, Top-Tk and Top-Tk, pRL-CMV Renilla plasmids were previously described (Hendriksen et al., 2005). pEG-FP-N1 was obtained from Clontech.

In vitro binding studies
GST-ß-catenin binding studies were performed as described before (Hendriksen et al., 2005).

Luciferase reporter assays
NCI-H28 cells were cultured in 12-wells plates and transfected with 200 ng Top/Fop-Tk, 1 ng pRL-CMV Renilla and 25 and 100 ng GFP-ß-catenin. Luciferase activity was measured 48 h after transfection using the Dual-luciferase reporter assay system (Promega).

Western blotting
To detect GFP-ß-catenin 25 µg cell lysate was analyzed by SDS-PAGE. Western blotting was performed as described before (Hendriksen et al., 2005).

Cell culture, transfection and photo bleaching experiments
All cell lines were cultured in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco-BRL) and were transfected using Fugene-6 (Roche) or Lipfofectamine (Invitrogen) as instructed by the supplier. For FLIP experiments, 3.105 cells were grown on 40 mm coverslips and transfected with 100 ng pEGFP, GFP-ß-catenin or ΔGSK3-ß-catenin, up to 500 ng in total using pcDNA3 as stuffer DNA. FRAP experiments were performed 24 hours after transfection using a live cell chamber at 37°C in bicarbonate-buffered saline (containing: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 23 mM $NaHCO_3$, 10 mM glucose and 10 mM HEPES at pH 7.2).

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Photo bleaching and imaging was done on a confocal laser scanning microscope (SP2 TCS AOBS, Leica). Cells expressing low amounts of tagged-ß-catenin were selected and imaged at 4% laser power using the 488 laser line of the 20 mW argon laser and bleached at 100% laser power. The cytoplasm was bleached uniformly by using 4 point-bleaches of 3 seconds each. Using Leica time lapse, 2 images were taken before bleach using an interval of 1.68 seconds, after bleach 50 frames were imaged using a 3 second interval. Averaged intensities of regions of interest were measured using Image J and recovery curves and taus were determined using R software. LMB was used at 50 nM for 30 min up to 1.5 hours (Wolff et al., 1997). To control for LMB activity, cells were transfected with 1 µg of NLS-Rev-NES-GFP per 40 mm coverslip (Henderson and Eleftheriou, 2000).

Data analyis

Statistical analysis was done using the R software package (R Development Core Team, 2005). Nuclear and cytoplasmic decay curves were fitted to a single exponential function $I = I0 - A * (1 - exp(-t/tau))$, using the optim() function in R. Quality of the fit was assessed by "goodness of fit" $(R2) = 1-$ (sum of squared residuals) /(sum of squared differences from mean), where 1 equals a perfect fit and 0 no fit. A small number of fits with an R2 of < 0.8 were not used in further analysis. The low quality of these fits could be traced back to movement of cells during recording or very low signal to noise. The mean R2 of data excluding these outliers was 0.97.

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Chapter 6

Summary and Discussion

"Great minds discuss ideas,
average minds discuss events,
small minds discuss people"

