Intracellular routing of β-catenin

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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-

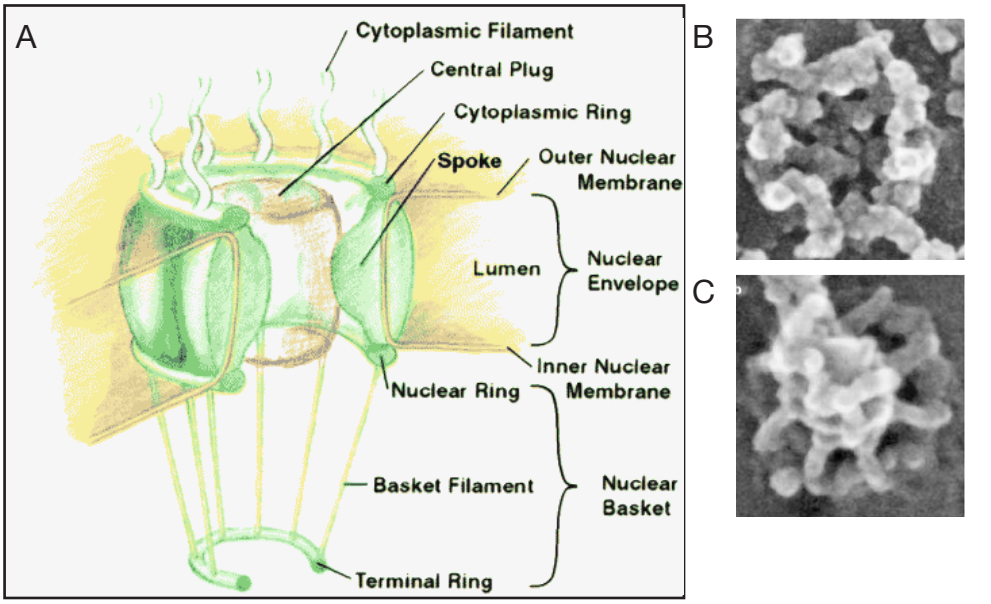

Figure 1. A. Schematic representation of a nuclear pore complex. Indicated are the spoke, nuclear and cytoplasmic ring structures and the nuclear basket and cytoplasmic filaments. From Ohno et al., 1998. B and C. Scanning electron microscopy images of the cytoplasmic (B) and nuclear (C) side of a NPC, 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-

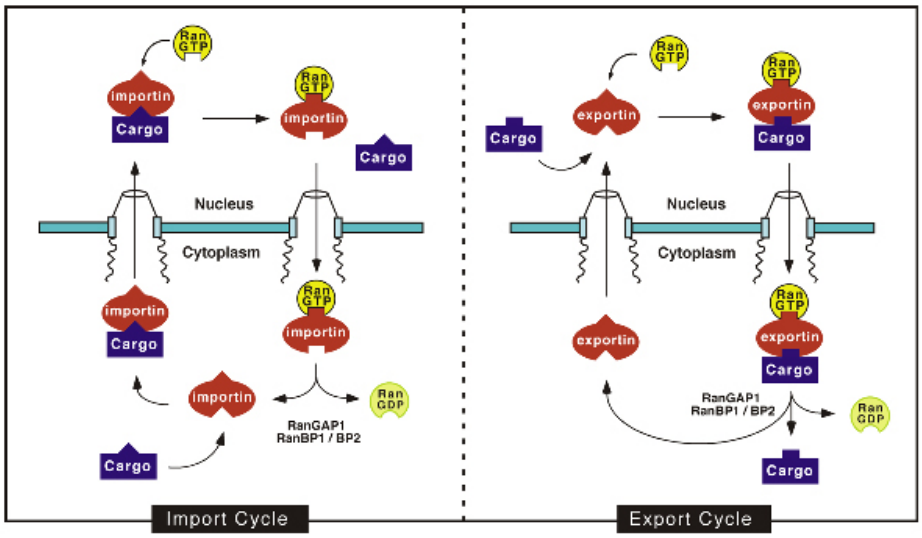

Figure 2. Schematic overview of a nucleocytoplasmic import (left) and export cycle (right). Adapted from (Ohno et al., 1998).

