Unravelling the molecular mechanisms of the canonical Wnt signalling pathway

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Unravelling the molecular mechanisms of the canonical Wnt signalling pathway

Ontraveling van de moleculaire mechanismen van de *canonical* Wnt-signalerings cascade

(met een samenvatting in het Nederlands)

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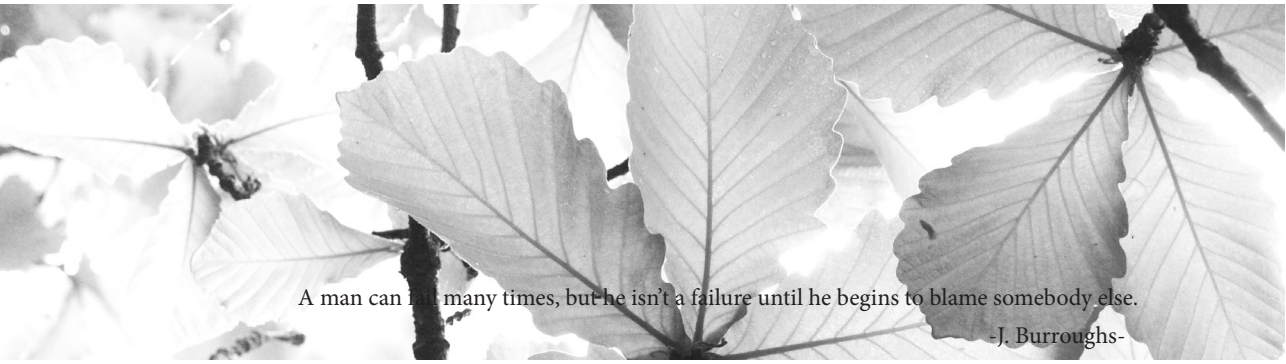

1. Wnt signalling pathway

Wnt mediated signalling is a highly conserved signalling pathway and a fundamental mechanism that directs a myriad of events during embryonic development and adult-self renewing tissue homeostasis and regeneration[1]. Wnt signalling plays a critical role in cell proliferation, differentiation, morphology, motility and cell fate determination. As a result, mutations of Wnt pathway components or deregulation of the pathway often cause abnormal development or lethality in embryos, as well as cancer and other diseases in adults[2, 3].

Wnt signalling can be divided into two major sub-pathways, the canonical Wnt pathway and the non-canonical Wnt pathway, according to its downstream effects. Wnts activate the downstream cascade through binding to their cell-surface receptors, a seven-pass transmembrane receptor from the Frizzled (Fz/Frz) family and low-density lipoprotein receptor related protein5/6 (LRP5/6). The crucial and most established Wnt pathway is the canonical Wnt pathway, which functions through regulating the protein stability and nuclear localization of cytosolic β-catenin. β-catenin is a key transcriptional co-activator of the DNA-bound T-cell factor/Lymphoid enhancer-binding factor (Tcf/Lef) family, leading to activation of Wnt target gene expression. Therefore the canonical Wnt pathway is also referred to as the Wnt/β-catenin pathway. Wnts also induce Tcf/Lef-β-catenin-independent cellular responses such as increased calcium flux, cytoskeletal rearrangements, planar cell polarity and suppression of Tcf/Lef-β-catenin-mediated transcription in LRP5/6 receptor-absent cells[4-7]. These β-catenin-independent Wnt mediated signalling activities are collectively termed as part of the non-canonical Wnt pathway. How Wnts activate different signalling pathways with strict specificity in a temporally and spatially controlled manner is not fully understood.

In this thesis we will focus specifically on the molecular events governing the canonical or Wnt/β-catenin signalling pathway.

2. Outline of the Wnt/β-catenin signaling pathway

The pivotal regulatory step in the Wnt/β-catenin pathway is the precise stabilization of the downstream effector cytoplasmic protein β-catenin (Figure 1).

In the absence of Wnt, the cytoplasmic pool of β-catenin is constantly eliminated by a complex that is normally referred to as the β-catenin destruction complex. Scaffolding protein Axis inhibition protein (Axin) mainly recruits adenomatous polyposis coli (APC), glycogen synthase kinase 3alpha/beta (GSK3α/β), casein kinase 1 alpha (CKIα) and β-catenin to form the dedicated destruction complex[2]. Within the complex, β-catenin is sequentially phosphorylated first by CKIα on serine 45 and then by GSK3 on threonine 41, serine 37 and lastly serine 33[8]. Only serine 33 and 37 phosphorylated β-catenin can be recognized by β-TrCP, an F-box-containing E3 ubiquitin ligase,

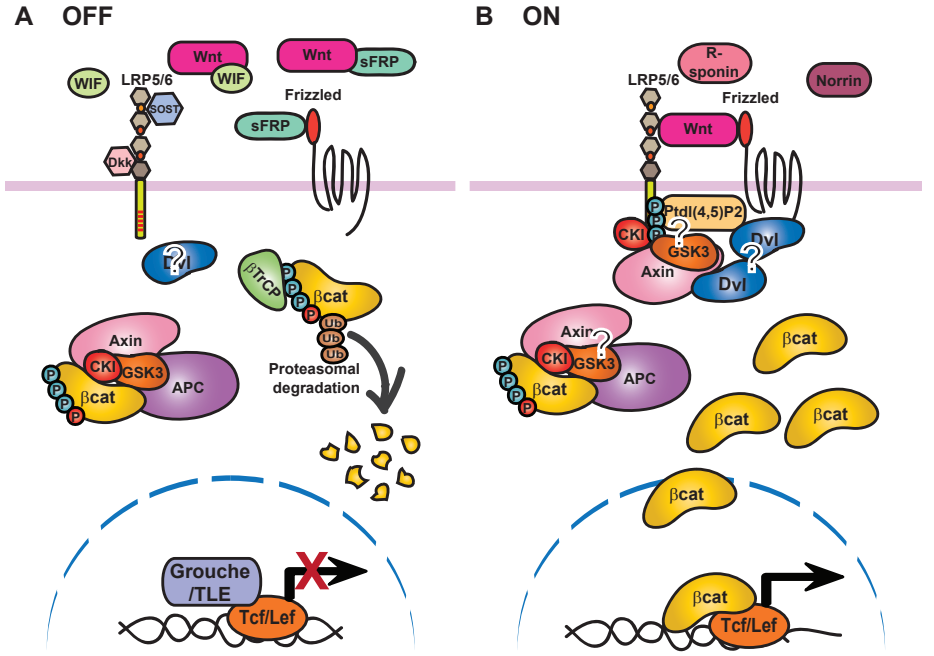

Figure 1 Overview of Wnt/β-catenin signalling pathway.

(A) In the absence of Wnt stimulation, cytoplasmic β-catenin levels are kept low via the destruction complex, which is mainly composed of APC, Axin, GSK3 and CKIα. β-catenin is first phosphorylated by CKIα (red) and then GSK3 (blue). Phopshorylated β-catenin is ubiquitinated by the E3 ubiquitin ligase β-TrCP, leading to its proteosomal degradation. In the nucleus, Tcf/Lef transcription factors associate with transcriptional repressors to block target gene activation. **(B)** In the presence of Wnt stimulation, phosphorylation of LRP5/6 is induced followed by membrane recruitment of Axin. Through unclear mechanisms the destruction complex is inactivated leading to β-catenin stabilization and accumulation in the nucleus to activate transcription of Tcf target genes.

which ubiquitinates β-catenin leading to its proteasomal degradation[8-10]. As a result, cytosolic β-catenin protein levels remain low, preventing β-catenin to accumulate and translocate into the nucleus. Therefore in the absence of nuclear β-catenin, DNA bound Tcf/Lef family proteins repress the transcription of Wnt target genes via interaction with co-repressors such as groucho/TLE[11] (Figure 1A).

Under physiological conditions, Wnt ligands bind to receptors Fz and LRP5/6 and turn on the downstream Wnt cascade. It is thought that formation of the Wnt-Fz-LRP5/6 complex may activate another downstream component of the pathway, the cytoplasmic protein Dishevelled (Dvl) [12, 13]. This results in the phosphorylation of LRP5/6 on its intracellular PPPSPXS (P, proline; S, serine or threonine; x, avariable residue) motifs[14] and Axin is recruited to LRP5/6[15]. Through undefined mechanisms, these events lead to interruption of the continued destruction of β-catenin by the dedicated destruction complex. This ultimately results in β-catenin accumulation in the

cytoplasm and its shuttling to the nucleus, where it complexes with Tcf/Lef transcription factor family and activates Wnt target gene transcription[11] (Figure 1B).

3. Modulation of Wnt/β-catenin signaling pathway

The Wnt morphogen and most of its signalling transduction components were first identified in *Drosophila melanogaster* and later were found to be highly conserved. The precise temporal and spatial regulation of Wnt signalling is essential for proper embryonic patterning and regeneration or renewal of the adult tissues in all metazoan animals[1, 2]. How Wnt and its resulting downstream signal transduction pathway is modulated has been the subject of much research since discovery of this important protein. The Wnt/β-catenin pathway can be modulated on five different levels, (1) Biogenesis and transportation of the Wnt ligand; (2) Antagonists and agonists of Wnt and its receptor in extracellular matrix; (3) β-catenin destruction complex; (4) β-catenin nuclear translocation; and (5) β-catenin-Tcf/Lef transcriptional activation. This thesis focuses on two modes of regulation of Wnt/β-catenin signalling; the β-catenin destruction complex and β-catenin-Tcf/Lef transcriptional regulation.

3.1 Wnt and its receptors

The name of cysteine rich morphogen, Wnt (19 known family members in humans) is the combination of Wingless (Wg, *Drosophila*) and INT-1(mouse) [16]. Two distinct Wnt receptor families were found to be critical for Wnt/β-catenin signalling: the serpentine receptor of Frizzled (Fz) family (10 family members in humans)[1] and a member of the low-density lipid receptor family (LRP5/6) [17]. A single Wnt can bind with different Frizzled receptors and vice versa. Most Frizzled receptors have variable capacities to activate Wnt/β-catenin signalling when co-overexpressed with Wnt and LRP5/6[18] and functional redundancy among Frizzled members is likely prevalent[1]. Although lacking *in vivo* confirmation, most *in vitro* data support the model that Wnt induces the formation of Frizzled-LRP5/6 complex to initiate the signalling cascade[19-21].

Studies in *Drosophila* and *nematode* Wnt proteins and mammalian Wnt3A protein has demonstrated that, precise acylation by palmitoyl and palmitoleoyl groups and N-linked glycosylation is essential for Wnt proteins' proper secretion and biological activity (for more comprehensive review please refer to Hausmann, G. and others review at 2007) [22-25]. Once released into the extracellular environment, Wnt competes with some secreted protein families such as Secreted Frizzled Related Proteins (sFRPs), Wnt inhibitory protein (WIF), Dickkopf (Dkk) and Wise/SOST family to interact with its co-receptor Fz -LRP5/6. WIF and sFRPs both bind to Wnt, and sFRPs additionally bind to Frizzled and thereby function as Wnt antagonists for both Wnt/β-catenin and non-canonical Wnt signalling[26, 27]. Dkk and Wise/SOST family are the antagonists for LRP5/6.

They both can suppress Wnt/β-catenin signalling by disrupting the Wnt-induced Fz-LRP complex formation through binding with LRP5/6[28-30]. In addition to antagonists, other proteins Norrin and R-spondin were identified as agonists for Wnt/β-catenin signalling. Norrin was identified as a specific ligand for Frizzled 4 and acts through Frizzled 4 and LRP5/6 during retinal vascularisation[31]. The R-spondin family exhibit synergy with Wnt and its receptors[32-35] and show genetic interaction with LRP6 during embryogenesis[36], but the biochemical mechanism behind this synergism remains undefined.

Once Wnt binds to Fz and LRP5/6, a first key event is the induction of LRP5/6 phosphorylation on its intercellular five reiterated PPPSPxS motifs[37]. This phosphorylation on LRP6 PPPSPxP motif is essential for LRP6 function, Wnt/β-catenin signalling downstream activation, and also the generation of a docking site for Axin1[37-39], thereby recruiting Axin1 or the Axin1 complex to LRP6 upon Wnt stimulation[15]. Moreover, LRP6 activity is particularly sensitive to the PPPSPxS copy number[14, 40] which indicates its potential role in amplification of Wnt signalling. CKI[38], GSK3[18, 35, 39, 41, 42] and phosphatidylinositol 4, 5-bisphosphates (PtdIns(4,5)P2)[42] were all reported to phosphorylate the LRP6 intercellular domain including the PPPSPxS motif upon Wnt induction. How Wnt induces LRP5/6 phosphorylation is not fully understood yet, but Fz and Dvl function and their interaction with each other is thought to be crucial for this activation[12, 43, 44]. Artificially forced Fz-LRP6 association is sufficient to trigger LRP6 phosphorylation[12], and a heterologous receptor with PPPSPxS motif is able to activate the Wnt/β-catenin signalling[14, 37, 39]. In addition to the Fz-Dvl interaction, the Dvl-Axin1 interaction via the DIX domain is also required for LRP6 activation and recruitment of Axin1 to the membrane[13, 44]. The recruitment of Axin1 to the membrane is proposed to lead the Axin1-GSK3 complex to LRP6 and result in LRP6 phosphorylation by GSK3[12, 39]. Other proteins such as Caprin-2[45] and microtubule actin cross-linking factor 1 (Macf1) [46] were also shown to facilitate LRP6 phosphorylation and Axin1 membrane shuttling to LRP6 respectively. All these observations have lead to the "signalsome" or " amplification" model. According to this model, Wnt induces Dvl (and Axin1) polymerization/aggregation via their DIX domains, which in turn facilitates weak but dynamic protein interactions between Fz-Dvl and Dvl-Axin1. The Fz-Dvl and Dvl-Axin1 interaction then sequentially recruits PtdIns(4,5)P2, CKI and GSK3 kineases to LRP6, and thus initiate LRP6 phosphorylation leading to full phosphorylation of the LRP6 PPPSPxS motif. However, the unambiguous evidence for such aggregation under physiological conditions without over-expression remains to be shown.

In short, through unresolved mechanisms, when Wnt binds to Fz and LRP5/6 it activates LRP5/6 by inducing its PPPSPxS motif phosphorylation, facilitated by Fz and Dvl. Axin1/Axin1 complex is then recruited to LRP6. Through unclear mechanisms this sequence of events results in the degradation of Axin1 protein levels, and therefore inhibition of the β-catenin destruction complex function to initiate the downstream signalling cascade.

3.2 β-catenin destruction complex

The essence of Wnt/β-catenin signalling is thought to be stabilization of cytosolic β-catenin through inhibition of β-catenin phosphorylation within the destruction complex leading to a block in its degradation by the proteasome. So far, the underlying mechanisms of how Wnt modulates the dedicated destruction complex have been speculated but not fully substantiated and could be summarised as below:

3.2.1 Disassembly of β-catenin destruction complex upon Wnt stimulation

Thus far, most studies probing Wnt mediated inhibition of the β-catenin destruction complex support a regulatory model in which upon Wnt stimulation, the β-catenin destruction complex is disassembled. Disassembly of the destruction complex prevents phosphorylation of β-catenin by CKIα and GSK3 resulting in its stabilization and accumulation in the cytosol and its subsequent shuttling into the nucleus where it complexes with the Tcf/Lef transcriptional family to activate Wnt target genes. There are two main alternative but non-exclusive biochemical mechanisms proposed, which describe the molecular events that lead to Wnt mediated disassembly of the destruction complex leading to stabilization of β-catenin:

3.2.1a Membrane translocation and degradation of Axin1 protein upon Wnt activation

The scaffolding protein of the β-catenin destruction complex, Axin is a highly conserved multi-domain protein. Axin uses separate domains to interact with all core components of the complex, APC, GSK3, CKIα and β-catenin and coordinates sequential phosphorylation of β-catenin by kinases CKIα and GSK3 (Figure 2A). There are two Axin homologues in metazoan, Axin1 and Axin2/Axil/Conductin. Axin1 was originally identified as an inhibitor of the Wnt pathway by characterization of the mouse *fused* locus product, whose disruption results in duplication of the axis (thereby named as Axis inhibitor protein (Axin)) and embryonic lethality[47, 48]. Axin1 is ubiquitously expressed in almost all tissues from early embryonic development through adult state[48] and its expression levels are believed to be much lower than other components in the complex[49]. Therefore, Axin1 is considered as the limiting component of the destruction complex. Very differently, Axin2, which was originally cloned by virtue of its interaction with β-catenin[50, 51] and found later to possess high homology with Axin1, is a Wnt target gene and expressed in a developmental and tissue specific manner[52-54]. Thus, Axin2 is thought to act as a negative feedback loop of Wnt/β-catenin signalling.
The first key event in Wnt/β-catenin signalling activation is the Wnt-induced LRP5/6

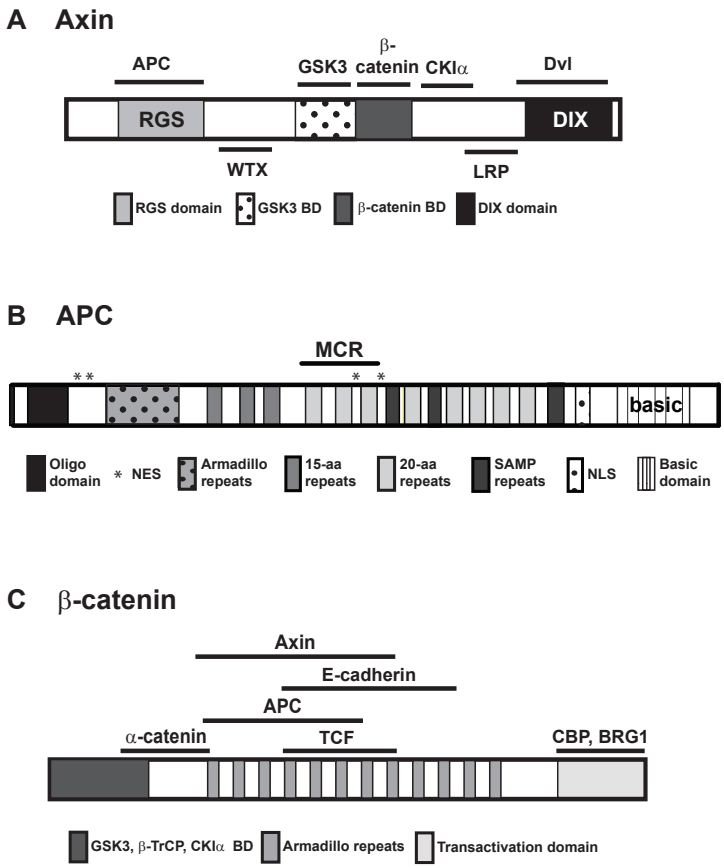

Figure 2. Schematic representation of Axin, APC and β-catenin proteins.