Eleanor Roosenveld

Summary and Discussion

The research described in this thesis focuses on the behaviour, localization and routing of the ß-catenin protein in Wnt signaling. ß-Catenin is a multifunctional protein that binds E-cadherin at the cell surface to regulate cellular adhesion (Daugherty and Gottardi, 2007). In addition, ß-catenin is the central signaling molecule of the Wnt pathway, which delivers the Wnt signal from the cytoplasm to the nucleus (Clevers, 2006). In the nucleus, ß-catenin regulates the transcription of target genes in complex with TCF/Lef transcription factors (van de Wetering et al., 1997). The Wnt pathway regulates numerous cellular processes which, when deregulated, lead to the development of cancer (Giles et al., 2003). Knowledge of the behaviour and regulation of ß-catenin is therefore important for cancer research. The constant and rapid degradation of ß-catenin in the cytoplasm is considered to be a major regulatory mechanism of the Wnt pathway. The cytoplasmic degradation complex composed of APC, Axin and the protein kinases GSK3 and CK1, traps and phosphorylates ß-catenin on its N-terminus, marking it for ubiquitinization and subsequent degradation by the proteasome (Hart et al., 1998; Hart et al., 1999; Liu et al., 2002; Amit et al., 2002; Yanagawa et al., 2002). Engagement of the Frizzled and LRP5/6 receptors on the cell surface by Wnt results in the phosphorylation of the intracellular domain of LRP5/6, which serves as a docking site for Axin that is recruited to the receptor complex (Cliffe et al., 2003; Davidson et al., 2005; Zeng et al., 2005; Zeng et al., 2008). Axin recruitment hampers the degradation complex allowing ß-catenin to accumulate in the cell and to enter the nucleus. Nuclear localization of ß-catenin has been considered an indicator of active Wnt signaling. However, correlations with *in vitro* assays are poor and nuclear ß-catenin is rarely detected in human colorectal adenomas (Kobayashi et al., 2000; Anderson et al., 2002).

The hypothesis that the signaling capacity of ß-catenin is a direct consequence of an increased half-life of ß-catenin upon activation of Wnt signaling has been questioned. Guger and Gumbiner showed that in *X. laevis* embryos, enhanced signaling of N-terminal ß-catenin mutants is not accounted for by accumulation of either total or cadherin-free ß-catenin (Guger and Gumbiner, 2000). Staal and colleagues showed

that interference with ubiquitinization, and therefore ß-catenin breakdown, does not result in an increase in transcriptional activation of TCF reporter genes. Furthermore, they showed that only bona fide Wnt signals specifically increase the levels of N-terminally dephosphorylated ß-catenin in the nucleus (Staal et al., 2002). Finally, Chan et al. have shown that ∆45 ß-catenin, which cannot be phosphorylated on its N-terminus, is more transcriptionally active, stimulates cell growth and survival, binds less to E-cadherin and is enriched in the nucleus (Chan et al., 2002). In summary, these studies suggest that not all ß-catenin is qualitatively equal with respect to transducing the Wnt signal.

In Chapter 2 we describe a new role for Ran-binding protein 3 (RanBP3) as a negative regulator of nuclear ß-catenin activity. RanBP3 is a nuclear protein that functions as a cofactor in CRM1-mediated export (Englmeier et al., 2001; Lindsay et al., 2001; Noguchi et al., 1999; Taura et al., 1998). Overexpression of RanBP3 inhibits Wnt signaling in both human cells and *X. laevis* embryos. Conversely, reduction of RanBP3 levels results in increased Wnt signaling in human cells and *D. melanogaster* embryos. RanBP3 binds directly to ß-catenin in a RanGTP-stimulated manner and this is important for its inhibition of Wnt signaling (Hendriksen et al., 2005). This suggests a possible function in nuclear transport of ß-catenin. Two different export mechanisms have been proposed for ß-catenin. In the first, ß-catenin can exit the nucleus on its own, using interactions with the nucleoporins to pass through the NPC (Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). In the second, ß-catenin exits the nucleus via the normal CRM1 pathway, but since it does not have an NES, it binds and uses APC or Axin to exit the nucleus (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). We find several lines of evidence that favor the first mechanism. These include the finding that RanBP3 binds directly to ß-catenin, while in a CRM1 export complex, RanBP3 would be expected to bind ß-catenin via CRM1. Furthermore, we demonstrate that RanBP3 still affects ß-catenin-mediated transcription in cell lines expressing truncated APC, that lacks ß-catenin binding sites. Furthermore, we show that depletion of RanBP3 results in a specific accu-

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mulation of dephospho-ß-catenin in the nucleus. Dephospho-ß-catenin is considered to be the signaling-competent form of ß-catenin and is present in low amounts in the cell (Staal et al., 2002). SW480 colon carcinoma cells, however, express relatively high levels of dephospho-ß-catenin (Korinek et al., 1997). Using these cells, we show that RanBP3 overexpression clears dephospho-ß-catenin from the nucleus, leaving total ß-catenin unaffected. We find that dephospho-ß-catenin reflects only a small proportion of total ß-catenin levels. This is in line with the study of Staal et al., showing that cells with hampered phosphorylation and degradation of ß-catenin contain only a small fraction of ß-catenin in an active, dephosphorylated state. Our finding that RanBP3 specifically exports the dephosphorylated form of ß-catenin out of the nucleus, underscores the importance of this form of ß-catenin for Wnt signaling activity.

