Chapter 6

Summary and Discussion

“Great minds discuss ideas, average minds discuss events, small minds discuss people”

Eleanor Rooseneveld
Summary and Discussion

The research described in this thesis focuses on the behavior, localization and routing of the β-catenin protein in Wnt signaling. β-Catenin is a multifunctional protein that binds E-cadherin at the cell surface to regulate cellular adhesion (Daugherty and Gottardi, 2007). In addition, β-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, β-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 behavior and regulation of β-catenin is therefore important for cancer research. The constant and rapid degradation of β-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 β-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 β-catenin to accumulate in the cell and to enter the nucleus. Nuclear localization of β-catenin has been considered an indicator of active Wnt signaling. However, correlations with in vitro assays are poor and nuclear β-catenin is rarely detected in human colorectal adenomas (Kobayashi et al., 2000; Anderson et al., 2002).

The hypothesis that the signaling capacity of β-catenin is a direct consequence of an increased half-life of β-catenin upon activation of Wnt signaling has been questioned. Guger and Gumbiner showed that in X. laevis embryos, enhanced signaling of N-terminal β-catenin mutants is not accounted for by accumulation of either total or cadherin-free β-catenin (Guger and Gumbiner, 2000). Staal and colleagues showed that interference with ubiquitination, and therefore β-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 β-catenin in the nucleus (Staal et al., 2002). Finally, Chan et al. have shown that Δ45 β-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 β-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 β-catenin activity. RanBP3 is a nuclear protein that functions as a cofactor in CRM1-mediated export (Engelmeier 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 β-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 β-catenin. Two different export mechanisms have been proposed for β-catenin. In the first, β-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, β-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 β-catenin, while in a CRM1 export complex, RanBP3 would be expected to bind β-catenin via CRM1. Furthermore, we demonstrate that RanBP3 still affects β-catenin-mediated transcription in cell lines expressing truncated APC, that lacks β-catenin binding sites. Furthermore, we show that depletion of RanBP3 results in a specific accu-
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 transcriptional 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 (Derkens 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 molecular 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 regard 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-
dition, β-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 β-catenin is a mechanism to terminate Wnt signaling, we decided to study the nuclear export of β-catenin (Chapter 5). To do so, we used photobleaching techniques to monitor the kinetics of GFP-tagged β-catenin. GFP-β-catenin was very mobile in both the cytoplasm and nucleus. Furthermore, we found that GFP-β-catenin exits the nucleus very rapidly and that inhibition of the CRM1 pathway does not influence the nuclear export of β-catenin, which is in support of previous studies (Wiechens and Fagotto, 2001; Eleftheriou et al., 2001). Additionally, the nuclear export of GFP-β-catenin exceeded that of the free diffusion of GFP alone, suggesting that GFP-β-catenin exits the nucleus via an active transport pathway. Our data suggest that β-catenin may use a similar mechanism as the transport receptors to pass the nuclear pore complex. Indeed, we found that β-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 β-catenin and FG repeat nucleoporins (Suh and Gumbiner, 2003). In that study, however, β-catenin was compared to importin-β with respect to binding to nucleoporins. Importin-β displays the strongest interaction to FG nucleoporins, whereas the interaction between β-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 β-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 β-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 β-catenin (Kriehoff et al., 2006). In that study, however, full recovery of YFP-β-catenin export was observed after 8 minutes, i.e. three times slower than our GFP-β-catenin. In addition, the authors found that export of YFP alone was faster than that of YFP-β-catenin. Both observations could be explained by differences in expression levels of tagged β-catenin and/or the use of a different fluorescent marker. We did not map the binding domain of RanBP3 on β-catenin, although RanBP3 interacted less well to arm1-12 compared to full-length β-catenin. The specificity of RanBP3 to dephospho-β-catenin implies that the binding domain overlaps the N-terminus of β-catenin plus part of the arm repeats. Future studies narrowing down the RanBP3 binding site on β-catenin may answer whether RanBP3 is sensitive to the presence of negative phosphate on the N-terminus of β-catenin.

It is intriguing that whereas C. elegans uses different β-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 β-catenin, the different β-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 β-catenin. HelixC forms an α-helix that packs on armadillo repeat 12 to shield the hydrophobic residues and that extends the superhelical core of β-catenin (Xing et al., 2008). Strikingly, HelixC is absent from C. elegans Hmp-1, which is involved in adhesion, while the two β-catenin proteins involved in signaling retain HelixC (Schneider et al., 2003). Drugs targeting HelixC in β-catenin could therefore specifically inhibit the tumour promoting signaling function of β-catenin without affecting the tumour suppressive adhesion functions of the protein.

References


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