(A) Axin contains two highly conserved domains, the N-terminal RGS domain and the C-terminal DIX domain. The APC, GSK3, WTX, CKIα, LRP5/6, Dvl and β-catenin binding regions of Axin are as indicated. Axin DIX domain is also essential for Axin oligomerization. **(B)** APC can be divided into three fragments: the N-terminal, Central and the C-terminal fragments. The N-terminal fragment contains the Armadillo repeats and two nuclear export signals (NES). The central fragment contains three 15-aa repeats and seven 20-aa repeats that are responsible for β-catenin binding; three SAMP motifs that bind Axin; two NES and two nuclear localization signals (NLSs). The C-terminal fragment of APC contains a basic domain and several conserved binding regions for different proteins such as Dlg1, Bub1, EB1 etc. The Mutation Cluster Region (MCR) is indicated. **(C)** β-catenin consists of three essential domains, the N-terminal, Central and the C-terminal regions. The N-terminal region contains binding sites for multiple proteins such as GSK3, CKIα and β-TrCP. The central region contains 12-repeats of 42 amino acids known as Armadillo repeats. The C-terminal region harbors a potent transactivation domain. The APC, Axin, β-catenin, TCF, E-cadherin, CBP and BRG1 binding regions of β-catenin are indicated.

phosphorylation. This phosphorylation is essential not only for LRP5/6 function but also for recruitment of Axin1 to LRP5/6[17]. Due to the limited amounts of Axin1 protein in the cells, recruitment of Axin1 to LRP5/6 and its sequestration to the membrane upon Wnt stimulation, is thought to reduce the functional cytoplasmic Axin1-β-catenin

destruction complex and therefore result in β-catenin accumulation in the cytoplasm[15, 19]. Moreover, this membrane translocation of Axin1 is shown to be important for Axin1 protein dephosphorylation and destabilization in response to Wnt activation[15, 39]. As well, endocytic adaptor disable-2 (Dab2), which interacts with Axin1 and prevents its translocation to the membrane, could block PP1 (protein phosphatase 1) interaction with and dephosphorylation of Axin1 leading to stabilization of Axin1 protein levels[55]. In addition, stabilization of Axin1 protein levels by small molecule inhibition of the poly-ADP-ribosylating enzyme family member tankyrase 1 and tankyrase 2 were reported to selectively inhibit β-catenin mediated transcription. Tankyrase, the target of the small molecule XAV939 identified in a screen for inhibitors of Wnt signaling was shown to mediate Axin1 degradation[56]. Moreover, SUMOylation of Axin1 on its C6 motif KVEKVD was reported to protect it from polyubiquitination, thus increasing the stability of Axin1[57]. All these observations support a regulatory model in which Wnt interrupts the β-catenin destruction complex by degradation of Axin1 protein levels. It is important to note, however, that Wnt-induced Axin1 degradation lags significantly behind β-catenin stabilization[58-60]. Thus Axin1 degradation is unlikely to be a primary response to Wnt stimulation but may be important to sharpen and amplify of the effect of the Wnt signal.

3.2.1b Interruption in interaction of Axin1 and/or APC with GSK3 or β-catenin

A multi-functional tumor suppressor, APC is another core component of β-catenin destruction complex. *APC* was identified as the gene responsible for a heritable predisposition to colorectal cancer, referred to as familial adenomatous polyposis coli (FAP), and is also mutated in most sporadic colorectal cancers[61, 62]. *APC* encodes a large multifunctional protein with several structural domains and is expressed ubiquitously (Figure 2B). The central fragment of APC contains three successive 15-amino acid (aa) repeats followed by seven related but distinct 20-aa repeats, both of which are known to bind β-catenin independently[63]. Within the 20-aa repeat region there are SAMP (Serine-Alanine-Methionine-Proline) motifs shown to mediate APC binding to Axin1/2. APC-Axin1 complex is thought to modulate the activity of the GSK3 kinase. Binding of GSK3 to Axin1 and thereby to APC was shown to be necessary for the efficient down-regulation of β-catenin.

Both APC and Axin1 activities correlate with their phosphorylation state, which was shown to modulate their protein stability and binding affinity to β-catenin *in vitro*. The kinases CKI and GSK3 were reported to phosphorylate Axin1 and APC in resting cells and enhance their binding affinity to GSK3 and β-catenin and thus promote β-catenin phosphorylation and degradation. Interestingly, both phosphorylated Axin and APC were shown to compete with each other for the same β-catenin interaction interface, leading to the hypothesis that APC might function to remove phosphorylated β-catenin from Axin1 for ubiquitination. According to this model, as a result of APC-mediated removal of phosphorylated β-catenin, the Axin1

complex would become free for further rounds of β-catenin phosphorylation[64, 65]. Although the precise biochemical mechanism remains elusive and a specific phosphatase for Axin1 remains to be identified, both Axin1 and APC are dephosphorylated upon Wnt activation. The catalytic subunits of both PP1 and PP2C were shown to directly bind to and dephosphorylate Axin1, promoting the disassembly of the Axin1-β-catenin destruction complex[66, 67]. In addition to modulation of the phosphorylation state of Axin1 and APC, some proteins such as GBP/Frat were reported to compete with Axin1 for binding to GSK3, thereby disrupting the β-catenin destruction complex[68, 69]. Another example is the protein Dishevelled, another key component of the Wnt signalling cascade, which binds Axin1 directly and interrupts the formation of the β-catenin destruction complex upon Wnt activation[70, 71]. While these studies point to the importance of the composition of the destruction complex for its proper function, they largely rely on over-expression approaches.

Thus far, studies probing how Wnt induces a change in composition of the endogenous β-catenin destruction complex and leads to its inactivation are lacking.

3.2.2 Inactivation of GSK3 kinase activity

GSK3 is a global highly conserved serine/threonine protein kinase involved in a wide range of signal transduction cascades, which include Wnt signalling. In mammals there are two isoforms of GSK3, a 51kDa GSK3α and 47kDa GSK3β. Unlike most protein kinases, GSK3 is active in unstimulated, resting cells and its activity is diminished during cellular responses[72]. GSK3 shows a preference for target proteins that are pre-phosphorylated at a "priming" residue located C-terminal to the site of GSK3 phosphorylation. This priming phosphorylation is not absolutely required but enhances the phosphorylation efficiency of most GSK3 substrates[73]. The consequence of GSK3 phosphorylation is usually inhibition of its substrates, which include NFAT, Ci155/Gli and β-catenin [74].

Only GSK3 kinase mediated serine 33 and serine 37 phosphorylated β-catenin is recognised by the ubiquitin E3 ligase β-TrCP, which ubiquitinates β-catenin leading to its proteasomal degradation. The well established GSK3 kinase itself has been shown to be inhibited by PKB/Akt phosphorylation. PKB/Akt inhibits GSK3 kinase activity by phosphorylating serine 21 on GSK3α and serine 9 on GSk3β[75, 76]. The well established PKB/Akt inhibition of GSK3 has led to the hypothesis that there may be cross talk between the PI3K/Akt pathway and the Wnt pathway via regulation of GSK3 kinase activity. In addition to direct inhibition of GSK3 by the growth factor activated Akt kinase, Dishevelled has also been shown to be important in Wnt inhibiton of GSK3 kinase activity by recruiting activated Akt to the β-catenin destruction complex in a Wnt-dependent manner[70].

Another potential mechanism of how Wnt inhibits GSK3 mediated β-catenin phosphorylation relies on the observation that phosphorylated LRP6 cytoplasmic domain or individual phosphor-PPPSPxS peptides can directly inhibit

GSK3 phosphorylation of β-catenin *in vitro*[77-79]. An observation that indirectly supports the notion that phosphorylated LRP6 intracellular domain mediates GSK3 kinase inhibition of dephosphorylated β-catenin is the appearance of GSK3 at the plasma membrane close to activated LRP6 and Axin1 upon Wnt3A stimulation[80].
However, the underlying mechanisms of how Wnt blocks GSK3 kinase phosphorylation of β-catenin are not yet well defined.

3.2.3 β-catenin phosphorylation and dephosphorylation

β-catenin is a 92kDa protein which was originally identified as a protein that interacts with the cytoplasmic domain of E-cadherin, important for cell-cell adherence junctions[81, 82] (Figure 2C). Later studies revealed that β-catenin is a core player in the Wnt signalling pathway through modulating Tcf/Lef transcriptional activation[83, 84].
There are at least two distinct β-catenin pools present in the cells, the E-cadherin bound membrane β-catenin pool and the cytosolic β-catenin pool. It was determined that only the cytosolic β-catenin pool participates in Wnt/β-catenin signalling[85].
The cytosolic β-catenin protein levels are controlled by the β-catenin destruction complex as described above. One of the key steps in this regulation is the phosphorylation of β-catenin leading to its ubiquitination and degradation. CKIα and GSK3 are two well established kinases for β-catenin. β-catenin is first phosphorylated by CKIα on serine 45 followed by GSK3 on threonine 41, serine 37 and serine 33. Phosphorylation on serines 33 and 37 provides a docking site for ubiquitin E3 ligase binding and therefore is essential for regulation of cytosolic β-catenin levels[8]. Dephosphorylation of phoshphorylated β-catenin by phosphatase is another potential mechanism of Wnt-dependent-β-catenin stabilization. Studies have suggested that one of the functions of APC may be to protect β-catenin from dephosphorylation by protein phosphatase 2A (PP2A) [86]. Their finding is consistent with the observation that Axin1/2 overexpression is capable of inducing β-catenin degradation even in cells lacking APC function[50]. Nevertheless, more direct *in vivo* evidence is needed to support this hypothesis.

3.3 β-catenin ubiquitination, degradation

Ubiquitination and proteasomal degradation of phosphorylated β-catenin is the essential step for preventing β-catenin nuclear translocation. Although β-catenin was found to be ubiquitinated by the E3 ligase Fox/WD40-repeat protein (FWD1), the mouse homologue of Slimb/β-TrCP[9], the nature of the interaction between the β-catenin destruction complex with the ubiquitin ligase complex and proteasome is uncharacterized and their regulation by Wnt is not fully understood yet. Recently, WTX (Wilms tumor gene on the X chromosome) was identified as a novel component of the destruction complex, which interacts with β-catenin, Axin, APC and β-TrCP to promote β-catenin ubiquitination[87]. This finding provided a new angle to understand how the

FWD1/β-TrCP-ubiquitin ligase complex may interact with the β-catenin destruction complex and facilitate ubiquitination and promote degradation of β-catenin.

3.4 β-catenin nuclear translocation

β-catenin stabilization in the cytoplasm results in its higher nuclear levels and interaction with Tcf/Lef transcriptional factors, but the shuttling and retention of β-catenin in the nucleus is not yet well understood[88, 89]. Earlier studies by Henderson and Fagotto suggested that β-catenin enters the nucleus via direct interaction with nuclear pore proteins in an NLS (nuclear locatlizaiton signal)-and importin-independent fashion[88]. Interestingly, APC[88] and Axin[90] were reported to play a role in β-catenin nuclear export. Ran binding protein 3(RanBP3), which binds to β-catenin in a Ran-GTP-dependent manner was also reported to participate in β-catenin nuclear transport[91]. However, live cell imaging experiments suggested that while APC and Axin can enrich β-catenin in the cytoplasm, Tcf and β-catenin co-activators such as BCL9 and Pygopus can increase nuclear β-catenin, thus indicating that they likely only participate in β-catenin cytoplasmic or nuclear retention rather than shuttling[92]. Moreover, a recent finding of Wu and the others at 2008 proposed that stabilization of β-catenin in the cytoplasm may not be sufficient for its nuclear accumulation; Wnt activation of Rac1 and Jun N-terminal kinase2 (JNK2) was suggested to promote β-catenin nuclear translocation by interacting with and phosphorylating β-catenin at serines 191 and 605[93]. Further studies are required to clarify whether or how these factors regulate β-catenin localization.

3.5 Tcf/β-catenin transcription

The main nuclear partners of β-catenin-mediated gene expression are the Tcf/Lef family of transcription factors. While there is one Tcf member protein found in *Drosophila* (dTcf/pangolin) and nematodes (Pop-1), four Tcf/Lef family members exist in mammals (Tcf1, Lef, Tcf3 and Tcf4). Tcf/Lef family proteins contain a high mobility group (HMG) box, which binds DNA in a sequence-specific manner[94, 95]. The DNA sequence is characterised by CCTTTGXX (X: represents either T or A) which is normally termed as the Wnt responsive element (WRE)[96]. The HMG box containing proteins bind DNA in the minor groove and bend the double helix and thus may fashion in the context of chromatin. In the absence of β-catenin, Tcf/Lef transcription factors are thought to suppress gene expression by interacting with and recruiting repressors such as Groucho (TLE1 in human), which promotes histone deacetylation and chromatin compaction of target genes[97]. Nuclear β-catenin is then thought to replace Groucho by binding to the N-terminus of Tcf/Lef proteins and mediate the recruitment of other co-activators to activate the target gene expression[98, 99].
There is a large number of Tcf variants with distinct properties found produced through alternative splicing and promoter usage, particularly extensive alternative

splicing at the C-terminus[100]. The different spatial expression of different Tcf isoforms is one of the major strategies for Tcf/β-catenin transcription regulation. For example, the N-terminal β-catenin binding domain can be omitted in Tcf1 and Lef1 by starting transcription from a second intronic promoter, downstream of exon II, leading to generation of dominant-negative forms of Tcfs (dnTcfs). For instant, dnTcf1 was found to antagonize Tcf4 in intestinal stem renewal and thus acts as a tumor suppressor. Another example of Tcf variants is Tcf3 and Tcf4 isoforms, which depending on the isoform expressed could contain a CtBP binding motif in their C-terminal region and result in CtBP binding and recruitment of histone deactelases (HDACs) that prevent chromatin from being transcribed[101]. The second DNA-binding domain, 'C-clamp'/'CRARF' domain which recognizes an additional GC element downstream of the consensus Tcf/Lef recognition motif, is only found in Tcf1 and Tcf4 isoforms and may regulate different sets of target genes[102]. In *Drosophila*, mutation of an alanine residue to a valine in the C-clamp domain of dTcf abrogates Wingless signalling[102].

In addition to the different expression variants, post-translational modifications of Tcf/Lef proteins have also been shown to modulate Tcf/Lef protein function, thereby affecting Tcf/β-catenin transcription[103, 104]. These modifications which include sumoylation, phosphorylation, acetylation and ubiquitination are often specific to individual Tcf/Lef proteins and confer differential regulation. For instance, SUMO ligase PIASy sumoylates Lef1 and represses its activity but enhances Tcf4/β-catenin transcription. In *Drosophila* and *nematode*s, CBP acetylated Tcf results in transcriptional repression. Another good example is the phosphorylation of Tcfs by different kinases; CKIε and CKII phosphorylate Tcf3 and Lef1 respectively to enhance their binding with β-catenin and decrease Lef1 interaction with Groucho/TLE. Nemo-like kinase (NLK) also phosphorylates Lef1 and Tcf4 but this phosphorylation leads to diminished Tcf/β-catenin complex binding to DNA and to Lef1/Tcf4 degradation.

Dynamic modulation of the interaction of Tcf with β-catenin and their binding with co-activator or co-repressor complexes plays a crucial role in the precise expression of specific Wnt target genes during embryonic development and adult tissue homeostasis. Formation of Tcf/β-catenin complexes and recruitment of co-activators by β-catenin is another modulation target in Tcf/Lef transcription. Mainly through its carboxyl terminal region, β-catenin has been shown to recruit a plethora of co-activators to Tcf/β-catenin complexes. For example, in *Drosophila*, β-catenin binds with BCL9 and results in the recruitment of Pygopus to the complex, which interacts with the Mediator complex to facilitate transcription initiation[105]. Moreover, when Pygopus forms a complex with BCL9, its PHD (plant homology domain) domain binds preferentially to dimethylated Histone H3K4 (lysine4), a mark of active transcription[106]. Components of the two major classes of chromatin modifying complexes, ATP dependent chromatin remodelers, and enzymes that covalently modify histones, have been implicated in β-catenin mediated transcriptional regulation. BRG-1, the catalytic subunit of SWI/SNF chromatin remodeling factors can interact with β-catenin

to activate transcription of certain TCF target genes[107]. ISWI, another chromatin remodeling enzyme, and the mixed lineage leukemia H3K4 histone methyltransferase, MLL were found to bind to the C-terminus of β-catenin[108]. The acetyl transferases CBP and p300 and have also been implicated in acetylating histones in target gene promoters, allowing access of transcriptional activators to DNA[109]. As well, β-catenin was reported to interact with TBP, TRRAP/TIP60 and the elongating PAF1 complex, thus providing a link of the activation complex to the transcription machinery[109-112]. Chibby[113] and ICAT[114] are two other proteins, which were shown to bind to β-catenin but disturb the interaction between β-catenin with Tcfs. These repressors were reported to act as antagonists of β-catenin in the nucleus by promoting β-catenin nuclear export and thus suppressing Tcf/β-catenin transcription[115, 116].

Despite the identification of numerous TCF4 and β-catenin co-activators, few transcriptional activators are known to be essential and unique to the regulation of the Wnt transcription program. The identification and study of known and discovery of novel TCF4/β-catenin co-activator complex components dedicated to this transcriptional program will be critical to clarify the mechanisms that mediate proper regulation of Wnt target gene transcription and identify how misregulation of the Wnt signaling pathway causes cancer.

4. Cancer and the destruction complex

Deregulation of the Wnt/β-catenin signaling pathway, particularly resulting from activating mutations of components of the β-catenin destruction complex is often found in various types of cancers. Although the mutations occur on different components of the destruction complex, most result in stabilization of cytoplasmic β-catenin, its nuclear translocation and activation of the Wnt target genes such as the oncogene c-Myc[117, 118].

Mutations in both alleles of the tumor suppressor gene, *APC* have been linked to Familial adenomatous polyposis (FAP) and sporadic colon cancer[61, 62, 119]. Somatic mutations in the APC gene have been detected in about 80% of sporadic colorectal polyps and carcinomas[120]. Most APC mutations in colorectal cancer are clustered in a so called Mutation Cluster Region (MCR), which result in a protein that is truncated at its C-terminus missing the Axin binding SAMP motifs and several but not all β-catenin binding 20-aa repeats[121, 122].

Mutations in the substrate and key component of the complex, β-catenin are found in almost 50% of colorectal cancers without APC mutations, which represents 10% of all colorectal cancers[123]. These β-catenin activating mutations frequently occur in or near the N-terminus serine/threonine phosphorylation sites of CKIα and GSK3, which prevent β-catenin phosphorylation and block its degradation (reviewed in[124]). Mutations of β-catenin are also reportedly found in 16-24% of ovarian cancers[125-127],

20% of hepatocellular cancers and 40-89% of hepatoblastoma[128-132].
Alterations in both Axin1 and Axin2 have also been detected in several different types of tumours such as hepatocellular cancer, endometrial cancer, adenoid cystic carcinoma and colorectal cancer etc. Very different from APC and β-catenin, mutations found in the Axin1 coding sequence are scattered throughout the whole gene[133]. Silent mutations and polymorphisms of Axin are also often found. In colorectal and hepatocellular cancers, most Axin1 mutations lead to expression of the inactivated truncated protein eliminating the APC, GSK3 and β-catenin binding domains, while Axin2 mutations more often result in elimination of the DIX domain in proteins[134-140]. Moreover, deletion of chromosome 17, where Axin2 is located has been found in 45% of squamous cell carcinomas as well[141].
In addition to β-catenin, APC and Axin, mutation of other destruction complex components such as WTX has been reported and linked to cancer[142]. WTX was found to be mutated in 30% of pediatric kidney cancers, Wilms tumours, 50% of which contain constitutive activation of Wnt/β-catenin signaling[142-144]. Noteworthy that, only approximately 10% of Wilms tumours express oncogenic mutated β-catenin[144].

5. Axin1, the most regulatory, Wnt-specific component of the destruction complex

Among the core components within the destruction complex, the limited scaffold protein Axin1 is believed to be most specific in the Wnt signaling pathway, when compared to APC, GSK3 and CKIα. The other core components of the complex, APC and the two kinases GSK3 and CK1 have been shown to be involved in many other biological processes. In addition to its role in mediating β-catenin degradation, APC is involved in a variety of other cellular functions. Consistent with its role as a tumor suppressor, overexpression of APC blocks cell cycle progression from the Go/G1 to the S phase. Via interaction with the protein Dlg1, APC plays an important role in transducing the cell cycle blocking signal[145]. APC also regulates microtubules stability by moving along and accumulating at microtubule growing plus ends. This movement of APC is believed to be essential for its regulation of cell migration. Kap3, a member of the kinesin superfamily-associated protein mediates APC interaction with the kinesin superfmaily, microtubule plus-end-directed motor proteins, thereby important for APC microtubule movement and its role in cell migration[146]. APC is also reported to interact with Asef, a Rac-specific guanine nucleotide exchange factor (GEF), and thus regulates the actin-cytoskeletal network, cell morphology and migration[147]. APC binds directly to the kinetochore protein Bub1, previously implicated in chromosomal instability, and mediates attachment of microtubules to kinetochores through EB1[148]. The interaction of APC with the protein tyrosine phosphatase PTP-BL may indirectly modulate the steady state levels of tyrosine phosphorylation of associated proteins, such as β-catenin, playing a major role in the

regulation of cell division, migration and cell adhesion.