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; Paraskeva et al., 1999). Ran-binding protein 3 (RanBP3) 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).

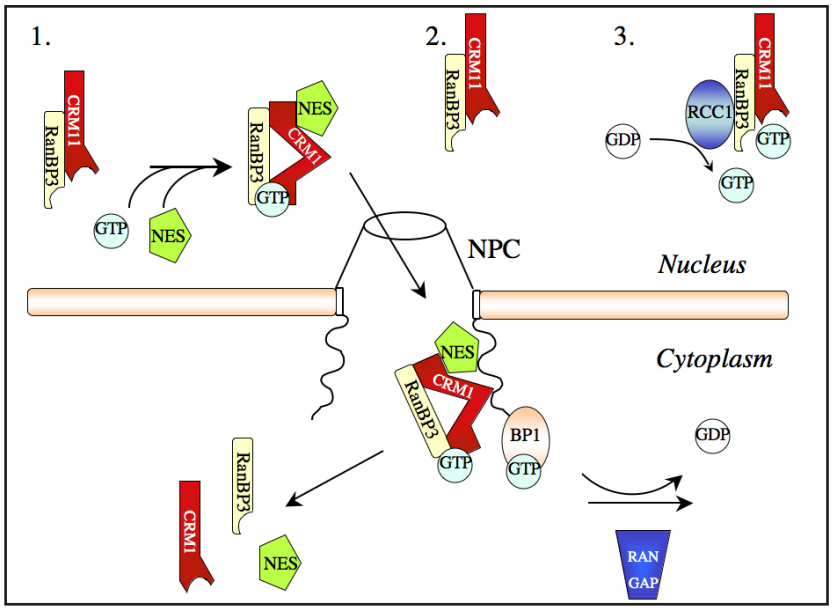

Figure 3. Schematic representation of RanBP3 function. 1. RanBP3 stimulates the stability and export of CRM1-NES export complexes. 2. RanBP3 functions as a nuclear anchor keeping CRM1 concentrations high in the nucleus. 3. RanBP3 stimulates RanGEF activity to increase the efficiency of CRM1 loading with 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-

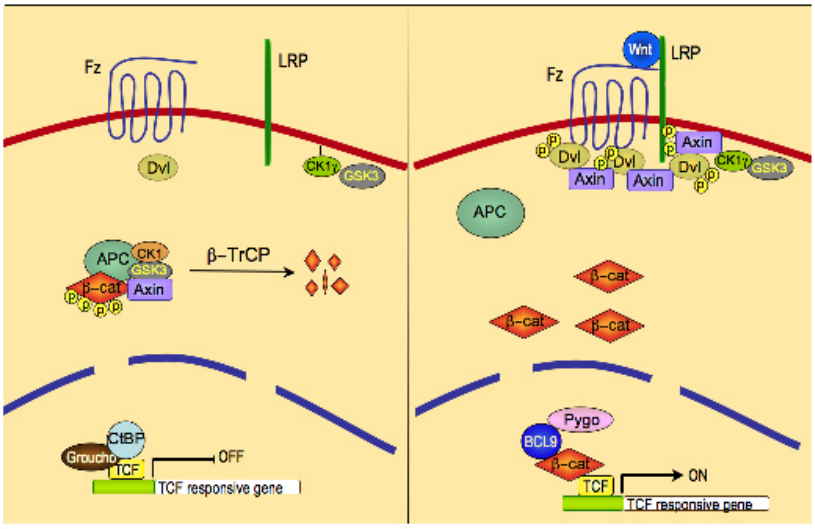

Figure 4. Schematic representation of the canonical Wnt signaling cascade. Left panel; without Wnt, ß-catenin is trapped by the degradation complex and phosphorylated by GSK3α/ß and CK1. Phosphorylated ß-catenin is recognized by the E3 ubiquitin ligase ßTrCP and rapidly degraded by the proteasome. In 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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