In Chapter 3 we used a panel of colon carcinoma cell lines to study dephospho-ß-catenin in more detail. Staal et al. had shown that dephospho-ß-catenin correlates well with trancriptional activity and that it is restricted to the nucleus of Hek293T cells after stimulation with Wnt (Staal et al., 2002). In our panel of colon carcinoma cell lines, we found that dephospho-ß-catenin was mainly localized to cell-cell contacts. Few cell lines showed prominent nuclear dephospho-ß-catenin, which correlated with absence of E-cadherin expression. We also tested the localization in Hek293T cells before and after Wnt3a stimulation. As these cells express E-cadherin, dephospho-ß-catenin was found at the cell-cell contacts and not in the nucleus (our unpublished results). Our results indicate that at least part of the pool of dephospho-ß-catenin at the plasma membrane functions in cellular adhesion and, therefore, not all dephospho-ß-catenin is signaling competent.

Close inspection of ß-catenin at the plasma membrane revealed that dephospho-ß-catenin is present at the apico-lateral site, whereas total ß-catenin decorates the whole lateral membrane. This correlates with the expression of E-cadherin, which is enriched at the adherens junctions that localize apico-laterally in the plasma membrane (Takeichi et al., 1990). We stained human small intestine and found a similar specific localization of dephospho- and total ß-catenin. Interestingly, these differences in localization were only found in the crypt area of the colon, not in the villi. As Wnt signaling is active in the colon crypts

to stimulate the regeneration of the epithelium (Korinek et al., 1998), it is tempting to speculate that part of the pool of dephospho-ß-catenin at the plasma membrane is active in Wnt signaling. A possible scenario is that plasma membrane localized dephospho-ß-catenin might have a storage function, allowing the cell to quickly respond to incoming Wnt signals. Communication between E-cadherin and Fz/LRP receptors after Wnt induction might occur in the signalosome aggregates, although this remains highly speculative. Release of ß-catenin from the adherens junctions has been described to occur during epithelial-to-mesenchymal transitions (EMT), but it is not observed under normal circumstances (Behrens et al., 1993; Piedra et al., 2001). We performed fractionation experiments on our panel of colon carcinoma cell lines and found that neither E-cadherin-bound nor free dephospho-ß-catenin is predictive of Wnt signaling output. This indicates that the activity of ß-catenin in Wnt signaling is regulated on multiple levels, in support of previous studies (Guger and Gumbiner, 2000; Staal et al., 2002). Based on these results, we emphasize the need for an E-cadherin-negative background in studying the Wnt-responsive dephosphorylated pool of ß-catenin.

In Chapter 4, we have used an E-cadherin negative background to study dephospho-ß-catenin in Wnt signaling. We used the mouse mammary carcinoma cell line Kep1 that does not express detectable levels of ß-catenin and has no TCF reporter activity (Derksen et al., 2006). Interestingly, stimulation of these cells with Wnt3a protein resulted in the appearance of distinct dots of dephospho-ß-catenin at the plasma membrane. These dephospho-ß-catenin dots colocalized with APC, Axin and the activated (i.e. phosphorylated) co-receptor LRP6. These dots strongly resemble the recently described LRP-signalosomes, which are large protein aggregates that occur at the plasma membrane upon Wnt induction (Bilic et al., 2007; Schwarz-Romond et al., 2007a; Schwarz-Romond et al., 2007b; Zeng et al., 2008). We complemented these recent studies, by showing that dephospho-ß-catenin is also recruited to the plasma membrane upon Wnt stimulation. We find in both human cells and *X. laevis* embryos that Wnt-induced ß-catenin is transcriptionally more competent than overexpressed ß-catenin. Furthermore, in response to Wnt stimulation, a pool of dephospho-ß-catenin is recruited to the LRP5/6 receptor at the plasma membrane, independently of E-cadherin. We propose that optimal transcriptional activity of