The two essential kinases, GSK3 and CKIα are involved in many different biological processes through their phosphorylation of a variety of substrates. GSK3, in addition to β-catenin also phosphorylates Glycogen Synthase and other metabolic enzymes, transcription factors such as CBP (CREB Binding Protein), c-Myc, c-Jun and the translation initiation factors eIF2 and eIF2B[149]. GSK3 phosphorylation of these transcription factors can cause ubiquitination, nuclear exit, or a decrease in DNA binding leading to decreased transcription[150]. In hedgehog signalling, GSK3, in combination with CKI and the priming kinase PKA, phosphorylates the pathway's key component, Ci155 to target it for proteolytic processing in the absence of a hedgehog signal[151, 152]. CKIα was also reported to phosphorylate MDMX thereby inhibiting p53 transcriptional function[153]. Moreover, CKIα-mediates GPCR phosphorylation and plays a role in receptor coupling to the MAPK pathway[154]. CKIα also phopshorylates the synaptic vesicle-specific protein SV2[155]. In addition, CKIα was shown to be a bifunctional regulator of the transcription factor, NF-κB[156].

Thus, the core subunits of the β-catenin destruction complex, APC, GSK3 and CKIα are involved in a variety of cellular functions and signalling pathways in addition to their role in the canonical Wnt signaling pathway. Axin, as the scaffold of the complex, directly contacts each core component, APC, GSK3, CKIα as well as β-catenin. Biochemical studies on *Xenopus* egg extracts estimated that the intracellular concentrations of Axin1 are approximately 1000 times lower than those of other destruction complex components, suggesting that Axin1 is the limiting factor in this pathway[49]. Because of its limited availability, Axin1 may therefore play a critical regulatory role within the destruction complex, and its degradation may be important in amplifying and sharpening of the Wnt signal. Axin1 also appears to be the most specific component of the destruction complex for Wnt signalling in that Axin1 has not been shown to be involved in many other cellular functions. Thus far, in addition to canonical Wnt signalling, Axin1 was reported to be involved in the TGFβ and SAPK/JNK signaling cascades. In TGFβ signalling, Axin1 may act as an adaptor for Smad3 to facilitate its phosphorylation and transcriptional activity[157]. Interaction of Axin1 with MEKKs was shown to place Axin1 as a positive modulator of SAPK/JNK (stress activated protein kinase/Jun N-treminal kinase) signalling pathway mediated-cell apoptosis[158-160]. The importance of this interaction and subsequent SAPK/JNK activation has been validated by the identification of Axin mutations in cancer cells resistant to apoptosis[135, 140, 161, 162]. Thus, among the core components of the β-catenin destruction complex, Axin1 is the least abundant, and believed to be the most specific and potentially most regulatory to the function of the complex.

Outline of the thesis

The aim of this thesis was to illustrate and address a number of questions related to the modulation of Wnt/β-catenin signalling transduction. In **chapter 2** we rule out the possible cross-talk between PI3K/Akt pathway and Wnt/β-catenin pathway via GSK3 kinase. This study validates that the Axin-bound GSK3 pool, which participates in the Wnt/β-catenin signal transduction pathway is shielded from PKB kinase, thus PI3K/PKB pathway could not modulate the Wnt/β-catenin pathway via GSK3 kinase inactivation within the β-catenin destruction complex. In **chapter 3**, we report the identification of the MAP kinase, MAP kinase kinase kinase 1 (MAP3K1) as one of the Axin1 binding partners in Ls174T cells, and we demonstrate its positive regulatory role in the Wnt/β-catenin pathway. A loss of function study with the use of different mutants of MAP3K1 as well as siRNA mediated depletion shows the essential role of its ubiquitin E3 ligase function but not its kinase activity on Wnt/β-catenin driven transcription. In **chapter 4** we probe the mechanisms of functional inactivation of the β-catenin destruction complex in response to Wnt stimulation. We examine the composition of the complex in various constitutively Wnt active colon cancer cell lines and probe its dynamic change in composition in response to Wnt in the Wnt-inducible HEK293T cells. We find that Wnt stimulation blocks the ubiquitination of phosphorylated inactive β-catenin within the β-catenin destruction complex. Thus the destruction complex is saturated with phosphorylated β-catenin and newly synthesized β-catenin is able to accumulate in the cytoplasm and translocate to the nucleus to activate the Wnt transcriptional program. In the addendum chapter (**chapter 5**) we briefly described and summarized the Axin1 and Axin2 immunoprecipitation coupled to Mass Spectrometry identification of a number of previously known and novel interesting interaction partners of Axin1 and Axin2 is both Ls174T CRC as well as HEK293T cells. We also present the Mass Spectrometry identification of several possibly regulatory phosphorylation sites for Axin1, APC and WTX. The identification of novel players and possible modes of regulation through protein modifications provide new angles to study the regulation of the β-catenin destruction complex. Lastly, in **chapter 6,** we describe the identification of the kinase Tnik as an essential Tcf4/β-catenin co-activator, which phosphorylates Tcf4 and specifically activates of Wnt-dependent target gene transcription.

CHAPTER 2

Phosphatidylinositol 3-Kinase (PI3K) Signaling Does Not Activate the Wnt Cascade

Adapted from:

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A man can succeed at almost anything for which he has unlimited enthusiasm.
-C. M. Schwab-

Abstract

Mutational activation of the phosphatidylinositol 3-kinase (PI3K) pathway occurs in a wide variety of tumors, whereas activating Wnt pathway mutants are predominantly found in colon cancer. Because GSK3 is a key component of both pathways, it is widely assumed that active PI3K signalling feeds positively into the Wnt pathway by Protein Kinase B (PKB)-mediated inhibition of GSK3. In addition, PKB has been proposed to modulate the canonical Wnt signaling through direct stabilization and nuclear localization of β-catenin. Here we show that compartmentalization by Axin of GSK3 prohibits cross-talk between the PI3K and Wnt pathways and that Wnt mediated transcription activity is not modulated by activation of the PI3K/PKB pathway.

Introduction

Developmental signaling cascades typically transduce signals from the cell surface onto regulatory sequences of nuclear target genes. In the simplest model, signals transduced through different pathways are integrated at the level of the regulatory elements of individual genes. Such regulatory elements may be viewed as assemblies of cis-acting response elements that are tailored to create each gene's unique expression pattern. However, numerous studies propose that signaling pathways may interact at any stage between the plasma membrane and the nucleus. One mechanism by which such cross-talk may occur involves the sharing of a common component between two different pathways. It is often tacitly assumed that such shared components are equally accessible to all pertinent pathways.

Glycogen Synthase Kinase 3-α and -β, collectively termed GSK3, are constitutively active serine/threonine kinases[163]. GSK3 features in two signaling pathways that are of particular importance in cancer. GSK3 is a downstream component of the Phosphoinositide 3-OH kinase (PI3K) pathway[164, 165]. Growth signals, activated Ras proteins or loss of PTEN (Phosphatase and tensin homolog) all activate PI3K, which in turn phosphorylates and activates Protein Kinase B (PKB)[165]. Active PKB phosphorylates GSK3α on Ser21[75] and GSK3β on Ser9[76], in both cases leading to inhibition of the constitutive kinase activity. GSK3 is also a component of the Wnt cascade[2]. GSK3 is bound by Axin[166] and phosphorylates β-catenin, thus targeting it for ubiquitination and degradation by the proteasome. Wnt signaling is assumed to block GSK3-mediated β-catenin phosphorylation, leading to the accumulation and nuclear translocation of β-catenin[2]. It remains unclear how the Wnt cascade controls the activity of the dedicated, Axin1-bound GSK3 pool. A recent genetic experiment has demonstrated that removal of the inhibitory serines from the two GSK3 proteins has no effect on Wnt signaling[167].

Although an early study proposed that the two pathways do not crosstalk at the

level of GSK3[168], a multitude of papers have since appeared which are based on the premise that a single pool of GSK3 is targeted by both signals (see Table S1). Moreover, direct stabilization of β-catenin by the PI3K/PKB pathway has been claimed in several additional studies (see Table S1). Mutational activation of the Wnt pathway through loss of Adenomatous polyposis coli protein (APC), of Axin1/2, or through point mutations in β-catenin occurs in a limited diversity of cancers, most notably of the intestine[2], and is characterized by stabilized β-catenin and constitutive transcriptional activity of β-catenin/TCF complexes in the nucleus. This can be readily read out by the constitutive activity of β-catenin/TCF reporters such as pTOPFlash[169]. Mutational activation of the PI3K pathway occurs in a wide variety of tumors through mutational activation of any of the Ras genes, *v-raf* murine sarcoma viral oncogene homolog B1 (*BRAF*), *PI3K* or loss of *PTEN*[165]. If GSK3 would indeed represent a focal point of crosstalk between the two pathways, β-catenin/TCF-driven transcription would be activated in tumors harbouring PI3K-activating mutations. This has major implications for our thinking on the molecular pathogenesis of cancer.

Results and Discussion

When we screened a large panel of breast cancer cell lines for constitutive β-catenin/TCF reporter (pTOPFlash) activity, the hallmark of active Wnt signalling, we found only a single positive cell line to be responsive to Wnt[85]. This cell line, DU4475, turned out to carry a mutation in the Wnt pathway tumor suppressor *APC*, a mutation typically found almost exclusively in colon cancers. We have since determined the mutational status of the three *RAS* genes (*KRAS, HRAS, NRAS*), the *BRAF* kinase gene, the *PTEN* gene and *PI3K (PIK3CA)* in a much larger panel of breast cancer cell lines[171]. We conducted TCF reporter assays for the entire breast cancer cell line panel. Moreover, we subjected two well-defined prostate cancer cell lines, both of which harbor inactivating PTEN mutations[172]. No TOPFlash activity was observed in any of the cell lines. LiCl is a well-established inhibitor of GSK3 kinase activity and as such represents a pharmacological activator of the Wnt cascade at the level of GSK3[173]. Nine of thirteen cell lines subjected to LiCl treatment responded by a potent activation of the TOPFlash reporter, indicating the presence of an intact intracellular Wnt pathway. Table 1 displays the identities of the mutations present in the individual cell lines, the presence of a constitutively active or inactive Wnt pathway as measured in the TOPFlash assay, as well as the inducibility of the Wnt pathway by LiCl which was tested in a subset of the cell lines. The underlying TOPFlash values are given in Table S2.

We next investigated the presence of a complete and functional Wnt pathway in the PTEN-mutant prostate cancer cell line PC3 and in the PTEN-mutant breast cancer cell line BT549 by transfection of Wnt1 or Wnt3A. While modest responses were observed in the PC3 cells, the BT549 responded vigorously to the Wnt proteins (Figure 1A/B top panels). We concluded that these two cell lines harbored fully functional Wnt pathways,

which were not affected by the mutational status of the PI3K pathway but could readily be activated by Wnt proteins. To examine the effect of inhibition of the PI3K pathway on Wnt-induced transcriptional activity, we applied the pharmacological PI3K inhibitor wortmannin. Western blot analysis of phospho-PKB and phospho-GSK3β confirmed efficient inhibition of the PI3K pathway in these cell lines in response to wortmannin treatment (Figure 1 A/B bottom panels). TOPFlash reporter assays revealed no effect of wortmannin on Non-Wnt-stimulated cells (Figure 1A/B, top panel) or on Wnt1 or Wnt3A-stimulated cells (Figure 1A/B, top panels). In an independent experiment, we applied the PKB inhibitors *AKT inhibitor IV* and *VIII*. While these inhibitors readily blocked phosphorylation of PKB and GSK3β (Supplementary Figure S1), no statistically significant differences were noted on LiCl-stimulated TOPFlash reporter activity in BT549 cells (Supplementary Figure 2). We extended these results by examining the effect of PI3K pathway inhibition on Wnt-induced transcription in two other prostate and breast cancer cell lines, LNCaP and MDA-MB-468 respectively. Both cell lines were transcriptionally induced in response to β-catenin/TCF4 expression or LiCl treatment (Figure 1C/D top panels). However, addition of PI3K pathway inhibitors Wortmannin and *AKT inhibitor IV*, at concentrations which efficiently blocked PKB and GSK3

Table 1

Cell lines	Affected Gene	Constitutive TCF/β-catenin Signaling activity	Activation after LiCl treatment
Breast Cell lines [171]			
BT20	***PIK3CA***	-[85]	ND
BT549	***PTEN***	-[85]	+
CAMA-1	***PTEN***	-[85]	ND
EVSA-T	***PTEN***	-[85]	ND
Hs578T	***HRAS***	-[85]	ND
MCF-7	***PIK3CA***	-[85]	ND
MDA-MB-144VI	***KRAS***	-[85]	ND
MDA-MB-231	***BRAF,***	-[85]	ND
MDA-MB-435s	***BRAF***	-[85]	ND
MDA-MB-453	***PTEN,***	-[85]	ND
OCUB-F	***PIK3CA***	-[85]	ND
SK-BR-5	***PIK3CA***	-[85]	ND
T47D	***PIK3CA***	-[85]	ND
DU4475	***BRAF, APC***	+[85]	ND
BT483	***PIK3CA***	-	+
HCC1937	***PTEN***	-	-
MDA-MB-361	***PIK3CA***	-	-
MDA-MB-415	***PTEN***	-	+
MDA-MB-468	***PTEN***	-	+
SK-BR-7	***KRAS,***	-	+
SUM159-PT	***PIK3CA,***	-	+
SUM185PE	***PI3KCA***	-	+
UACC893	***PI3KCA***	-	-
ZR-75-1	***PTEN***	-	-
Prostate Cancer Cell lines [172]			
LNCaP	***PTEN***	-	+
PC3	***PTEN***	-	+

Table 1. None of the tested cell lines with an active PI3K/ PKB and Ras-pathway display constitutive Wnt activity, except DU4475 which harbours a mutated APC protein. The TCF/ β-catenin transcription activity in all cancer cell lines were tested with the standard pTOPFlash assay. In the case when 20 mM LiCl responses were tested, LiCl was added 16hrs after transfection and the cells were lysed in PLB 24hrs later. (See also supplementary Table S2). ND means not done.

phosphorylation (Figure 1C/D bottom panels), had no significant effect on LiCl or TCF4/β-catenin induced transcriptional activation. Comparison of differences between TOPFlash activities was performed using two-tailed student t-test (where equal variance between groups was assumed).

We further confirmed our observations in an *in vivo* system, the nematode *C.elegans*. In the nematode *C.elegans*, canonical Wnt/β-catenin signaling regulates the left-right asymmetric migration of the Q descendants[174, 175]. On the left side, egl-20 triggers the expression of the Hox gene *mab-5*, which directs the migration of the Q descendants (QL.d) towards the posterior[176]. On the right side, *mab-5* is not expressed and as a result the QR descendants (QR.d) migrate in the default anterior direction. Mutations that disrupt Wnt/β-catenin signaling, such as mutations in *mig-1/Fz*, *bar-1/β-catenin* or *pop-1/Tcf*, lead to loss of *mab-5* expression in QL and anterior migration of the QL.d[175, 177, 178]. Conversely, mutation of negative regulators of Wnt/β-catenin signaling, such as *pry-1/Axin*, leads to ectopic expression of *mab-5* in QR and posterior migration of the QR.d[177, 179]. The final positions of the Q descendants therefore provide a sensitive measure of Wnt/

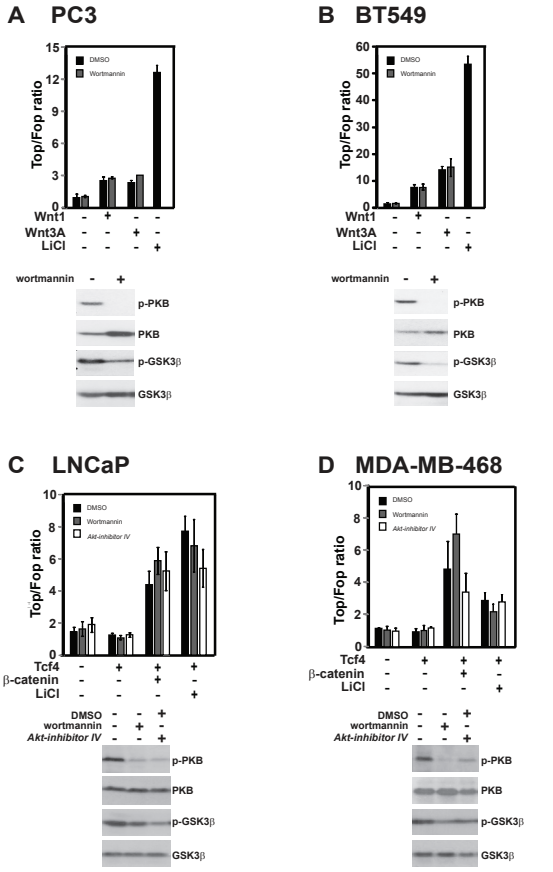

Figure 1. Wnt ligands, β-catenin and LiCl induce TCF/β-catenin-dependent transcription in PC3 (A), BT549 (B), LNCaP(C) and MDA-MB-468(D) cell lines in a PI3K/Akt-independent manner. Cells were plated at 50%-60% confluency in 24 wells plates and transfected with pTOPFlash reporters, with or without Wnt1, Wnt3A, β-catenin or TCF4 plasmids as indicated. 20mM LiCl, 100nM (BT509) or 250nM (PC3, LNCaP and MDA-MB-468) PI3K-inhibitor wortmannin, and 50 nM *Akt inhibitor IV* were added as indicated 16hrs following transfection, and measurements were done after 24hrs. The TOPFlash data (top panel) are representative of at least two independent experiments. Error bars represent ± standard deviation (STDEV). Effective inhibition of the PI3K/PKB pathway in response to wortmannin and *Akt inhibitor IV* treatment is shown by Western blotting as indicated in the panels below the pertinent bar graphs.

β-catenin pathway activity. We investigated whether mutation of the *C. elegans PTEN* ortholog *daf-18* [180] affects Q descendant migration. The deletion allele *daf-18(ok480)* is expected to represent a strong loss of function or the null phenotype. We found that *daf-18(ok480)* has no effect on either QL.d or QR.d migration compared to wild type (Table 2, in each case, n=50), indicating that loss of PTEN does not affect Wnt/β-catenin signaling in *C. elegans*.

Table 2

Genenotype	Affect on Q descendant migration
Control[muls32(mec-7::gfp)]	100% wild type, n=50
daf-18(ok480); muls32#2	100% wild type, n=50
daf-18(ok480); muls32#13	100% wild type, n=50
daf-18(ok480); muls32#16	100% wild type, n=50

Table 2. The mutation of the nematode *C.elegans* PTEN ortholog daf-18 has no effect on Q descendant migration. All assays were performed at 20°C and the final positions of the Q descendants was scored using a mec-7::gfp (muls32) reporter transgene.

We then turned to a physiological model for insulin/PI3K signaling. HEK293T cells respond to insulin by activating PI3K, which phosphorylates and activates PKB/AKT [164], leading to inhibitory phosphorylation of GSK3β on Ser9. As shown in Figures 2B and 2C, addition of insulin lead to a rapid phosphorylation of total cellular PKB and GSK3β ("input", compare lanes 1-3). Inhibition of PI3K abolished PKB and GSK3β phosphorylation at 30 min (Figure 2B "input", compare lanes 1 and 4), 1 hour and 4 hours post wortmannin treatment (Figure 2D, compare lanes 1-3).

To study a potential effect of PI3K/PKB pathway activation on Wnt/β-catenin target gene expression in HEK293T cells, we used the pTOPFlash Wnt reporter assay. As shown in Figure 3, Wnt-mediated transcriptional activity is not influenced by PI3K/PKB pathway activation, but could readily be blocked by the soluble Wnt inhibitor Dkk1 in HEK293T cells.

Axin is believed to be the central scaffolding component in the cytoplasmic destruction complex of the Wnt cascade, as it carries independent interaction domains for the Wnt pathway components APC, GSK3, CK1, Dsh and β-catenin[2, 166]. Moreover, it is by far the least abundant core component of the Wnt pathway[49]. The pool of GSK3β that is dedicated to Wnt signaling is stably bound to Axin[2, 166]. To be able to investigate whether the endogenous Axin-bound pool of GSK3β was targeted by insulin-activated PKB, we generated monoclonal antibodies against Axin1 (Figure 2A). Figure 2B (lanes 9 - 12) and demonstrates results obtained with immunoprecipitations of endogenous Axin from the same lysates as used in input lanes respectively. Endogenous GSK3β readily co-immunoprecipitated with Axin (Lane 9 -12). This Axin/GSK3 complex was robust, as it even resisted 3% NP40/TritonX100 (not shown). From multiple experiments, we estimate that 3-5% of total cellular GSK3β resides within the Axin complex.

When the blots were probed with anti–phospho GSK3β antibodies, the phosphorylation level was found to remain unchanged upon both insulin signaling (Figure 2B/C) and treatment with Wortmannin (Figure 2B/D). Furthermore, (phospho-) PKB was not co-precipitated. We conclude that, while insulin rapidly activated the PI3K

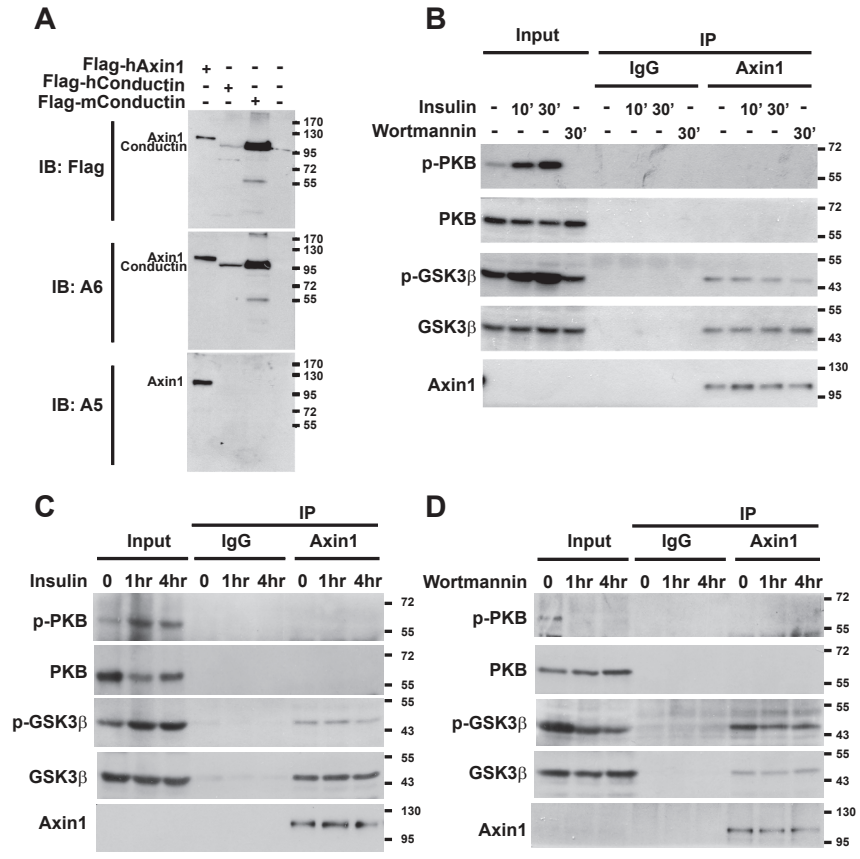

Figure 2. The PI3K/PKB pathway activity does not modulate Wnt/β-catenin signaling through GSK3β. **(A)** The mouse monoclonal A5 antibody is Axin1 specific whereas A6 antibody recognizes both Axin1 and Conductin. 0.5ug of DNA/well were transfected into HEK293T cells in a 6-well plate. All samples were lysed in 2xSDS sample buffer after 24hrs and Western blot analysis was performed using anti-Flag, A6 and A5 antibodies respectively. **(B-D)** Activation of PI3K/PKB pathway does not influence the phosphorylated or total amount of GSK3β protein which is bound to Axin. HEK293T cells were cultured in 5%FCS/RPMI to 80% confluency and then starved in serum free medium for at least 12hrs before treatment with 10mg/L Insulin for 10 and 30 min **(B)** and 1hour and 4 hours **(C)** or 200nM Wortmannin for 30 min **(B)**, 1 hour and 4 hours **(D)**. Cells were lysed and immunoprecipitated with either control mouse IgG or anti-Axin antibody. Resulting immunocomplexes were subjected to immunoblotting using the indicated antibodies.

pathway as evidenced by sequential phosphorylation of PKB and GSK3β, the small axin-bound pool of GSK3β is protected from activated PKB. Thus, activation or inhibition of PI3K/PKB does not target the Axin bound GSK3β pool. These results were further confirmed in PC3 prostate cancer cells, which have previously been used to implicate a role for PI3K signaling in the Wnt pathway (Supplementary Figure 3)[181].
This result supports the hypothesis that different pools of GSK3 kinases exist in cells,

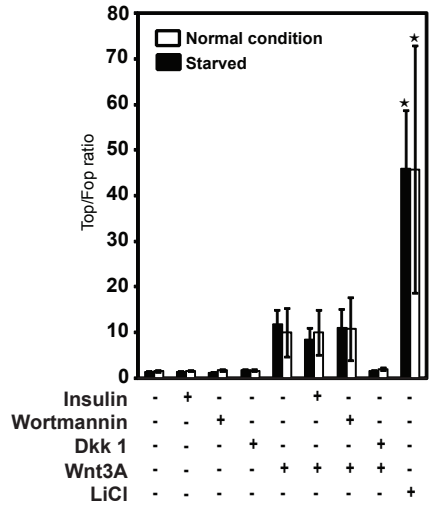

Figure 3. Tcf/β-catenin transcription activity is not modulated by activation of PI3K/PKB pathway. HEK293T cells were plated at 30%-40% confluency in 24 wells plates and transfected with pTOPFlash reporters, Wnt3a, Dkk1 (Wnt inhibitor) plasmids as indicated.16hrs after transfection, half of the cells were starved with serum free medium for at least 12hrs before treatment with 20mM LiCl, 10mg/L Insulin or 200nM Wortmannin as indicated. Measurement was done another 16-24hrs later. Incubation periods of the non-starved cells were the same for the starved cells. Data are from three independent duplicate experiments (except*, N=2). Error bars indicated mean ±STDEV.

participating separately in the PI3K/PKB pathway or the Wnt/β-catenin pathway as originally proposed by Frame and Cohen[182]. The existence of Axin-GSK3 complexes appears to serve two functions: First, the Axin complex allows GSK3 to target β-catenin specifically by providing docking sites for both proteins[2]. Second, when GSK3 is complexed to Axin, it is shielded from activated PKB. This molecular arrangement allows Wnt and PI3K inputs to have independent effects on the biological outputs of the cells that receive these signals.

These findings also help to clarify why mutations in canonical Wnt components (i.e. APC, Axin/conductin, or β-catenin) are mutually exclusive in colorectal cancer, while individual cases of colorectal cancer frequently combine an activating Wnt pathway mutation with a mutation in a component of the PI3K pathway [183]. Similar observations are made e.g. for Ovarian Endometroid Adenocarcinoma[184]. If activating PI3K pathway mutations would activate the Wnt pathway, such mutations should not occur in combination with activating Wnt pathway mutations in cancer.

Material and Methods

Q descendants migration count in C.elegans

The final positions of the Q descendants was scored using a mec-7::gfp (muIs32) reporter transgene[170]. All assays were performed at 20°C. The *daf-18(ok480)* allele (provided by the *C. elegans* gene knockout project at the Oklahoma Medical Research Foundation) was detected by PCR using the following primers: dag-18int-in (CAA CGC AGT ACA TCT CGA AGC C) and daf-18int-out (CCA GCT GAT ACC GAT GAT GTT GAT).

Cells and Cell culture

HEK293T cells were maintained in RPMI 1640 (Invitrogen) supplemented with 5% fetal calf serum (FCS). All cancer cell lines used in this study are listed in Table1. The prostate cancer cell lines, LNCaP and PC3 were kind gifts of Dr J. Trapman and were cultured in RPMI with10% FCS. The breast cancer cell lines, EVSA-T, and SK-BR-5/7 were kind gifts of Dr. N. DeVleeschouwer (Institute Jules Brodet, Brussels, Belgium), Dr. H. S. Smith (California Pacific Medical Center, San Francisco, CA), and Dr. E. Stockert (Sloan-Kettering Institute for Cancer Research, New York, NY), respectively. The SUM-series cell lines were generated in the Ethier laboratory (available at http://www.asterand.com). Cell lines OCUB-F was obtained from Riken Gene Bank (Tsukuba, Japan). All other cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were grown according to the suppliers' recommendations and are unique and monoclonal as shown by extensive analysis of nearly 150 polymorphic microsatellite markers. Wnt3A producing L-Cells were a kind gift from R. Nusse, R and Wnt3A/control condition medium were generated by using the standard protocol provided by ATCC.

Reporter Gene Assays

TCF reporter constructs were both modified to contain 10 optimized and 10 mutated TCF-binding sites as previously described. cDNA of Dkk1(kind gift from C. Niehrs), mutated β-catenin (S33A) (kind gift from K. Kinzler), Wnt1 and Wnt3a (both kind gifts from R. Nusse, R) were subjected to PCR amplification and then cloned into pcDNA4TO. HCC1937, MDA-MB-361, MDA-MB-468, SUM159, SUM185, UACC893 and LNCaP were transfected with Fugene-HD transfection reagent (Roche); BT483, BT549, MDA-MB-415, SK-BR-7, PC3 and 293T were transfected with polyethylenimine (polysciences); and ZR-75-1 was transfected with electroporation. The amount of DNA in each transfection was kept constant (250ng/well in 24-wells plate for Fugene-HD or P-Pei transfection and 1.1µg/well in 6-well plates for electroporation) by the addition of an appropriate amount of empty expression vector, pCDNA4TO and the ratio of reporter plasmid (TOP10 or FOP10) with internal control plasmid (pCMV-Rennilla) was kept constant at 10:1. 16hrs after TOPFlash transfection, the cells were treated with condition medium or inhibitors (wortmannin, Akt inhibitor IV and Akt inhibitor VIII both from SIGMA) and the measurements were performed 12-16hrs later. All experiments were performed in duplicate or triplicate and repeated at least twice.

Protein analysis

The expression construct for human flag tagged Axin1 was produced by PCR amplification of the coding region using the IMAGE clone ID 5809104 as a cDNA template and this PCR product was cloned into pCDNA3. HEK293T cells were cultured to 80% confluence and starved at least 12hrs before treatment with 10 mg/L Insulin (Invitrogen) for10min, 30 min, 1 hour and 4 hours or with 200 nM wortmannin for 30min, 1 hour and 4 hours except control (0'). Cells were washed with cold PBS and lysed in cold lysis buffer containing 300 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EDTA, 0.1% NP-40, 0.1mM PMSF, 0.5 mM DTT, protease inhibitor cocktail tablets (EDTA-free)(Roche) and phosphatase inhibitor cocktail1 and 2(SIGMA). A cycle of freeze - thawing was used for lysis. After clarification by centrifugation (18,000 rcf for 30 min at 4°C), equal amounts of the total protein from each samples were incubated with anti-Axin1 antibody or mouse IgG immobilized on Protein G PLUS-Agarose

beads (Santa Cruz biotechnology) at 4°C overnight. After washing the immune-complexes with cold lysis buffer six times, they were resuspended in 2xSDS sample buffer. Immunoblottings were done by reprobing the same blot after stripping (Pierce) using anti-phospho GSK3β (S9) (cell signaling), anti-phospho PKB (S473) (cell signaling), anti-GSK3β (cell signaling), anti-PKB (cell signaling) and anti-Axin antibodies.

Generation of Axin specific antibodies

Flag-tagged mouse Axin2/Conductin was a gift from Jürgen Behrens, and flag-tagged human Axin2 was cloned from IMAGE clone ID4053244 to pCDNA4TO vector by PCR. Antibodies directed against Axin were produced by immunizing Balb/c mice with the N-terminal part (amino acid 3-326) of mouse Axin1. Hybridomas were generated using standard procedures.

Supplementary Data

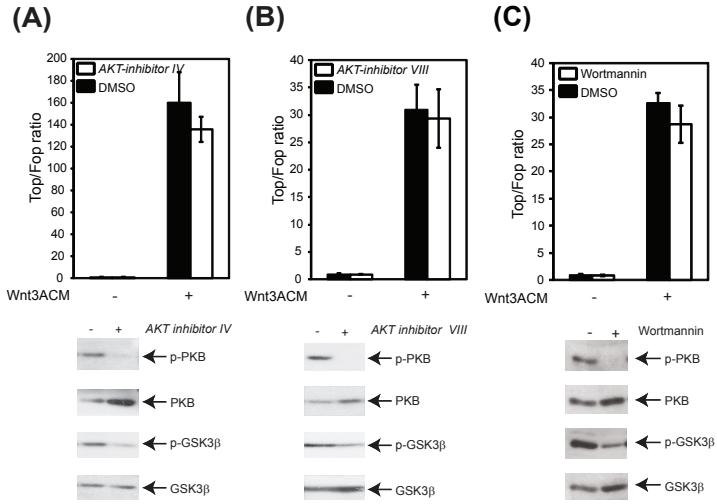

Figure S1. Neither of the PKB inhibitors (*Akt inhibitor IV* and *Akt inhibitor VIII*) nor PI3K inhibitor (wortmannin) have an effect on Wnt3A activated Tcf/β-catenin transcription activity. BT549 cells were plate at 50%-60% confluency in 24 wells plates and transfected with pTOPFlash reporters. Wnt3A Conditioned medium (Wnt3ACM) or Control medium (CM) with the indicated combination of inhibitors were added 16hrs after tranfection. The measurements were done 12-16hrs later. Error bars represent ± standard deviation (STDEV). The Western blots below the graphs indicate that the inhibitors are active in inhibiting the indicated phosphorylation events.

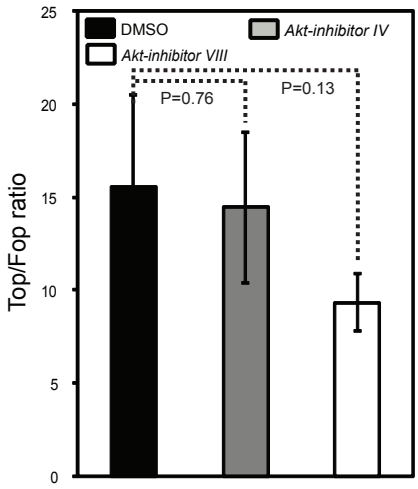

Figure S2. LiCl-induced Tcf/β-catenin-dependent transcription in BT549 cells is not affected by PI3K/PKB. Cells were plated at 50%-60% confluency in 24-wells plates and transfected with pTOPFlash reporters. LiCl (20mM) alone, with combination of *Akt inhibitor IV* (50nM) or *Akt inhibitor VIII* (5μM) were added to the cells after 16hrs transfection. Cells were collected 16hrs later. Error bars represent ± standard deviation (STDEV).

Table S1 The list of references which propose that the PI3K/PKB pathway directly modulates canonical Wnt pathway through GSK3 and/or β-catenin.

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Table S2

Breast Cell lines													
BT483	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	820	**125067**	620	**92304**	956	**104353**	899	**93802**	0.006556	0.006717	0.009161	0.009584	**0.71**
20mM LiCl	5854	**128984**	4247	**74356**	977	**108625**	671	**48693**	0.045385	0.057117	0.008994	0.013780	**4.50**
HCC1937	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	2568	**99144**	2745	**55639**	7204	**316022**	12235	**305512**	0.025902	0.049336	0.022796	0.040048	**1.20**
20mM LiCl	3115	**88384**	1826	**76920**	6795	**266066**	7268	**259807**	0.035244	0.023739	0.025539	0.027975	**1.10**
MDA-MB-361	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	4358	**18880**	7681	**29656**	20374	**39954**	23658	**47070**	0.230826	0.259003	0.509936	0.502613	**0.48**
20mM LiCl	2181	**15212**	2727	**15852**	4754	**16332**	4800	**17453**	0.143374	0.172029	0.291085	0.275024	**0.56**
MDA-MB-415	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	628	**46158**	595	**57511**	572	**40963**	677	**35472**	0.013605	0.010346	0.013964	0.019085	**0.72**
20mM LiCl	4112	**58908**	2742	**66026**	628	**40413**	497	**56243**	0.069804	0.041529	0.015540	0.008837	**4.57**
SK-BR-7	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	9443	**440294**	10547	**422424**	8254	**441791**	8480	**376253**	0.021447	0.024968	0.018683	0.022538	**1.13**
20mM LiCl	26092	**462789**	20180	**460992**	1477	**449786**	1147	**432418**	0.056380	0.043775	0.003284	0.002653	**16.87**

SUM159	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	1677	**161813**	1832	**185638**	2661	**288459**	3432	**388789**	0.010364	0.009869	0.009225	0.008827	**1.12**
20mM LiCl	50233	**369831**	40012	**269710**	1841	**369553**	2658	**376422**	0.135827	0.148352	0.004982	0.007061	**23.60**

SUM185	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	1460	**99345**	1282	**103419**	878	**64741**	799	**74651**	0.014696	0.012396	0.013562	0.010703	**1.12**
20mM LiCl	12097	**135769**	17035	**253257**	916	**234164**	678	**184635**	0.089100	0.067264	0.003912	0.003672	**20.62**

UACC893	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	1960	**183559**	1715	**162507**	3160	**200779**	3000	**183227**	0.010678	0.010553	0.015739	0.016373	**0.66**
20mM LiCl	1277	**147402**	1340	**162229**	2339	**206998**	2924	**239113**	0.008663	0.008260	0.011300	0.012229	**0.72**

ZR-75-1	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	26028	**109646**	22812	**108649**	25239	**137893**	28925	**152620**	0.237382	0.209961	0.183033	0.189523	**1.20**
20mM LiCl	26062	**197203**	20530	**169559**	25997	**186458**	21013	**194227**	0.132158	0.121079	0.139426	0.108188	**1.02**

MDA-MB-468	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	893	**532230**	941	**592303**	2341	**1159267**	2735	**1381350**	0.001678	0.001589	0.002019	0.001980	**0.82**
20mM LiCl	3774	**689245**	3639	**693907**	658	**742807**	644	**875620**	0.005476	0.005244	0.000886	0.000735	**6.61**

BT549	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	877	**694875**	865	**597979**	697	**504802**	878	**742787**	0.001262	0.001447	0.001381	0.001182	**1.06**
20mM LiCl	41220	**764430**	51363	**1157585**	990	**994563**	1060	**1216901**	0.053923	0.044371	0.000995	0.000871	**52.66**

Prostate Cancer Cell lines

LNCaP	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	163070	**2143959**	149179	**3060777**	143459	**2367420**	105169	**2302621**	0.076060	0.048739	0.060597	0.045674	**1.17**
20mM LiCl	248321	**2310734**	242465	**2331631**	63981	**1630144**	60532	**1771073**	0.107464	0.103989	0.039249	0.034178	**2.88**

PC3	TOP1		TOP2		FOP1		FOP2		TOP		FOP		**Average TOP/FOP**
	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	RLU1	**RLU2**	1	2	1	2	
Control	1080	**147424**	988	**150502**	739	**128879**	631	**123366**	0.007326	0.006565	0.005734	0.005115	**1.28**
20mM LiCl	2781	**156080**	2220	**141304**	700	**155728**	680	**161615**	0.017818	0.015711	0.004495	0.004208	**3.85**

Table S2. Raw data of pTOPFlash assay in tested cancer cell lines. All cells except ZR-75-1 were plated at 50-60% confluency in 24-wells plate and transfected with reporter as indicated by polyethyleneimine or Fugene-HD. ZR-75-1 cells were transfected by electroporation and cultured in 6-wells plates. LiCl was added 16-24hrs after transfection and another 24hrs later measurement was performed. All reporter assays were performed in duplicate and repeated at least twice. Data from a single experiment is given in the table.