dephospho-ß-catenin requires routing to and activation at the receptor complex at the plasma membrane. Activation of ß-catenin at the Fz/LRP receptor complex would allow Wnt and ß-catenin to adopt a 1:1 stoichiometry. This scenario would be far more efficient than the current models in which Wnt signaling input is titrated against the activity of the degradation complex in the cytoplasm. Moreover, activation of ß-catenin at the plasma membrane would put the Wnt pathway in line with other signaling pathways, in which the transcriptional activator is licensed for signaling at the plasma membrane.

It should be noted however, that elevating ß-catenin levels to supraphysiological levels results in transcriptional activation as well, suggesting that ß-catenin activity is regulated at multiple levels. Future studies using inducible GFP-tagged ß-catenin to mimic endogenous ß-catenin may help to clarify whether ß-catenin is translocated to the receptor complex before entering the nucleus. Alternatively, ß-catenin routing could be studied by using biochemical tagging. For instance, the LRP receptor could be modified by an intracellularly fused biotin ligase domain and ß-catenin by addition of an avidine tag. If the systems works, detection of biotinylated ß-catenin in the nucleus would hint for routing of ß-catenin to the nucleus via the receptor complex at the plasma membrane.

It is very important to determine the molecular signature of the highly active pool of ß-catenin that is recruited to the signalosomes at the plasma membrane upon Wnt induction. This pool is very small and independent of E-cadherin. Furthermore, the lack of phosphate groups at positions 33, 37, 41 and 45 alone is not sufficient to identify signaling competent ß-catenin in the cell. It is feasible that dephospho-ß-catenin is marked by post-translational modifications leading to increased interaction with transcriptional activators, such as Legless/BCL9. Possible Wnt-induced modifications on dephospho-ß-catenin remain to be identified and could be addressed by using immunoprecipitated dephospho-ß-catenin in mass spectrometric analysis. Identification of the molecular signature of active ß-catenin would provide a highly useful tool to study ß-catenin in human cancer and to develop medicines that specifically inhibit its signaling function.

Gottardi and Gumbiner (2004) have suggested a model in which ß-catenin exist in different mo-lecular conformations that determine whether the protein acts in signaling or adhesion. The activity of dephospho-ß-catenin could therefore be regulated by such Wnt-induced conformational changes in ß-catenin. Gottardi and Gumbiner showed that in the absence of Wnt, ß-catenin binds equally well to E-cadherin and TCF. After Wnt induction, however, a monomeric form of ß-catenin is generated that binds TCF but not E-cadherin. The authors suggested that the monomeric transcriptionally active form of ß-catenin may be regulated by the C-terminus that folds back to interact with its final arm repeats, thereby overlapping the E-cadherin binding domain (Cox et al., 1999; Piedra et al., 2001; Castano et al., 2002; Gottardi and Gumbiner, 2004). The presence of such a conformation at the C-terminus of ß-catenin is not supported, however, by the recently published structure of full-length zebrafish ß-catenin (Xing et al., 2008). Both the N- and C-termini of ß-catenin were demonstrated to be unstructured, and interact with the armadillo repeat domain in a highly dynamic and variable manner. It should be noted that bacterially produced proteins were used to solve the crystal structure. Therefore, it may still be possible that Wnt-induced post-translational modifications contribute to an *in vivo* stabilization of the C-terminus (Gottardi and Peifer, 2008).

We and others have shown that N-terminally dephosphorylated ß-catenin correlates with Wnt signaling activity (Staal et al., 2002; van Noort et al., 2002; This thesis, Chapters 2-4). Gottardi and Gumbiner (2004) found no evidence for a contribution of the N-terminus of ß-catenin with regards to binding selectivity towards TCF and E-cadherin. It is possible that the key site of activation of ß-catenin is located in the armadillo repeats which is the site where most interaction partners bind. Future studies mapping the ß-catenin domain that is necessary for Wnt-induced, E-cadherin-independent plasma membrane localization should help to clarify these issues.