Chapter 3

MAP3K1 functionally interacts with Axin1 in the canonical Wnt signalling pathway

Adapted from:

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For man is man and master of his fate.
-A. Tennyson-

Abstract

A central point of regulation in the Wnt/β-catenin signalling pathway is the formation of the β-catenin destruction complex. Axin1, an essential negative regulator of Wnt signalling serves as a scaffold within this complex and is critical for rapid turnover of β-catenin. To examine the mechanism by which Wnt signalling disables the destruction complex, leading to stabilization and nuclear localization of β-catenin, we used an immunoprecipitation–coupled proteomics approach to identify novel endogenous binding partners of Axin1. We found mitogen-activated protein kinase kinase kinase 1 (MAP3K1) as an Axin1 interactor in Ls174T colorectal cancer (CRC) cells. Importantly, confirmation of this interaction in HEK293T cells indicated that the Axin1-MAP3K1 interaction is induced and modulated by Wnt3A stimulation. siRNA depletion of MAP3K1 specifically abrogated TCF/LEF-driven transcription and Wnt 3A-driven endogenous gene expression in both HEK293T as well as DLD-1 colorectal cancer cells. In addition, MAP3K1 depletion abrogated Wnt3A dependent expression of endogenous Wnt target genes in HEK293T cells as well as DLD-1 CRC. Expression of ubiquitin ligase mutants of MAP3K1 abrogated TCF-LEF transcription, whereas kinase mutants had no effect in TCF-driven activity, highlighting the essential role of the MAP3K1 E3 ligase activity in regulation of the Wnt/β-catenin pathway. These results suggest that MAP3K1, previously reported as an Axin1 interactor in c-Jun NH_2-terminal kinase (JNK) pathway is also involved in the canonical Wnt signalling pathway and positively regulates expression of Wnt target genes.

Introduction

A central point of regulation in Wnt/β-catenin signalling is the stabilization of β-catenin. In the absence of Wnt, low levels of β-catenin are maintained and tightly controlled by the β-catenin destruction complex. The β-catenin destruction complex contains glycogen synthase kinase-3β (GSK-3β), the adenomatous polyposis coli protein (APC), casein kinase I (CK1) and the scaffolding protein Axin1, among others, and catalyzes the phosphorylation of β-catenin, leading to its proteosomal degradation. Upon Wnt signalling, activated Wnt receptors, Frizzled and LRP5/6, are thought to block the function of the β-catenin destruction complex through yet unclear mechanisms, leading to the accumulation of β-catenin in the cytosol and subsequent nuclear translocation[2]. Upon transfer to the nucleus, stabilized β-catenin forms a complex with DNA-binding TCF/LEF transcription factors and serves as a transcriptional co-activator, thus switching on the Wnt transcriptional program[98, 99]

Abnormal activation of the Wnt pathway, either by functional loss of the inhibitors Axin[48, 138], APC[169], WTX[7] or activating point mutations in β-catenin[185] leads to lethal or abnormal development of embryo and tumourigenesis in the adult.

Within the β–catenin destruction complex, Axin1 is the least abundant limiting component[49], and therefore functionally critical to the complex. There are two Axin homologues, Axin1 and Axin2/Conductin, which are highly conserved on their N-terminal RGS (regulator of G protein signalling)[48] and C-terminal DIX (dishevelled and axin binding domain) domains[186]. Axin1 is ubiquitously expressed in almost all tissues from early embryonic development through adult state[48], Axin2/Conductin, a Wnt target gene product, is expressed in a developmental and tissue specific manner[54]. Studies indicate that Axin1 interacts directly with other Wnt/β-catenin signalling pathway components including APC, GSK3, CK1α and β-catenin, and is essential for degradation of β-catenin in the Wnt/β-catenin signalling cascade[2]. We previously reported the generation of monoclonal antibodies, which specifically recognize Axin1[187].

In addition to Axin1, CK1, GSK3β and APC, other proteins including PP2A, and WTX have been proposed to be involved in regulation of the β-catenin destruction complex[87]. However, how the interplay between the destruction complex components results in the phosphorylation and degradation of β-catenin, and the mechanism by which Wnt stimulation disables this complex, remain elusive.

To gain insight into the mechanism by which Wnt stimulation regulates the function and composition of the β-catenin destruction complex, we immunopurified the endogenous Axin1 complexes from Ls174T CRC using Axin1 antibodies and IgG as control, followed by mass spectrometry (MASS SPEC). MAP3K1(MEKK1), a serine/threonine protein kinase, and member of the MAPK signalling pathway, was found to specifically interact with Axin1 in Ls174T CRC.

MAP3K1 contains a highly conserved C-terminal kinase domain belonging to the STE11 family[188]. At its N-terminus, MAP3K1 contains a plant homeo domain (PHD), whose structure is closely related to the RING finger domain containing seven cysteines and histidine spatially arranged in a C4HC3 consensus sequence (Figure1B, Figure5A-C), a structure shown to mediate E3 ubiquitin ligase activity[189, 190]. MAP3K1 has been shown to phosphorylate several different MAP kinase kinases (MKKs). However, its strongest activity is directed towards MKK4, an upstream kinase for c-Jun NH_2-terminal kinases (JNKs)[191]. Phosphorylation of MKKs by MAP3K1 in turn activates the MAP kinase activity. Recently, MAP3K1 was reported to be the only MEKK protein that contains an E3 ubiquitin ligase PHD domain, implicated to be important for its role as a negative regulator through ubiquitination and degradation of ERK1/2 and c-Jun proteins[192, 193].

Although MAP3K1 was previously reported as an Axin1 interactor involved in the JNK pathway, we found that MAP3K1 interaction with Axin1 is modulated by Wnt stimulation. Confirmation of the MAP3K1 interaction with Axin1 in HEK293T cells showed increased binding between MAP3K1 and Axin1 in response to Wnt treatment. In addition, siRNA mediated depletion of MAP3K1 suppressed not only TCF/LEF-driven reporter activity, but also the expression of endogenous Wnt target genes in both HEK293T as well as DLD-1 CRC containing truncated inactive APC. Interestingly, HEK293T cells expressing with inactive E3 ubiquitin ligase but not kinase-dead

mutants of Rattus Map3k1 (rMap3k1) were able to suppress TCF/LEF-driven reporter activity. This inhibition was not longer observed in HCT116 CRC which contains activating point mutated β-catenin, thereby placing MAP3K1 upstream of β-catenin during Wnt/β-catenin pathway signal transduction.

Results

MAP3K1 co-immunoprecipitates with Axin1 in Ls174T CRC cells

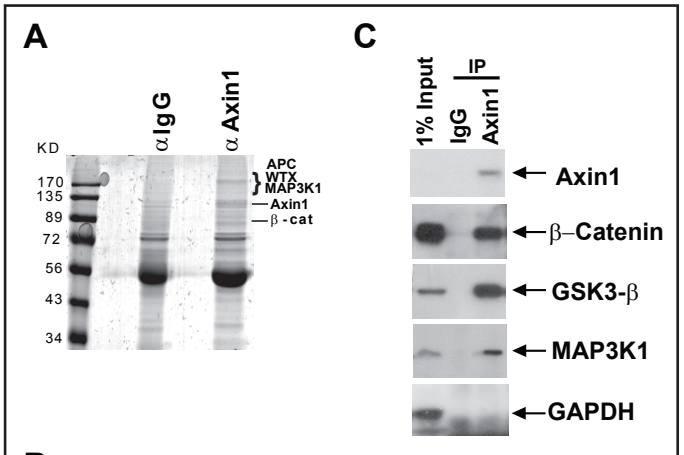
To identify potentially novel components within the β-catenin destruction complex, we immunopurified Axin1 complexes from CRC Ls174T cells (carrying a wild type APC and oncogenic point mutation in β-catenin) using a monoclonal antibody against Axin1 and non-immune IgG as control. The bound proteins were subjected to SDS-PAGE and ComassieBlue staining (Figure 1A), followed by mass spectrometric identification. As expected, we readily detected Axin1 itself (data available from the authors upon request). We were also able to detect known components of the β-catenin destruction complex, such as APC, β-catenin, WTX and Axin2 (data available from the authors upon request), serving as positive controls to the experimental set-up indicating that we successfully immunoprecipitated the β-catenin destruction complex. Amongst the proteins that specifically co-precipitated with Axin1 from Ls174T cells, the kinase MAP3K1 was identified (Figure 1B). To confirm the interaction, we immunoprecipitated Axin1 from Ls174T cells and probed for its association with endogenous MAP3K1 by western blotting using a MAP3K1 antibody (Figure 1C). The MAP3K1 was co-immunoprecipitated specifically in the Axin1 complex and not the non-immune IgG control. As expected, β-catenin and GSK3β were also present in the Axin1 complex, whereas GAPDH as control did not bind Axin1.

MAP3K1 interacts with Axin1 in Wnt-dependent manner.

To examine whether the Axin1-MAP3K1 association could modulate by the Wnt/β-catenin pathway activity, we employed the Wnt inducible HEK293T cell line in which the Wnt pathway is present, yet not mutationally activated.

Upon Wnt stimulation, the Wnt co-receptor LRP6 is phosphorylated, leading to accumulation of β-catenin. Dkk specifically blocks the Wnt/β-catenin pathway via binding to the LRP6 receptor[194, 195]. Therefore, we tested the specificity of the Wnt3A conditioned medium using the TOPFlash assay (Figure 2A) (Promega, Madison, WI, USA) in the presence or absence of DKK.We also probed the dynamic downstream activation of the Wnt/β-catenin cascade by checking the accumulation of free β-catenin levels in the cytosol, and the phosphorylation status of LRP6 in the membrane (Figure 2B). As shown in Figure2B, dramatic increase of the β-catenin protein levels as well as phosphorylation of LRP6 occurred at 30 min, 2 h, and plateaued after 4 h of treatment with Wnt3A-conditioned medium (Wnt3A+R-spondin). Therefore,

we decided to examine the interaction between MAP3K1 and Axin1 before and after 2 h of Wnt3A stimulation in HEK293T cells. Axin1 was immunoprecipitted from HEK293T lysates and probed for interaction with MAP3K1 by Western blotting (Figure 2C). Although present in the Axin1 complex in the absence of Wnt sinalling, the interaction of MAP3K1 with Axin1 increased significantly in response to Wnt treatment. As expected, binding of the known interactors, LRP6 and Dishevelled3 with Axin1 was induced in response to Wnt stimulation. Binding of GSK3β to Axin1 did not change in response to Wnt stimulation. Our data suggested that MAP3K1 functions as a regulator of the Wnt/β-catenin pathway.



B

IPI00787127 **Mass:** 195863 **Score:** 74 **Queries matched:** 1 **emPAI:** 0.01
Tax_Id=9606 Gene_Symbol=MAP3K1 similar to Mitogen-activated protein kinase kinase kinase 1

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HLALIFKIASATTAPSIPSHLSPGLRDVALRCLELQPQDRPPSRELLKHPVFRTTW

Figure 1. MAP3K1 co-immunoprecipitates with Axin1 in Ls174T cells. (A) Coomassie Blue stained gel of Axin1 complexes immunoprecipitated from Ls174T cells using antibody directed against Axin1. Proteins (Axin1, β-catenin, WTX, APC and MAP3K1) were identified by mass spectrometry (MS) and are indicated by arrows and parentheses (see material and methods section for details). **(B)** Peptide coverage of MAP3K1 in MS experiment and functional domains of MAP3K1. Amino acid sequences of MAP3K1 peptide detected in the Axin1 immunoprecipitate is given in white and highlighted in black; the PHD domain is highlighted in grey; the SWIM-type zinc finger domain and kinase domains are shown with underlined double and single line respectively. **(C)** Ls174T cell lysates were immunoprecipitated with antibodies directed against endogenous Axin1 and analyzed by Western blotting with the indicated antibodies.

Depletion of MAP3K1 by siRNA abolishes TCF4/LEF-driven transcription and expression of endogenous Wnt target genes in HEK293T and DLD-1 cells.

To address the potential function of MAP3K1 in the Wnt/β-catenin pathway, we depleted MAP3K1 using a siRNA approach. We examined the effect of MAP3K1

depletion on TCF/LEF-driven reporter activity in both HEK293T (Figure3A,B) and CRC DLD-1 cells (Figure 3C,D) using the TOPFlash assay. Significant depletion of MAP3K1 was accomplished at 96 h post-siRNA treatment in both HEK293T as well as in DLD-1 cells as shown by Western blotting (Figure 3). Removal of MAP3K1 resulted in specific suppression of Wnt-dependent TOPFlash activity not only in the Wnt stimulated HEK293T cells but also the DLD-1 cells.

As MAP3K1 appears to modulate the TCF/LEF transcription regulation, we therefore further examined the specific effect of MAP3K1 depletion on expression change of endogenous Wnt target genes. We chose the classical Wnt target genes *AXIN2*, and *TCF7*, as well as our recently identified Wnt induced HEK293T target genes *BPTF* and *ZCCHC12* [196] for quantitative RT-PCR determination of expression. As shown in Figure 4A, all the selected genes were upregulated in response to Wnt stimulation. Expression of selected target genes was abrogated to near basal levels upon MAP3K1 depletion, highlighting the important positive role played by MAP3K1 in Wnt target gene activation (Figure 4A). Similar suppression of Wnt target genes *AXIN2*, *TCF7* and *ASCL2*[197] was observed upon MAP3K1 depletion in DLD-1 cells (Figure 4B). Our data strongly implicate MAP3K1 as a positive regulator in the canonical Wnt signaling pathway and regulation of Wnt target genes.

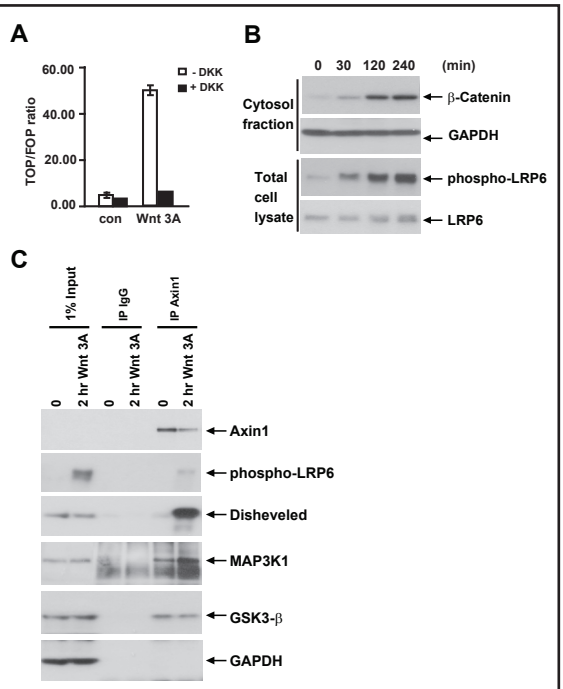

Figure 2. The PI3K/PKB pathway activity does not modulate Wnt/β-catenin signaling through GSK3β. (A) The mouse monoclonal A5 antibody is Axin1 specific whereas A6 antibody recognizes both Axin1 and Conductin. 0.5ug of DNA/well were transfected into HEK293T cells in a 6-well plate. All samples were lysed in 2xSDS sample buffer after 24hrs and Western blot analysis was performed using anti-Flag, A6 and A5 antibodies respectively. **(B-D)** Activation of PI3K/PKB pathway does not influence the phosphorylated or total amount of GSK3β protein which is bound to Axin. HEK293T cells were cultured in 5%FCS/RPMI to 80% confluency and then starved in serum free medium for at least 12hrs before treatment with 10mg/L Insulin for 10 and 30 min **(B)** and 1hour and 4 hours **(C)** or 200nM Wortmannin for 30 min **(B)**, 1 hour and 4 hours **(D)**. Cells were lysed and immunoprecipitated with either control mouse IgG or anti-Axin antibody. Resulting immunocomplexes were subjected to immunoblotting using the indicated antibodies.

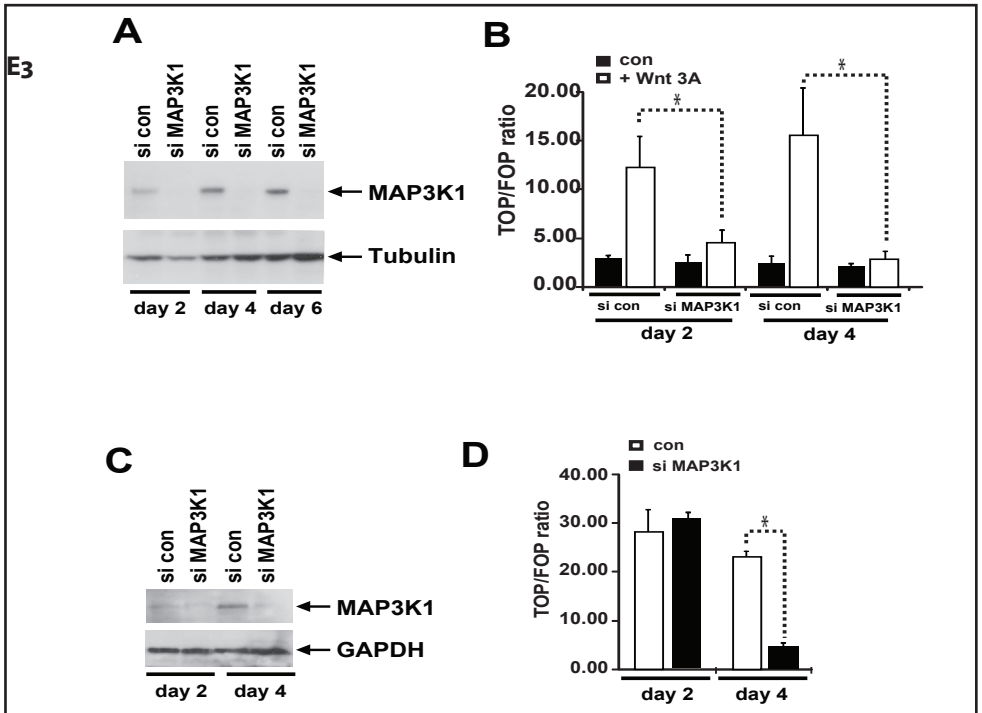

Figure 3. Depletion of MAP3K1 by siRNA in HEK293T and CRC DLD-1 cells abolished TCF4/LEF-mediated reporter activity. Suppression of TOPFlash activity upon MAP3K1 depletion in presence and absence of Wnt3A treatment in HEK293T cells **(B)** and in DLD-1 cells **(D)** at 2 and 4 days post-siRNA transfection. Activity of the TCF/LEF-driven TOPFlash and FOPFlash were measured 16 h post-transfection, normalized to an internal *Renilla* control and the data represented as the ratio between TOPFlash/FOPFlash. Error bars represent standard deviation; * p-value<0.05. Expression of MAP3K1 protein in HEK293T cells **(A)** and DLD-1 cells**(C)** was analyzed by Western blotting after 0, 2 and 4 days (DLD-1 cells) and after 0, 2, 4 and 6 days (HEK293T cells) post-siRNA transfection. More than 90% depletion of MAP3K1 protein was observed at 4 days post-siRNA transfection. Tubulin and GAPDH were used as the loading control.

Ubiquitin ligase PHD domain of MAP3K1 is required for its role as positive regulator of Wnt-driven transcription.

MAP3K1 is a well described kinase whose substrates include mitogen-activated protein kinase kinase 1, mitogen-activated protein kinase kinase 2 and mitogen-activated protein kinase kinase4[198, 199]. Recently, MAP3K1, which at its N-terminus also contains a conserved RING finger-like PHD domain(Figure 5A,B) was shown to exhibit E3 ubiquitin ligase activity toward ERK1/2, c-Jun and Fra-2[192, 193, 200]. To examine if the kinase or E3 ubiquitin ligase enzymatic activities of MAP3K1 are required for its regulation of Wnt/β-catenin pathway, we generated MAP3K1 mutants harboring point mutations in the kinase domain (R1349A/D1350A and D1369A/F1370A) as well as E3 ubiquitin ligase PHD domain (C433A and C478A) mutants of rMap3k1 (Figure5A-C).

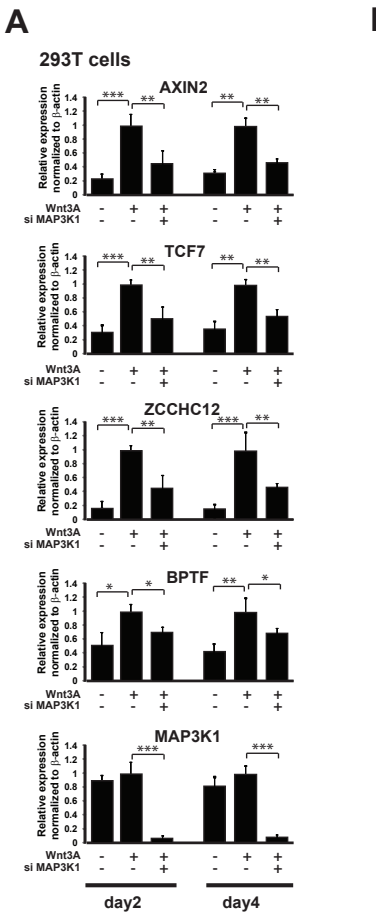

Figure 4. Depletion of MAP3K1 results in suppression of endogenous Wnt target genes in HEK293T and CRC DLD-1 cells. (A) Quantitative RT-PCR determination of expression of four selected endogenous Wnt target genes (Axin2, TCF7, ZCCHC12 and BPTF) upon Wnt3A treatment in the presence and absence of MAP3K1 in HEK293T cells at 2 and 4 days post-siRNA transfection. MAP3K1 expression was also examined by qRT-PCR. **(B)** Quantitative RT-PCR determination of expression of three selected endogenous Wnt target genes (Axin2, TCF7 and ASCL2) in the presence and absence of MAP3K1 in DLD-1 cells at 2 and 4 days post-siRNA transfection. MAP3K1 expression was also examined by qRT-PCR. * *p-value*<0.05; ** *p-value*<0.01; *** *p-value*<0.001.

Expression of wild type (WT) and mutant Myc-tagged rMap3k1 in HEK293T cells was examined by Western blotting (Figure 5D). We examined the effect of expression of mutant and WT Myc-rMAP3K1 in the Wnt-inducible HEK293T cells on TOPFlash activity in the presence or absence of Wnt (Figure 5E). As showed in Figure5E, expression of rMap3k1 E3 ubiquitin ligase PHD domain mutants (C433A and C478A) abolished Tcf-reporter TOPFlash activity. These results indicate that Tcf-driven transcription induced by addition of Wnt3A conditioned mdium is abolished by the exogenous expression of the C33A and C478A rMap3k1 containing point mutation in the conserved PHD domain, whereas overexpression of WT rMap3k1 alone is not sufficient to turn on the Wnt-driven transcription in HEK293T cells. Therefore, the E3 ubiquitin ligase activity of MAP3K1 is essential for its regulation of the Wnt/β-catenin pathway. We also examined the effect of WT and mutant rMap3k1 expression on TOP/FOP transcription in the HCT116 CRC cell which expresses WT APC but active point mutated β-catenin protein (data not shown), to gain insight into the requirement of MAP3K1 for Wnt/β-catenin pathway signaling transduction. In comparison to HEK293T, expression of

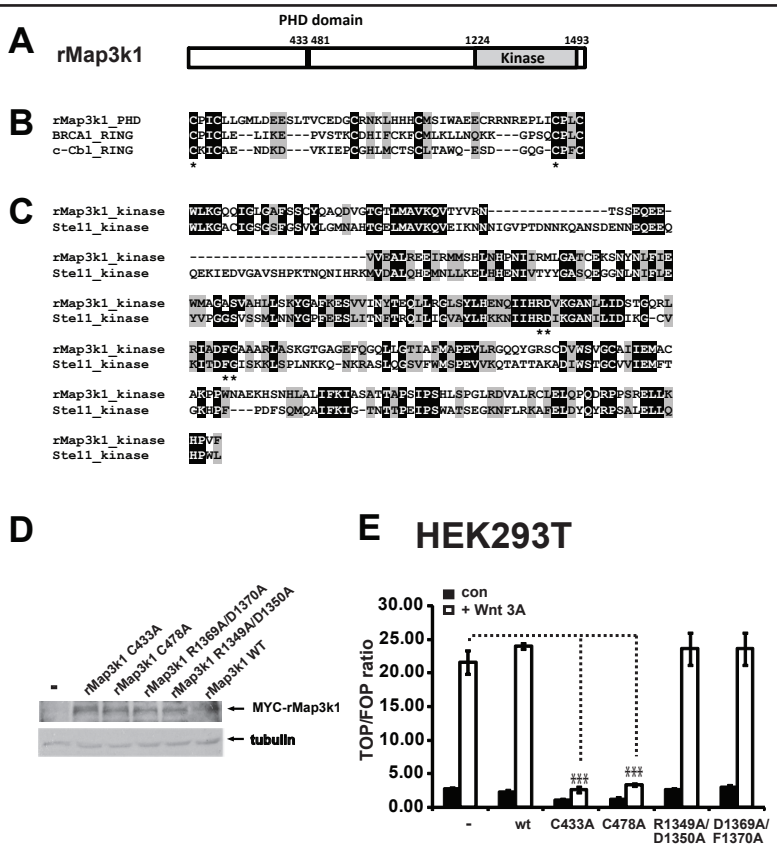

Figure 5. MAP3K1 E3 ubiquitin ligase PHD domain is required for TCF/β-catenin-mediated transcription. (A) The PHD domain (aa 433-481) and kinase domain (aa 1224-1493) of rMap3k1 in a schematic structure. **(B)** Alignment of the rMap3k1 PHD domain with RING finger domains of c-Cbl and BRCA1 E3 ligases. **(C)** Amino acid alignment of the C-terminal kinase domain of Rattus Map3k1 with Ser/Thr kinases STE11 kinase domain. Identical amino acids are shown in black and conserved amino acids are highlighted in grey. Asterisks indicate the conserved amino acids mutated to generate rMap3k1 mutants. **(D)** Western blot analysis indicates expression of MYC-tagged WT and mutant rMap3k1 proteins using anti-MYC antisera and Tubulin as control. **(E)** Expression of rMap3k1 PHD E3 ubiquitin ligase mutants (third and fourth panels) abrogates β-catenin/TCF-driven transcription, whereas overexpression of WT rMap3k1 (second panel) and rMap3k1 kinase domain mutants (fifth and sixth panels) has no effect on β-catenin/TCF-driven transcription in HEK293T cells. Cells were plated in 40-50% density and control or rMap3k1 expression constructs were transfected together with reporter vectors. Control conditioned medium (black bars) or Wnt3A-conditioned medium(white bars) was added 16 h post-transfection and luciferase activity was measured 24 h later. The data are represented as the ratio between TOPFlash/FOPFlash after normalization with internal *Renilla* control. Error bars represent the standard deviation from three independent experiments; *** *p-value*<0.001.

WT or mutant rMap3k1 in HCT116 cells containing stabilized β-catenin had no effect on TCF-mediated transcription (data not shown). Together, these data suggest that

MAP3K1 plays a role via its E3 ubiquitin ligase activity upstream of β-catenin during Wnt/β-catenin signal transduction.

Discussion

As the least abundant component within the β-catenin destruction complex, Axin is a critical focal point for investigating the regulatory mechanisms, which result in degradation of β-catenin, and its stabilization in response to Wnt signaling. To identify potential Axin interaction partners in a physiological *in vivo* setting, we previously generated Axin-specific antibodies, which efficiently immunoprecipitated endogenous Axin complexes[187]. Using this Axin1 specific antibody, we applied a combination of affinity purification and mass spectrometry to identify novel endogenous components within the Axin1 complex in Ls174T cells. MAP3K1, a serine/threonine protein kinase, and member of the RAS/RAF/MEK/ERK-signaling pathway, was identified in the list of candidates specifically interacting with Axin1.

In the past 10 years, aside from its involvement in Wnt signalling, and as an adaptor for Smad3 in the transforming growth factor β (TGFβ) pathway[157], Axin1 has been reported by several research groups to be involved in SAPK/JNK pathway during cell apoptosis[158, 160, 161]. The role of Axin1 in JNK pathway is thought to occur via its interaction with MAP3K1 (MEKK1) and MAP3K4 (mitogen-activated protein kinase kinase kinase4, MEKK4)[158, 159]. The kinase activity of MAP3K1 together with the homodimerization and sumoylation of Axin1 were shown to be important for Axin-induced SAPK/JNK pathway during cell apoptosis[158]. Moreover, MAP3K4 was shown to be able to compete with MAP3K1 to bind Axin1 and is also required in Axin-induced JNK activation. Thus, Axin might receive distinct signals via MAP3K1 or MAP3K4 to activate JNK[159].

Here, we have identified MAP3K1 as a component in the endogenous Axin1 complex in Ls174T CRC. This interaction is confirmed to be increased by Wnt signaling in HEK293T cells. siRNA-mediated depletion of MAP3K1 abrogates TCF/LEF-driven reporter activity and expression of endogenous Wnt target genes in both DLD-1 CRC and in HEK293T cells stimulated with Wnt3A. Examination of the effect of exogenous expression of WT and mutant rMap3k1 on TCF-driven transcription indicates that only mutations which disrupt the integrity of the PHD domain of MAP3K1 are able to abolish Wnt-mediated activation. Thus the E3 ubiquitin ligase activity of MAP3K1 is critical to its role as a positive regulator of Wnt signaling.

The importance of the E3 ubiquitin ligase functional domain of MAP3K1 in canonical Wnt signaling contrasts the previously reported requirement of the kinase activity of MAP3K1 in the non-canonical Wnt pathway. Thus, MAP3K1 appears to regulate the canonical and non-canonical Wnt pathways via different functional domains. We find that in HCT116 CRC, which express constitutively active stable β-catenin , TCF-driven

transcription is no longer effected by expression of E3 ubiquitin ligase inactive MAP3K1. This suggests that MAP3K1 acts as an upstream activator of β-catenin in the Wnt signaling cascade.Future studies are needed to determine the identity of the substrate(s) of MAP3K1 in the Wnt cascade and to elucidate the mechanisms by which this ubiquitination results in activation during Wnt/β-catenin signal transduction.

Material and Methods

Cell culture and DNA constructs

HEK293T, DLD-1, HCT116 and Ls174T cells were maintained in RPMI 1640 (Invitrogen, San Diego, CA, USA) supplemented with 5% foetal calf serum. Wnt3A producing-L-cells (kind gift from R. Nusse) or control-L-cells were cultured according to the manufacturer's instructions (ATCC, Manassas, VA, USA). Tcf reporter constructs were both modified to contain 10 optimized and 10 mutated Tcf-binding sites as previously described[201]. cDNA of Dkk1 (kind gift from C. Niehrs) was PCR amplified and cloned into pcDNA4TO.

Immunoprecipitation and mass spectrometry analysis

At 80% confluency, Ls174T cells and HEK293T cells were washed and collected with cold phosphate buffered saline (PBS) and then lysed in cold lysis buffer containing 150 mM NaCl, 30 mM Tris (pH 7.5), 1 mM EDTA, 1% TritonX-100, 10% Glycerol, 0.1mM PMSF, 0.5 mM DTT, protease inhibitor cocktail tablets (EDTA-free)(Roche Applied Science, Mannheim, Germany) and phosphatase inhibitor cocktail tablets (Roche). After clarification by centrifugation (12 000 *g* for 30 min at 4°C), the cellular lysates were pre-cleared with IgG-agarose beads (Sigma-Aldrich, St. Louis, MO, USA) for at least 6 h at 4°C. Immunoprecipitation of endogenous complexes were carried out by incubating the cellular lysates with anti-Axin1 antibody or mouse IgG immobilized on Protein G PLUS-Agarose beads (Santa Cruz biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Immunocomplexes were washed with cold lysis buffer six times, and resuspended in 2x SDS sample buffer. The immunoprecipitated material was subjected to SDS-PAGE and mass spectrometry analysis[196] or Western blot analysis by using anti-MAP3K1 (clone C-22, Santa Cruz), Dishevelled3(clone 4D3, Santa Cruz), GSK3β (clone 27C10, Cell Signaling Inc., Danvers, MA, USA), phosphor-LRP6 (Ser1490, Cell Signaling Inc.), GAPDH (Abcam, Cambridge, UK), Tublin (clone B-5-1-2, Sigma Aldrich) or β-catenin (BD Transduction Laboratories, San Diego, CA, USA) antibodies as described.

Fractionation

HEK293T cells were stimulated with Wnt3A-conditioned medium (Wnt 3A+R-spondin) or control conditioned medium as indicated and then washed and collected in ice-cold PBS. The cell pellets were then resuspended in hypotonic lysis buffer containing 10 mM KCl, 10 mM Tris pH7.5 and 2 mM EDTA with supply of protease inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets. Cell suspensions were incubate on ice for 30 min with multiple douncings in between. Lysis of the cells was controlled by checking under the

microscope. Nuclear proteins including the unlysed cells were pelleted by spinning at 1000 *g* for 2 min at 4°C centrifugation. The supernatant which contained both cytoplasm and membrane proteins was then centrifuged at 12 000 *g* for 30 min at 4°C. The supernatant was collected and resuspended in the 2x SDS sample buffer for further Western blotting analysis.

RNA interference

Pre-designed Dharmacon (Lafayette, CO, USA) siRNA pools targeting transcripts of the human MAP3K1 (L-003575-00-0005) and non-target control siRNA pool (D-001810-10-20) were used to knock down MAP3K1 in DLD-1 and HEK293T cells. siRNA was delivered into DLD-1 and HEK293T cells using the siLentFect Lipid Reagent (Bio-Rad, Hercules, CA, USA). In total, 15 nM siRNA were used to transfect $1x10^5$ cells, and protein levels were examined by Western blot analysis 48 or 96 h after transfection.

Transient transfection and reporter gene assays

In the siRNA knockdown experiments, HEK293T and DLD-1 cells were seeded at a density of $1x10^5$ cells/24-well plate and siRNA targeting the human MAP3K1 gene or control non-targeting siRNA was delivered the following day. After 24 or 72 h, cells were transfected with TCF luciferase-reporter constructs using polyethylenimine (Polysciences, Warrington, PA USA).

In TOPFlash assays examining the effect of rMap3k1 WT and point mutants in HEK293T and HCT116 cells, cells were plated in 40-50% density in 24-wells plate and 2.5 ng/well rMap3k1 constructs or control DNA were transfected together with reporter plasmid and internal control plasmid few hours after seeding.

The ratio of reporter plasmid (TOP10 or FOP10) with internal control *Renilla* plasmid was kept constant as 10:1. Control conditioned medium or Wnt3A conditioned medium were added to the HEK293T cells 16 h post-transfection. The cells were lysed after 24 h using luciferase lysis buffer (Promega Corp., Madison, WI, USA), and luciferase activities were measured using the Dual-Luciferase reporter assay system. Transfection efficiency was normalized using the co-transfected *Renilla* luciferase activity as an internal control. All transfection experiments were performed in either duplicates or triplicates.

Quantitative RT-PCR (qRT-PCR)

qRT-PCR experiments were analyzed in an iCycler iQ real-time PCR detection system(Bio-Rad, Hercules, CA, USA) using iQ Sybergreen Supermix (Bio-Rad). Values were normalized using β-actin as control. The following primer pairs were used for examining the effect of MAP3K1 depletion on Wnt target gene expression: human TCF7 forward primer: 5'GGT TCA CAG GCC TCT GCA GAC3'; human TCF7 reverse primer: 5'CTT GTG TCT TCA GGT TGC GG3'; human Axin2 forward primer: 5'CAG CAG AGG GAC AGG AAT C3'; human Axin2 reverse primer: 5'CAG TTT CTT TGG CTC TTT GTG3'; human BPTF forward primer: 5'GAT AGC AGC AAC ATG GCA GAG3'; human BPTF reverse primer: 5'AGG CTG GAT GGA GGT AGT GG3'; human ZCCHC12 forward primer: 5'ATT CCA GGG CTC AGT TTC CTT CCA3'; human ZCCHC12 reverse primer: 5'TGG CCT TCC TCA CCA CAA TAC GAA3'; human ASCL2 forward primer: 5'TCT ACA CAT TAA CTT GAG C3'; human ASCL2 reverse primer: 5'AGG TCA TCT TTA TTA CGC3'; human β-actin forward primer: 5'CGC AAA GAC CTG TAC GCC AAC3'; human β-actin

reverse primer: 5'GAG CCG CCG ATC CAC ACG3'; human MAP3K1 forward primer: 5'CGT CCA GAG GAA CGA ATG ATC3'; human MAP3K1 reverse primer: 5'GAC CTC TCC TGG AGA CTC AGC3'.

Site-directed mutagenesis

Rattus Map3k1 mutants rMap3k1 R1349A/D1350A, rMap3k1 D1369A/F1370A, rMap3k1 C433A and rMap3k1 C478A were generated using the Quick Change Site-Direted Mutagenesis kit (Stratagene, Cedar Creek, TX, USA). Wild type full-length Myc tagged rMap3k1 expression vector (a kind gift from Dr. Melanie H. Cobb) was used as a template for PCR based mutagenesis using the the following primers: rMap3k1 R1349A/D1350A forward primer: 5'GAG AAC CAG ATC ATT CAC GCA GCC GTC AAA GGG GCC AAT CTG C3'; rMap3k1 R1349A/D1350A reverse primer: 5'GCA GAT TGG CCC CTT TGA CGG CTG CGT GAA TGA TCT GGT TCT C3' ; rMap3k1 D1369A/F1370A forward primer: 5'GTC AGC GGC TGA GAA TTG CAG CCG CCG GTG CTG CCG CCA GGT TGG3'; rMap3k1 D1369A/F1370A reverse primer 5'CCA ACC TGG CGG CAG CAC CGG CGG CTG CAA TTC TCA GCC GCT GAC3'; rMap3k1 C433A forward primer: 5'GGA TGA AGA GGA GCA GAT GGC TCC CAT CTG CTT GTT GGG C3' ; rMap3k1 C433A reverse primer 5'GCC CAA CAA GCA GAT GGG AGC CAT CTG CTC CTC TTC ATC C3' ; rMap3k1 C478A forward primer: 5'GAA ATA GAG AAC CGT TAA TAG CTC CCC TTT GTA GAT CTA AG3' ; rMap3k1 C478A reverse primer 5'CTT AGA TCT ACA AAG GGG AGC TAT TAA CGG TTC TCT ATT TC3'.