Several studies have been published on the regulation of ß-catenin nuclear export. According to some, ß-catenin is co-exported out of the nucleus by the APC or Axin proteins, a mechanism that depends on the CRM1 nuclear export pathway (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000; Wiechens et al., 2004; Cong and Varmus, 2004). Other studies, however, have used very specific inhibitors of CRM1 and found no effect on ß-catenin export. In ad-

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dition, ß-catenin was shown to exit the nucleus on its own, independent of CRM1 and RanGTP (Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). As nuclear export of ß-catenin is a mechanism to terminate Wnt signaling, we decided to study the nuclear export of ß-catenin (Chapter 5). To do so, we used photobleaching techniques to monitor the kinetics of GFP-tagged ß-catenin. GFP-ß-catenin was very mobile in both the cytoplasm and nucleus. Furthermore, we found that GFP-ß-catenin exits the nucleus very rapidly and that inhibition of the CRM1 pathway does not influence the nuclear export of ß-catenin, which is in support of previous studies (Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). Additionally, the nuclear export of GFP-ß-catenin exceeded that of the free diffusion of GFP alone, suggesting that GFP-ß-catenin exits the nucleus via an active transport pathway. Our data suggest that ß-catenin may use a similar mechanism as the transport receptors to pass the nuclear pore complex. Indeed, we found that ß-catenin interacts with FG repeat nucleoporins, which is a prerequisite for facilitated transport of transport receptors.

Our results contradict a previous study in which the authors failed to detect an interaction between ß-catenin and FG repeat nucleoporins (Suh and Gumbiner, 2003). In that study, however, ß-catenin was compared to importin-ß with respect to binding to nucleoporins. Importin-ß displays the strongest interaction to FG nucleoporins, whereas the interaction between ß-catenin and FG nucleoporins that we observed was weak. It is therefore likely that under the conditions used in the study of Suh et al., the binding of ß-catenin to FG repeat nucleoporins was below the detection limit. It is important to note that weak interactions of transport receptors with FG repeats are important for efficient translocation through the inner channel of the NPC (Frey et al., 2006; Frey and Gorlich, 2007).

We suggest that ß-catenin mediates its own nuclear export and that its localization is regulated by retention via its interaction partners. Our results are supported by another study that similarly made use of photobleaching techniques to measure the nuclear export of ß-catenin (Krieghoff et al., 2006). In that study, however, full recovery of YFP-ß-catenin export was observed after 8 minutes, i.e. three times slower than our GFP-ß-catenin. In addition, the authors found that export of YFP alone was faster than that of YFP-ß-catenin. Both observations could be

explained by differences in expression levels of tagged ß-catenin and/or the use of a different fluorescent marker. We did not map the binding domain of RanBP3 on ß-catenin, although RanBP3 interacted less well to arm1-12 compared to full-length ß-catenin. The specificity of RanBP3 to dephospho-ß-catenin implies that the binding domain overlaps the N-terminus of ß-catenin plus part of the arm repeats. Future studies narrowing down the RanBP3 binding site on ß-catenin may answer whether RanBP3 is sensitive to the presence of negative phosphate on the N-terminus of ß-catenin.

It is intriguing that whereas *C. elegans* uses different ß-catenin proteins to regulate cellular adhesion and Wnt signaling, higher organisms have united these functions in one single protein (Korswagen et al., 2000). Combined with new structural information from zebrafish ß-catenin, the different ß-catenin proteins from *C. elegans* can provide helpful information. A new structural domain, called HelixC has been identified in the first part of the C-terminus of zebrafish ß-catenin. HelixC forms an α-helix that packs on armadillo repeat 12 to shield the hydrophobic residues and that extends the superhelical core of ß-catenin (Xing et al., 2008). Strikingly, HelixC is absent from *C. elegans* Hmp-1, which is involved in adhesion, while the two ß-catenin proteins involved in signaling retain HelixC (Schneider et al., 2003). Drugs targetting HelixC in ß-catenin could therefore specifically inhibit the tumour promoting signaling function of ß-catenin without affecting the tumour suppressive adhesion functions of the protein.

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Nederlandse Samenvatting

"Yesterday is history,
today is a gift,
tomorrow is an opportunity"

Adapted from Mystery Land ID&T

Nederlandse Samenvatting

De Wnt signaal transductie route vervult een belangrijke rol in cellulaire processen die cruciaal zijn tijdens embryonale ontwikkeling en tijdens het handhaven van de normale homeostase in volgroeide weefsels. Deregulatie van de Wnt route resulteert in een verstoorde balans tussen proliferatie en differentiatie wat kan leiden tot het ontstaan van ziekten zoals kanker. Wnt eiwitten worden gesecreteerd en functioneren als groeifactoren die communicatie tussen cellen bewerkstelligen. Binding van Wnt aan de Fz en LRP5/6 receptoren resulteert in de ontvangende cel in een activatie van de Wnt signaal transductie, die zal leiden tot een veranderding in transcriptie. Een centrale rol in de Wnt route wordt vervuld door het ß-catenine eiwit. ß-Catenine is een typisch voorbeeld van een eiwit dat verschillende functies heeft in de cel. Zo is het betrokken bij cel-cel adhesie aan de plasma membraan in een complex met E-cadherine, reguleert het het cytoskelet, en functioneert het als transcriptionele activator in de Wnt route. In een cel die niet gestimuleerd wordt door Wnt groeifactoren, is ß-catenine louter aanwezig in het E-cadherine complex in de cel-cel contacten. Vrij ß-catenine wordt namelijk efficiënt gebonden en afgebroken door een complex dat bestaat uit de 2 tumorsuppressor eiwitten APC en Axin, en uit de 2 kinases GSK3 en CK1. Dit complex phosphoryleert het N-terminale deel van het ß-catenine eiwit waardoor het gemarkeerd wordt voor afbraak door het proteasoom. Recente publicaties hebben laten zien dat na binding van Wnt aan de Fz en LRP6 receptor, er grote eiwit complexen worden gevormd aan de plasma membraan. Deze zogenaamde signalosomen bevatten verschillende eiwitten uit de Wnt route waaronder Fz, LRP6, Dvl, Axin en APC. Een mogelijk mechanisme is dat Wnt de Fz en LRP receptoren clustert waarna Dvl gerecruteerd wordt naar de Fz receptor. Dvl kan lange oligomeren vormen die ervoor zouden zorgen dat nog meer receptoren aggregeren. Axin wordt gebonden door Dvl waardoor ook de aan Axin bindende eiwitten APC, GSK3 en CK1 in de signalosomen gevangen worden. Hierdoor kunnen GSK3 en de membraan gebonden CK1γ het intracellulaire domein van LRP6 fosforyleren waar Axin vervolgens aan zal binden. Binding van Axin aan LRP remt de activiteit van het afbraakcomplex, waardoor ß-catenine accumuleert in de cel en naar de kern kan gaan om transcriptie te activeren. De regulatie van de stabiliteit van het ß-catenine eiwit wordt gezien als een cruciale stap in de Wnt route. Een aantal studies hebben echter laten zien dat niet alle ß-catenine moleculen even actief zijn in het reguleren van transcriptie. De aanwezigheid van ß-catenine dat niet gefosforyleerd is op de N-terminus, ook wel defosfo-ß-catenine genoemd, correleert veel beter met transcriptionele activiteit dan totaal aanwezig ß-catenine. Dit proefschrift beschrijft een aantal studies waarin in het bijzonder defosfo-ß-catenine bestudeerd is.