Statistical analysis

Comparison of differences between TOPFlash activities or qRT-PCR expression was performed using the two-tailed Student *t*-test (where equal variance between groups was assumed). A *p*-value<0.05 was considered as statistically significant.

Acknowledgements

We thank Dr. Melanie H. Cobb from University of Texas Southwestrn Medical Center for sending us the rMap3k1 expression constructs. T. Mahmoudi is funded by the Marie Curie Incoming International Fellowship (MC IIF 221108). V. Li is supported by the Croucher Foundation Fellowship. P. Hatzis was supported by a Human Frontier Fellowship.

Chapter 4

Wnt activation blocks the Axin1-β-catenin destruction complex by saturation with phosphorylated β-catenin

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Submitted

Ideas are like the stars – we never reach them, but like mariners, we chart our course by them.

-C. Schurz-

Abstract

Degradation of cytosolic β-catenin by the APC destruction complex is the key regulatory step of canonical Wnt signalling. However, how the endogenous β-catenin destruction complex is functionally inhibited upon Wnt activation is not understood. Taking advantage of a specific antibody against Axin1, the least abundant component of the destruction complex, we examined the composition of the endogenous Axin1 complex in APC-mutant and in β-catenin-mutant colorectal cancer (CRC) cell lines. We found that despite the constitutively activated Wnt signalling in these CRCs, the Axin1-β-catenin destruction complex is correctly assembled in these cells. In HEK293T cells which harbor a normal Wnt pathway, Wnt stimulation results in increased binding of phosphorylated β-catenin to the Axin1 complex. In disagreement with the current dogma, β-catenin is efficiently phosphorylated by GSK3 upon Wnt stimulation. Yet the next step, the ubiquitination of β-catenin, is blocked due to the dissociation of the ubiquitin E3 ligase, β-TrCP from the complex. Thus, inactivation of the destruction complex by Wnt stimulation is a consequence of its saturation by phosphorylated β-catenin. Our results suggest that the destruction complex is required not only for phosphorylation but also ubiquitination of β-catenin. As Wnt stimulation inhibits interaction with β-TrCP, the Axin1-β-catenin destruction complex becomes saturated with the phosphorylated, non-ubiquitinated form of β-catenin and consequently inactivated.

Introduction

The canonical Wnt (Wnt/β-catenin) signaling pathway is one of the most evolutionarily conserved signal transduction pathways in the animal kingdom. It controls many biological processes including cell fate determination, cell proliferation, differentiation and motility, and stem cell renewal and maintenance[2]. Deregulation of this pathway has been observed in different types of cancers, and underlies multiple hereditary syndromes[2, 3]. The key regulatory step of canonical Wnt signal transduction is the phosphorylation and consequent degradation of its downstream effector protein, β-catenin, by its dedicated cytoplasmic destruction complex. The destruction complex consists of the scaffold protein axis inhibition protein (Axin), and three other key core components, Adenomatous Polyposis Coli (APC) Glycogen Synthase Kinase-3 alpha/beta (GSK-3) and Casein Kinase-1 alpha (CKIα). Activating mutations in components of the β-catenin destruction complex, such as APC (mutated in over 80% of colon cancers), AXIN or β-catenin, have been shown to result in cancer[120, 138, 169, 185, 202], most notably of the colon.

In resting cells, despite the gene being continuously transcribed, vanishingly low

levels of free β-catenin protein are present in the cytosol. This pool of β-catenin is constitutively phosphorylated by CKIα at Ser45, priming GSK3 phosphorylation of β-catenin on the more N-terminal Thr41, Ser37 and Ser33 residues[8]. Phosphorylated β-catenin is then recognized and ubiquitinated by the F-box-containing protein β-TrCP ubiquitin E3 ligase and is subsequently degraded by the proteasome[9, 10].

During this degradation process, Axin1 has been reported to be the concentration- and rate-limiting factor of the β-catenin destruction complex and is therefore functionally critical to the complex[49]. Axin1 directly interacts with all core components of the destruction complex, β-catenin, APC, CKIα and GSK3, and is thus considered to be the central scaffold of the complex[8, 203-205]. As the least abundant component, Axin1 can regulate its rapid assembly and disassembly. For this reason, it has been proposed that degradation of Axin1 in Wnt activated cells may be the direct cause of β-catenin stabilization[15, 19]. Although multiple roles have been proposed for the genetically essential APC protein, there is no consensus as to its key activity.

Upon Wnt stimulation, Wnt ligands bind to the cognate frizzled (FZ/FZD) and low-density-lipoprotein-related protein 5/6 (LRP5/6) co-receptor complex to activate the canonical Wnt signalling pathway. Through an unresolved mechanism, activation of Frizzled and LRP5/6 receptors by Wnt ligands disrupts or functionally inactivates the β-catenin destruction complex leading to the stabilization, cytosolic accumulation and subsequent nuclear translocation of β-catenin. In the nucleus, β-catenin complexes with TCF/LEF transcription factors to bind to Wnt target genes and activate the Wnt transcriptional program[84, 206].

Models proposed to describe the events following Wnt receptor-ligand activation include inhibition of GSK3 activity or release of GSK3 from the Axin destruction complex, sequestration of Axin1/GSK3 and the cytosolic Wnt pathway component Dishevelled at the membrane via interaction with the membrane receptor LRP5/6 resulting in physical separation from APC and β-catenin, and degradation of Axin1 protein levels resulting in dissociation of the destruction complex[3]. All previously proposed models assume a physical dissociation or disruption of the β-catenin destruction complex, leading to stabilization of β-catenin. However, biochemical studies probing the dynamic states of the endogenous β-catenin destruction complex in response to Wnt activation are lacking.

Results and Discussion

Axin1-β-catenin destruction complex remains compositionally intact despite constitutive Wnt signalling.

To examine the mechanism by which Wnt activation functionally inhibits the endogenous β-catenin destruction complex, we used a monoclonal antibody we previously generated against Axin1[187], to probe the composition of the Axin1-

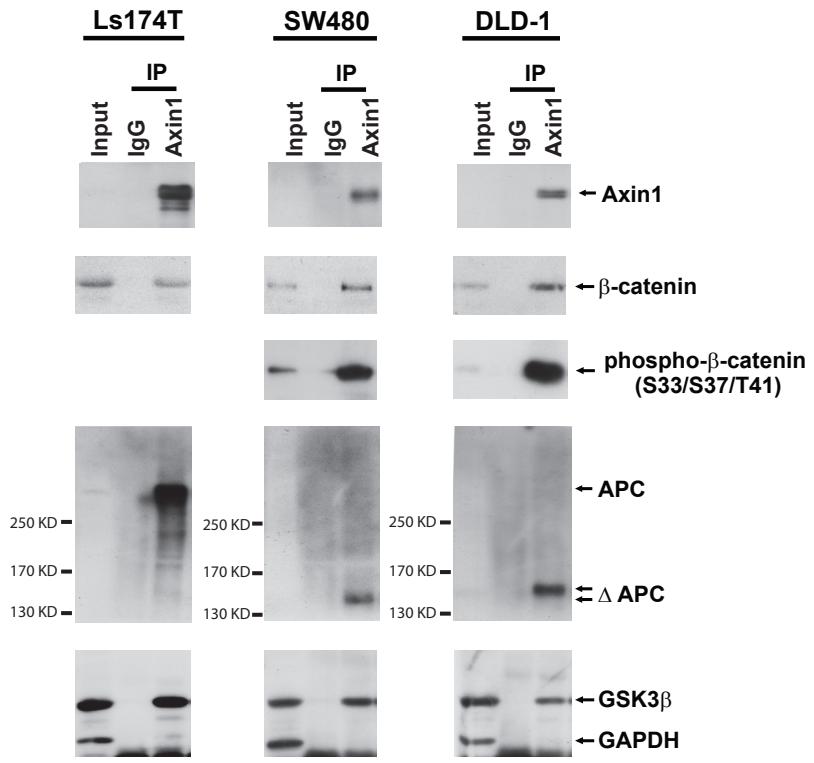

Figure1. The Axin1-β-catenin destruction complex remains compositionally intact despite constitutive Wnt signaling in various colorectal cancer cell lines.

Cell lysates from colorectal cancer cell lines Ls174T, SW480 and DLD-1, as indicated, were immunoprecipitated with a specific antibody directed against endogenous Axin1 and IgG as a negative control. The immunoprecipitated complex was analyzed by Western blotting using antibodies directed against Axin1, β-catenin, phosphorylated β-catenin, APC, GSK3β, and as a negative control, GAPDH. APC indicates the truncated form of APC expressed in SW480 (150 KD) and DLD-1 (140 KD) cells.

β-catenin destruction complex in Wnt active versus non-active states. We first examined the Axin1 complex in various colorectal cancer cell lines (CRC) in which the Wnt pathway is constitutively "on" by mutational activation. We immunoprecipitated Axin1 from Ls174T (mutant, active β-catenin; wt APC), and from DLD-1 and SW480, which express inactive truncated APC and wild type β-catenin. We then probed the Axin1-immunoprecipitated complexes for the presence of various components of the Axin1-β-catenin destruction complex by western blotting as indicated (Figure 1). To our surprise, in all CRCs examined, the Axin1-β-catenin destruction complex contained the core components, GSK3β, APC and β-catenin, despite the constitutive activity of the Wnt pathway, demonstrating that the destruction complex is not disrupted as a consequence of abnormal Wnt activation. More

interestingly, the Axin1 immunoprecipitated β-catenin destruction complex contained not only full length wild-type APC in the Ls174T CRCs, but also the mutationally truncated APC, which lacks the Axin binding SAMP motifs and several but not all β-catenin 20-aa repeats, in both SW480 and DLD-1 CRCs. The observation that the truncated form of APC still interacts with Axin1 complexes prompted us to probe whether GSK3 kinase might still phospohorylate β-catenin within the Axin1-β-catenin destruction complex in these APC-mutated CRCs. Indeed, there is a pool of Ser33/Ser37/Thr41 phosphorylated β-catenin co-immunoprecipitating with Axin1 in DLD-1 and SW480 CRCs (Figure 1). In Ls174T cells, β-catenin is mutated at Ser45 and cannot be phosphorylated by CKIα and subsequently by GSK3. β-catenin is thus not recognized by the antibody in these cells. The presence of the truncated form of APC together with GSK3 phosphorylated β-catenin in the dedicated destruction complex indicated that disruption of the interaction between APC and β-catenin is not the mechanism of tumorigenesis by the APC mutations in these colorectal cancers. These observations also raised the possibility that the Axin1-β-catenin destruction complex may be saturated by phosphorylated β-catenin in those CRCs due to

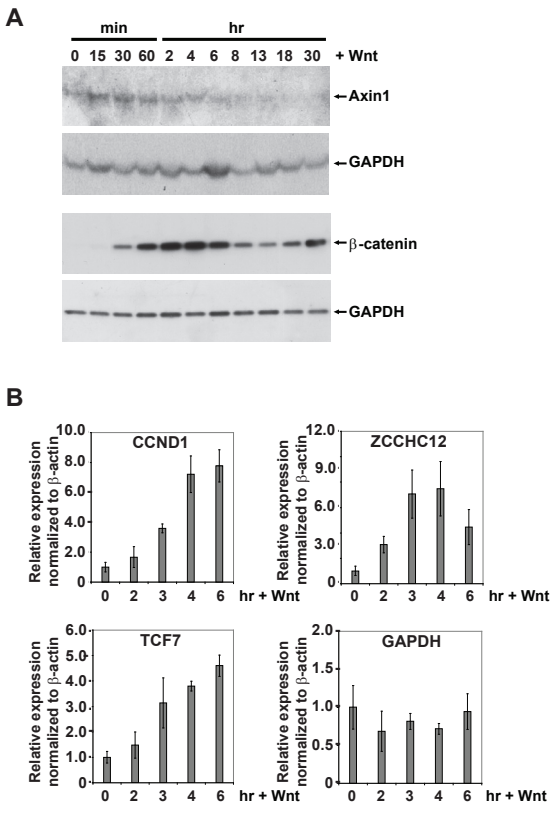

Figure 2. Wnt-induced stabilization of β-catenin occurs prior to and independently of Axin1 degredation.

HEK293T cells were stimulated with Wnt according to the indicated time points. **(A)** Cytosolic or whole cells lysates were generated for detection of expression of the indicated proteins. Top panel, significant degradation of Axin1 occurs much later at 6 hours post Wnt induction. Third panel, cytosolic β-catenin protein levels begin to accumulate 30 minutes after Wnt stimulation, peaking at approximately 2 hours. Second and last panel, GAPDH protein levels were used as loading control respectively. **(B)** Wnt induced activation of target genes, TCF7, ZCCHC12, CCND1 and as control GAPDH, was examined in HEK293T cells by quantitative RT-PCR at 0, 2, 3, 4 and 6 hours post Wnt stimulation. Time-course expression data is presented as fold induction over β-actin control in triplicate and is representative of at least two independent experiments. The error bars represent ± standard deviation.

improper function of APC.

Degradation of Axin1 is not the direct cause of β-catenin stabilization and Wnt-induced transcription upon Wnt stimulation.

To gain better understanding of the dynamics of the destruction complex in its different active states, we then analyzed the Wnt-inducible HEK293T system which harbours an intact Wnt pathway. Because Axin1 as the critical and scaffold subunit of the complex had been reported to be down-regulated at the protein level upon Wnt stimulation, we performed a Wnt stimulation time course experiment. We stimulated HEK293T cells with Wnt3A conditioned medium or control conditioned medium and monitored the correlation between degradation of Axin1 protein and accumulation of cytosolic β-catenin by western blot analysis (Figure 2A, third panel). While cytosolic accumulation of β-catenin was detectable as early as 30 minutes following Wnt treatment, we first observed a significant decrease in endogenous Axin1 protein levels at 6 hours post Wnt stimulation (Figure 2A, top panel).

Previously, we have reported detection of endogenous Wnt target gene expression by microarray analysis in HEK293T cells detectable as early as 4 hours post Wnt stimulation181. We examined the expression of the well-characterized endogenous Wnt target genes CCND1191, TCF7192 and ZCCHC12181 by quantitative RT-PCR in a time course response to Wnt treatment (Figure 2B). Importantly, a significant increase in mRNA expression levels for all endogenous target genes was already detected at 3 hours post Wnt stimulation (Figure 2B). Thus, several hours after efficient cytosolic accumulation of β-catenin and a detectable transcriptional response of endogenous Wnt target genes, Axin1 is still stably expressed in the presence of Wnt stimulation. Therefore, we excluded degradation of Axin1 as a possible mechanism for the functional inactivation of the destruction complex.

Accumulation of phosphorylated β-catenin in the endogenous Axin1-β-catenin destruction complex in response to Wnt activation.

Using the Wnt-inducible HEK293T system, we then asked how the interaction between Axin1 and other components of the Wnt cascade is influenced by Wnt stimulation. We immunoprecipitated the Axin1 complex and as a negative control IgG from HEK293T cells either untreated or treated for 0.5, 2 and 4 hours with Wnt conditioned medium and probed for the presence of various known components of the Wnt cascade by western blotting as indicated (Figure 3). As expected, in the absent of Wnt, Axin1 interacts with GSK3 (last panel) and APC (2nd panel) but not LRP6 (4th panel) as previously reported by other groups. In addition, as had been reported, phosphorylated LRP6 was only detected in the Axin1 complex in the presence of Wnt stimulation (3rd panel)[15, 19, 37]. Dishevelled 3 also co-immunoprecipitated with Axin1 in both the presence and absence of Wnt (5th panel). Importantly, in contrast to previous reports, we did not find a significant decrease in binding of Axin1 to either APC (2nd panel) or GSK3β (last panel) in response to Wnt stimulation. This observation is

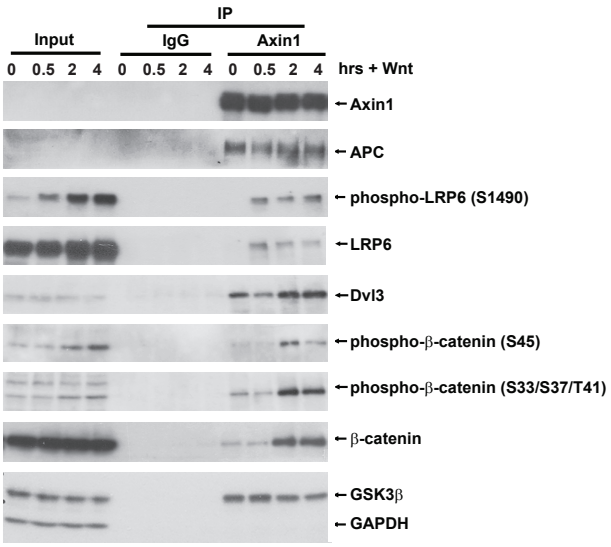

Figure 3. Accummulation of phosphorylated β-catenin in Axin1-β-catenin destruction complex upon Wnt activation. HEK293T cells were exposed to Wnt3A conditioned medium or control conditioned medium at different time points as indicated. Stimulated lysates were subjected to immunoprecipitation using an Axin1-specific antibody and IgG as control and subjected to SDS-PAGE followed by western blot analysis using the indicated antibodies.

inconsistent with models in which dissociation of the destruction complex, or modulation of Axin1 binding to GSK3 or APC mediate functional inactivation of the destruction complex and stabilization of β-catenin.

Interestingly, the amount of β-catenin co-immunoprecipitating with Axin1 was hardly detectable in the absent of Wnt stimulation, highlighting the dynamic nature of the interaction between β-catenin and Axin1 within a functional β-catenin destruction complex (Figure 3, 8th panel). Moreover, we found a significant Wnt-induced increase in β-catenin immunoprecipitated with Axin1. We further analyzed the β-catenin pool that interacted with Axin1 by western blotting using antibodies that specifically recognize Ser45 phosphorylated β-catenin or S33/S37/T41 phosphorylated β-catenin (Figure 3, 6th and 7th panel). We thus documented an increase in the presence of phosphorylated β-catenin in the Wnt stimulated Axin1 complex. Thus, as in the CRCs, the critical CK1α and GSK3β kinases are active and properly functioning within the destruction complex.

Wnt stimulation abrogates ubiquitination of phorphorylated β-catenin by disrupting the interaction of β-TrCP with the Axin1-β-catenin destruction complex.