De mechanismen die de activiteit van ß-catenine in de cel kern reguleren zijn niet geheel duidelijk. Hoofdstuk 2 van dit proefschrift beschrijft een nieuwe rol voor het Ran-bindingseiwit 3 (RanBP3) als een negatieve regulator van de activiteit van ß-catenine in de kern. RanBP3 is een nucleair eiwit waarvan bekend is dat het functioneert als een cofactor in de CRM1 nucleaire export route. Overexpressie van RanBP3 resulteert in een remming van Wnt signalering in zowel menselijke cellen als in *X. laevis* embryo's. Reductie van RanBP3 heeft het tegenovergestelde effect. De remmende werking van RanBP3 op ß-catenine is afhankelijk van de capaciteit van RanBP3 om RanGTP te binden. RanGTP is net als RanBP3 een co-factor die betrokken is bij export uit de cel kern. Het mechanisme waarmee RanBP3 ß-catenine reguleert zou dus mogelijk nucleaire export kunnen zijn. Wij vonden echter geen effecten op de lokalisatie van ß-catenine na manipulatie van RanBP3 hoeveelheden. Echter, toen we specifiek naar actief, oftewel defosfo-ß-catenine keken, vonden we dat RanBP3 de export van deze vorm van ß-catenine uit de kern bevordert. RanBP3 beïnvloedt de lokalisatie van defosfo-ß-catenine zelfstandig, onafhankelijk van de CRM1 export route. Onze resultaten passen in een model waarin RanBP3 export van defosfo-ß-catenine uit de cel kern stimuleert om zo een eind te maken aan het Wnt signaal. Het feit dat RanBP3 specifiek de export van niet gefosforyleerd ß-catenine uit de celkern bevordert, illustreert het belang van deze vorm van ß-catenine voor de Wnt route.

Hoofdstuk 3 beschrijft de lokalisatie van niet ge-fosforyleerd ß-catenine in een aantal colon tumor cellijnen en in normaal humaan darm weefsel. Het was eerder beschreven dat defosfo-ß-catenine correleert met ß-catenine dat transcription-eel actief is, en dat deze vorm van ß-catenine specifiek lokaliseert in de celkern. Onze data laat echter zien dat defosfo-ß-catenine vooral op de cel-cel contacten lokaliseert. Alleen wanneer cellen geen of weinig E-cadherine tot expressie brengen, vinden we defosfo-ß-catenine in de celkern. Het feit dat een vrij grote hoeveelheid defosfo-ß-catenine op de cel-cel contacten zit, suggereert dat het daar functioneert in cel adhe-sie. Nadere inspectie van ß-catenine aan de cel-cel contacten laat zien dat defosfo-ß-catenine specifiek op de apico-laterale zijde van de cel-membraan zit, terwijl totaal ß-catenine op de gehele baso-laterale membraan voorkomt. Dit verschil in lokalisatie vinden we in tumor celli-jnen en interessant genoeg ook in de regio van de humane darm waar de Wnt route actief is; de crypten. Deze bevinding suggureert dat een deel van het defosfo-ß-catenine op de apico-laterale membraan wellicht betrokken kan zijn in Wnt signalering. Het is echter onmogelijk om deze potentiële subgroep van actief ß-catenine aan de membraan te onderscheiden van het altijd aanwezige ß-catenine betrokken bij cel adhesie. Bovendien vinden we in ons panel van colon tu-mor cellijnen geen correlatie tussen Wnt activiteit en de aanwezigheid van ß-catenine in de kern of aan de cel-cel contacten. Wij benadrukken daarom het gebruik van een E-cadherine nega-tief systeem bij het bestuderen van de rol van niet gefosforyleerd ß-catenine in Wnt signalering.

In hoofdstuk 4 beschrijven we hoe we, door ge-bruik te maken van cellen die geen E-cadherine hebben, een kleine maar distincte hoeveelheid defosfo-ß-catenine aan de plasma membraan detecteren na stimulering met Wnt3a. Deze subgroep van defosfo-ß-catenine kan alleen gevisualiseerd worden in E-cadherine negatieve cellen omdat het in normale cellen gemaskeerd wordt door het ß-catenine aanwezig in cel-cel contacten. Defosfo-ß-catenine dat verschijnt op de celmembraan na Wnt stimulering in E-cad-herine negatieve cellen, colokaliseert zowel met de geactiveerde LRP6 receptor als met APC en Axin. In humane cellen en in *X. leavis* embryo's vinden we dat ß-catenine meer actief is wan-neer het gestabiliseerd wordt na een Wnt signaal dan wanneer het verhoogd tot expressie wordt gebracht. Onze data suggereert dat defosfo-ß-catenine samen met de rest van het afbraak-

complex naar de LRP6 signalosomen migreert na Wnt inductie. Daarnaast stellen we een model voor waarin het uiteindelijke resultaat van deze recrutering niet de remming van ß-catenine af-braak is, maar juist een activatie van ß-catenine aan de plasma membraan. Een dergelijk model maakt een 1:1 stoichiometrie mogelijk voor Wnt en ß-catenine, en plaatst de Wnt route in parallel met andere ontwikkelingsroutes waarbij het ef-fector eiwit aan de membraan geactiveerd wordt.

ß-Catenine reguleert transcriptie in de cel kern en dus zijn de import en export mechanismen van het eiwit mogelijk van belang voor de regu-latie van transcriptionele output. ß-Catenine is een vrij groot eiwit dat normaal gesproken een import en export signaal nodig zou hebben om via de transport routes te kunnen migreren tus-sen kern en cytoplasma. ß-Catenine migreert tussen kern en cytoplasma, maar import en ex-port signalen ontbreken. Er zijn op het moment twee theorieën over de export van ß-catenine: de eerste oppert dat ß-catenine zijn eigen export medieert door een interactie aan te gaan met de eiwitten van de kern porie. De tweede theorie stelt voor dat ß-catenine indirect via de normale CRM1 route migreert door te binden aan APC of Axin, die wel de juiste transportsignalen bevatten voor deze route. In hoofdstuk 5 hebben wij spec-ifiek de export van ß-catenine uit de kern bestu-deerd met een techniek die het mogelijk maakt om in levende cellen GFP (groen fluorescent eiwit) gelabelde ß-catenine te volgen. Uit onze metingen blijkt dat export van ß-catenine erg snel is, en nog steeds plaats vindt wanneer de CRM1 route volledig geblokkeerd is. Daarnaast laten we zien dat we de export van het vrije GFP molecuul uit de kern kunnen versnellen door het te koppelen aan ß-catenine, en dat er een *in vi-tro* interactie is tussen ß-catenine en de eiwitten van de kern porie. Deze resultaten suggereren dat ß-catenine zelfstandig de kern verlaat door te binden aan de kern porie en dat het wellicht zelf als transport receptor kan fungeren. Onze data impliceren dat ß-catenine vrij kan bewegen in de cel en dat lokalisatie gereguleerd wordt door retentie van eiwitten die ß-catenine binden.

Curriculum Vitae

Jolita Hendriksen werd geboren op 13 februari 1977 te Doetinchem. Na het behalen van haar VWO diploma in 1995 aan het st-Ludgercollege te Doetinchem, begon zij in datzelfde jaar de studie Biologie aan de Universiteit Utrecht. Tijdens deze studie liep zij haar eerste stage bij de vakgroep Moleculaire Microbiologie onder begeleiding van Dr. A. Pettersson en Dr. J. Thomassen aan de Universiteit Utrecht. Hier werd de immmunogeniteit en variabiliteit van de lactoferrine receptor eiwitten LbpA en LbpB onderzocht. De tweede stage werd gelopen bij de afdeling Tumor Biologie in het Nederlands Kanker Instituut. Onder begeleiding van Dr. Ir. I. Gaemers en Dr. Ir. J. Hilkens werden promoter studies verricht aan het MUC1 gen, dat een belangrijke rol speelt bij metastasering van borstkanker. De studie Biologie werd in maart 2001 afgerond, waarna zij als promovendus begon op de afdeling Tumor Biologie onder begeleiding van Dr. M. Fornerod. Het verzamelde werk heeft geresulteerd tot dit proefschrift.