To determine whether the β-catenin pool bound to Axin1 is still subject to normal ubiquitination and degradation, we combined the Wnt activation with the addition of the proteasome inhibitor MG132. This compound blocks the proteasome degradation function but not the ubiquitination of the E3 ubiquitin ligase substrate. Thus, if phosphorylated β-catenin is still capable of undergoing ubiquitination, it would be possible to detect the ubiquitinated phosphorylated β-catenin as an upper shift smear by western blot analysis. As shown in Figure 4A, there is a much stronger

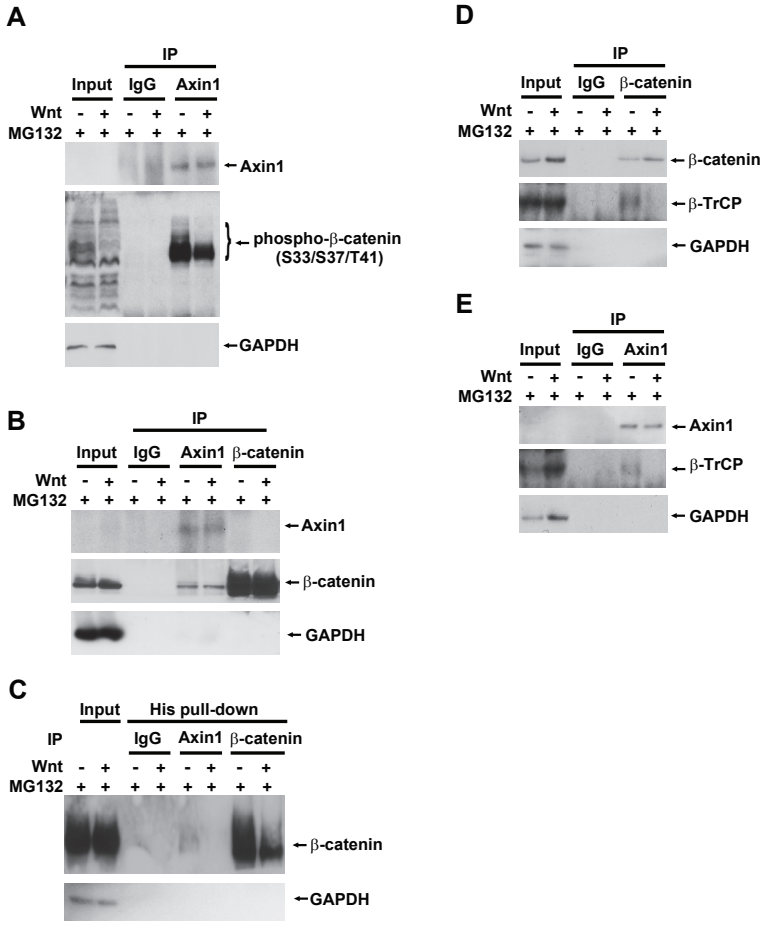

Figure 4. Wnt stimulation abrogates ubiquitination of phorphorylated β-catenin by disrupting the interaction of β-TrCP with the Axin1-β-catenin destruction destruction complex.

(A) HEK293T cells were treated with proteasome inhibitor, MG132 together with Wnt3A conditioned medium or control conditioned medium for 4 hours. Treated cells were collected, lysed and used for Axin1 immunoprecipitation, followed by SDS-PAGE and western blot analysis with anti-phosphor-β-catenin(Ser33/Ser37/T41 antibody. **(B, C)** HEK293T cells were transfected with His-tagged ubiquitin. After 12 hours, cells were treated with proteasome inhibitor, MG132 together with Wnt3A conditioned medium or control conditioned medium for 4 hours. Cell lysates were then used for immunoprecipitation with antibodies specific for Axin1, β-catenin, or control IgG as indicated **(B)**. Axin1 and β-catenin complexes were eluted and used in His-pull down assays. Samples were separated by SDS-PAGE and analyzed by western blotting with anti β-catenin antibody **(C)**. **(D, E)** Wnt treatment disrupts interaction of β-TrCP with endogenous Axin1-β-catenin destruction complex. HEK293T cells treated with MG132 together with Wnt3A or control conditioned medium for 4 hours were lysed and immunoprecipitated with an antibody against β-catenin **(D)** or Axin1 **(E)**. Immunoprecipitated complexes were analyzed by western blotting using antibodies directed against β-TrCP, β-catenin, Axin1 and GAPDH as control as indicated.

presence of higher molecular weight phosphorylated β-catenin in MG132 treated cells alone than in Wnt and MG132 treated cells that co-immunoprecipitated with Axin1(compare 1st -2nd lane for input and lane 5th-6th for Axin1 IP). This result suggested that Wnt stimulation interferes with ubiquitination of phosphorylated inactive β-catenin within the destruction complex.

To further examine the ubiquitination state of phospho-β-catenin within the destruction complex, we overexpressed His-tagged ubiquitin in HEK293T cells for at least 12 hours before treatment with MG132 and Wnt. Antibodies specific for Axin1, β-catenin, or control isotype-matched IgG were used to immunoprecipitate the specific complexes (Figure 4B). The immunoprecipitated complexes were washed and eluted from the beads followed by a His-pull down assay. The final His-pull down samples were analyzed by western blotting using an antibody specific for β-catenin (Figure 4C). We found significantly less ubiquitinated β-catenin in Wnt treated cells when comparing β-catenin immunoprecipitated complexes from Wnt treated and untreated samples (Figure 4C, lane7 and 8). We also found ubiquitinated β-catenin in the Axin1-immunoprecipitated complexes specifically from HEK293T cells treated with only MG132, and not MG132 together with Wnt (Figure 4C, lane 5 and 6). Our data demonstrated that Wnt stimulation interferes with the ubiquitination step of phosphorylated inactive β-catenin within the destruction complex. These observations further confirm that Wnt stimulation does not inhibit the β-catenin destruction through inhibition of GSK3 kinase activity or GSK3 dissociation from the complex and suggest that the β-catenin destruction complex is required not only for β-catenin phosphorylation but also its ubiquitination and possibly degradation.

We then examined β-TrCP, reported to be the E3 ubiquitin ligase which ubiquitinates β-catenin within the Axin1 destruction complex leading to its proteasomal degradation. We specifically asked whether Wnt stimulation has an affect on the interaction of β-TrCP with the destruction complex. As shown in Figure 4D, while β-TrCP is co-immunoprecipitated with both β-catenin (Figure 4D lane 5 and 6) and Axin1 (Figure 4E lane 5 and 6) in the presence of the proteasome inhibitor MG132 in non-Wnt-treated cells, Wnt treatment abrogated the interaction between β-TrCP and either β-catenin (Figure 4D) or Axin1 (Figure 4E) complexes.

Our data demonstrates that Wnt treatment disrupts the interaction between β-TrCP and the Axin1-β-catenin destruction complex, thus leading to a block in ubiquitination of phosphorylated inactive β-catenin within the complex. Our observations provide the possibility that upon Wnt stimulation, phosphorylated β-catenin is no longer ubiquintinated and degraded, and therefore remains "stuck", thereby saturating and functionally inactivating the destruction complex. On the basis of our data and considering Axin1 as a limiting factor in the destruction complex, we hypothesize that under normal physiological conditions, in response to Wnt signalling, association of phosphorylated β-catenin with Axin1 and other components in the complex saturates the limited β-catenin destruction complex

in the cells. Therefore newly synthesized β-catenin is able to accumulate in the cytoplasm and translocate to the nucleus to activate the Wnt transcriptional program.

Materials and Methods

Antibodies

APC (Milipore), Dishevelled3 (Santa Cruz), GSK3β (cell signaling), phosphor-LRP6 (S1490, cell signaling), LRP6 (cell signaling), phosphor-β-catenin S45 (cell signaling), phosphor-β-catenin S33/S37/T41 (cell signaling), β-catenin (BD transduction), β-TrCP (ITK Diagonistcs BV), and GAPDH (Abcam) were used in immunoprecipitations or western blot analysis.

Cell culture

HEK293T, DLD-1, SW480 and Ls174T cells were maintained in RPMI 1640 (Invitrogen) supplemented with 5% fetal calf serum (FCS). Wnt3A producing-L-cells (kind gift from R. Nusse) or control-L-cells were cultured according to the manufacturer's instructions (ATCC).

Fractionation

HEK293T cells were stimulated with Wnt3A conditioned medium or control conditioned medium as indicated. Cells were washed and collected in ice cold PBS. Cell pellets were then re-suspended in hypotonic lysis buffer (10mM KCl, 10mM Tris pH7.5 and 2mM EDTA) containing protease inhibitor and phosphatase inhibitor cocktail tablets. Cell suspensions were incubate on ice for 30 minutes and dounced multiple times. Lysis of cells was controlled by checking under the microscope. Nuclear proteins including the unlysed cells were pelleted by spinning at 2000rpm 2 minutes, 4°C centrifugation. The supernatant which contained both cytoplasm and membrane proteins was then centrifuged at 14,000rpm for 30 minutes at 4°C. The supernatant was collected and analyzed further by SDS-PAGE followed by western blotting.

Immunoprecipitation

At 80% confluency, HEK293T cells were treated with either Wnt3A or control conditioned medium in the absence or presence of 4mg/ml MG132 as indicated. HEK293T, Ls174T, DLD-1 and SW480 cells were washed and collected with cold PBS, lysed in cold lysis buffer containing 150mM NaCl, 30mM Tris (pH 7.5), 1mM EDTA, 1% TritonX-100, 10% Glycerol, 0.1mM PMSF, 0.5mM DTT, protease inhibitor cocktail tablets (EDTA-free)(Roche) and phosphatase inhibitor cocktail tablets (Roche). After clarification by centrifugation (14,000rpm for 30 minutes at 4°C), the cellular lysates were pre-cleared with IgG-agarose beads (Sigma) for at least 6 hours at 4°C. Immunoprecipitation of endogenous complexes was carried out by incubating the cellular lysates with anti-Axin1 antibody, mouse IgG, or β-catenin antibody (BD transductions) immobilized on Protein G PLUS-Agarose beads (Santa Cruz biotechnology) at 4°C overnight. Immunocomplexes were washed with cold lysis buffer six times, resuspended in SDS sample buffer, and subjected to SDS-PAGE and western blot

analysis.

His-pulldown assay

HEK293T cells were transfected with a His-ubiquitin expression construct (kind gift from Maurice, M. M.) for at least 12 hours before treatment with Wnt3A or control conditioned medium for 4 hours. Lysates were immunoprecipitated using antibodies directed against Axin1, β-catenin or control IgG. The immunoprecipitated complexes were washed three times with cold PBS and eluted in buffer A (6 M Guanidinium-HCl+ 0.1 M Na_2HPO_4 + 0.1 M NaH_2PO_4 + 0.01 M pH8.0 Tris/HCl + 20 mM Imidazole + 10 mM β-mercaptoethanol). The eluted supernatants were incubated with buffer A pre-washed Ni^{+2}-NTA agarose beads for 3 hours at room temperature (R.T.). After incubation and centrifugation (5000rpm at 30 seconds at R.T.), the pellets were washed with buffer B-E follow by elution in buffer I at R.T. as previously described[207]. The pull-down samples were subjected to SDS-PAGE for western blot analysis with β-catenin antibody (BD transduction).

Addendum Chapter 5

Potential new players in and mode of regulation of the β-catenin destruction complex in the Canonical Wnt pathway

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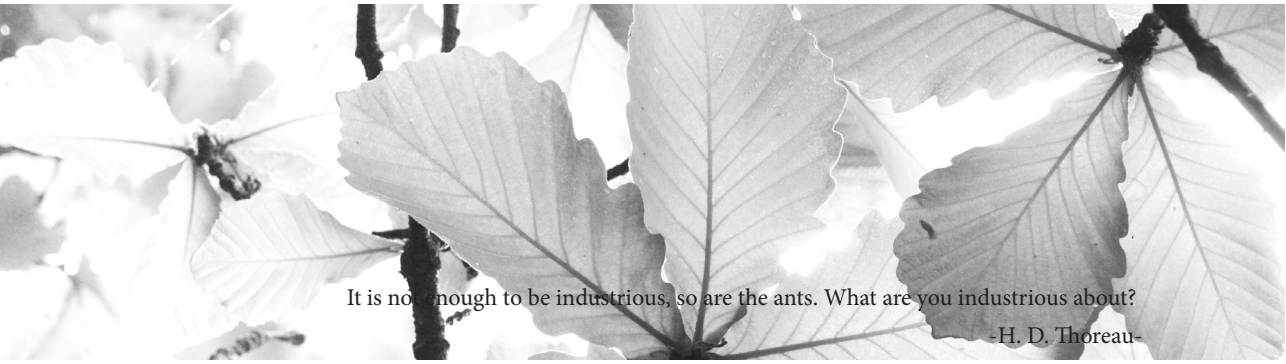

Abstract

In canonical Wnt signalling, Axis inhibition protein (Axin) functions as a negative regulator and scaffold protein within the β-catenin destruction complex, and is therefore critically important for proper regulation of Wnt signalling. We used an immunoprecipitation coupled to proteomics approach using previously generated Axin-specific antibodies to study the Wnt-dependent dynamic change in interaction between Axin and its previously known interactors, and to identify potentially novel interactors of Axin in Wnt-responsive HEK293T cells and Wnt activated Ls174T colorectal cancer cells. Using Mass Spectrometry, we also probed for the presence of Wnt-dependent dynamic changes in phosphorylation of Axin itself and its binding partners. Compare to relatively equal amount of Axin1 peptides we identified in MASS SPEC, we found no obvious change in the amount of either APC or GSK3 peptides co-immunoprecipitating with Axin1 in response to Wnt treatment. However, we found an increase in β-catenin, CKI alpha and WTX peptides recovered and identified in the Axin1 complex from Wnt-stimulated lysates. These results confirmed that the composition of the β-catenin destruction complex remains intact after Wnt stimulation, and contradicts models, which propose a Wnt-induced dissociation of the complex. In addition, we identified several phosphorylation sites within Axin1, APC and WTX which may potentially be important for function and regulation of the proteins. Moreover, several interesting and novel potential binding candidates were identified, including MAP3K1, RanBP2, DLG-1, CASK1,

Introduction

The key regulatory step of the canonical Wnt signalling cascade is the degradation of the downstream effector cytoplasmic protein, β-catenin by the β-catenin destruction complex. The dedicated destruction complex is mainly composed by scaffold protein axis inhibitor protein (Axin), adenomatous polyposis coli (APC), glycogen synthase kinase 3alpha/beta (GSK3α/β), casein kinase 1alpha (CKIα) and β-catenin[2]. In the resting cells, β-catenin is sequentially phosphorylated by CKIα and GSK3 within the dedicated complex. Phopshorylated β-catenin is ubiquitinated and degraded by proteasome. Thus eliminating the cytoplasmic β-catenin protein level and prevents its nuclear translocation[8-10]. In the Wnt stimulating cells, through unclear mechanisms, activation of Wnt receptors, Frizzled and low-density-lipoprotein-related protein 5/6 (LRP5/6) leads to interruption of the β-catenin destruction complex activity. Therefore, stabilizing cytosolic β-catenin and subsequently its association with Tcf/ Lef transcription factors in the nucleus and activating the Wnt target genes expression[2, 84]. Noteworthy, although the biochemical mechanism behind remain

undefined, R-spondin protein family exhibits synergy with Wnt and its receptors[32-35] and shows genetic interaction with LRP6 during embryogenesis[36].
Within the dedicated destruction complex, Axin serves as a scaffold thereby crucial for Wnt/β-catenin signal transduction. There are two Axin homologues in metazoan, Axin1 and Axin2 which is also named Conductin or Axil. Axin1 was originally identified as an inhibitor of the Wnt pathway by characterization of the mouse *fused* locus product. The disruption resulted in duplication of the axis and embryonic lethality[47, 48]. Later studies showed that, mutations of Axin1 have been associated with different tumors and cancers[134-137, 208, 209] Axin2/Conductin/Axil, which is related to Axin1, was cloned by virtue of interacting with β-catenin[50, 51]. Mutation of human Axin2 gene leads to familial tooth agenesis and predisposition to colorectal cancer[210]. All Axin proteins contain the highly conserved RGS (Regulator of G protein Signalling) and DIX (Dishevelled and axin binding domain) domains on their N-terminal and C-terminal domains respectively[211]. The RGS domain is essential for Axin to interact with APC[50, 204] and the DIX domain is believed to be required for the homo- or hector-dimer formation of proteins that contain DIX domains, for example as is the interaction between Dvl and Axin1/2[186, 212] .
Both Axin1 and Axin2 have been reported to directly interact with not only APC, Dvl and β-catenin but also the kinases in the destruction complex, GSK3 and CKIα[211]. Previous transgenic mice studies indicated that Axin1 and Axin2 could function equally *in vivo*[213]. Although both are thought to function as a negative regulator in Wnt signal transduction pathway, Axin1 and Axin2 have a very different expression pattern. Axin1 is ubiquitously expressed in almost all tissues from early embryonic development through adult state[48] and the expression level is believed to be much lower than other components of the destruction complex in the cells[49]. Therefore, Axin1 is consider as limiting factor and critical for Wnt signalling regulation. Very different from Axin1, Axin2 is a Wnt inducible target gene of the pathway. Thus expression of Axin2 is regulated by Wnt. As a result, Axin2 expresses in a developmental and tissue specific manner and is believed to act as a negative feedback loop of Wnt signalling[52-54].

We used Axin-specific antibodies[187] to immunoprecipitate endogenous Axin1 or Axin2 protein complexes from Wnt-responsive HEK293T cells and colorectal cancer cells Ls174T which expressed activated point mutated β-catenin, followed by Mass Spectrometry. We found a list of Axin1/Axin2 endogenous binding partners and potential regulatory phosphorylation sites on Axin itself as well as several interactor partners. Moreover, we found that Axin1 protein levels remain stable for hours post Wnt stimulation, while cytosolic β-catenin accumulates very quickly, highlighting inactivation of the destruction complex upon Wnt treatment. More interestingly, we observed a dramatic increase in the amount of β-catenin, CKIα and Wilms tumor gene on the X chromosome (WTX) but no obvious change in GSK3 and APC immunoprecipitating with Axin1. We conclude that the destruction complex is not disrupted following Wnt stimulation and rule out down regulation of Axin1 or

inactivation of GSK3 as the mechanisms for inactivation of the destruction complex upon Wnt stimulation.

Results and Discussion

To gain insight into the mechanism by which Wnt stimulation regulates the function and composition of the β-catenin destruction complex, we selected two well established cell lines, Wnt-responsible HEK293T cells and Wnt-constitutive active Ls174T cells (expressing point mutated oncogenic β-catenin) as our model systems. We first tested our Wnt3A conditional medium and R-spondin protein activity and specificity with TOPFlash assay in HEK293T cells to determine their effectiveness in activating TCF/LEF-driven transcription. As shown in Figure1A, combination of R-spondin plus Wnt 3A induced Wnt driven transcription more dramatically than Wnt3A conditional medium alone, while both could be efficiently blocked by expression of Dkk1, an inhibitor of Wnt receptor LRP5/6 as has been reported by other groups[32-36, 195]. Wnt3A conditional medium and R-spondin protein were used to stimulate HEK293T cells at different time points followed by monitoring the accumulation of cytosolic β-catenin and Axin1 protein expression levels by Western blot analysis. Comparison of Figures 1B and 1C demonstrates that degradation of Axin1 protein levels occurs much latter than inactivation of the destruction complex as shown by accumulation of β-catenin protein levels in the cytoplasm. Therefore, Axin1 protein degradation is not the mechanism for destruction complex inactivity at the early time points after Wnt stimulation. Moreover, cytosolic β-catenin protein levels are detectably increased at 45 minutes and almost saturated at 4 hous upon addition of Wnt3A+R-spondin (Figure 1B). Therefore, we decided to use 30 minutes and 2 hour time points of Wnt3A + R-spondin treatment in order to examine the composition of the Axin1 complex in the presence or absence of Wnt treatment. Taking advantage of Axin-specific antibodies we had previously generated (Supplementary Figure S1 A)[187], we immunopurified the endogenous Axin1 complexes from 0, 30minutes and 2 hours Wnt stimulated HEK293T cells. We also purified both Axin1 and Axin2 complexes from Ls174T CRC. Immuprecipitated complexes were separated by SDS-PAGE followed by Mass Spectrometry analysis (Supplementary Figure S1 B, C).

Changing composition of known and novel Axin binding proteins potentially involved in regulation of the b-catenin destruction complex.

We examined any potential change in composition of known β-catenin destruction complex components as well as novel interactors of Axin in HEK293T cells and their difference with pathological Ls174T cells (Table 1; coverage and score of most of the proteins please refer to Supplementary Figure S2). CRC Ls174T expressing Ser45 mutated β-catenin protein that no longer phosphorylated by CKIα and subsequently

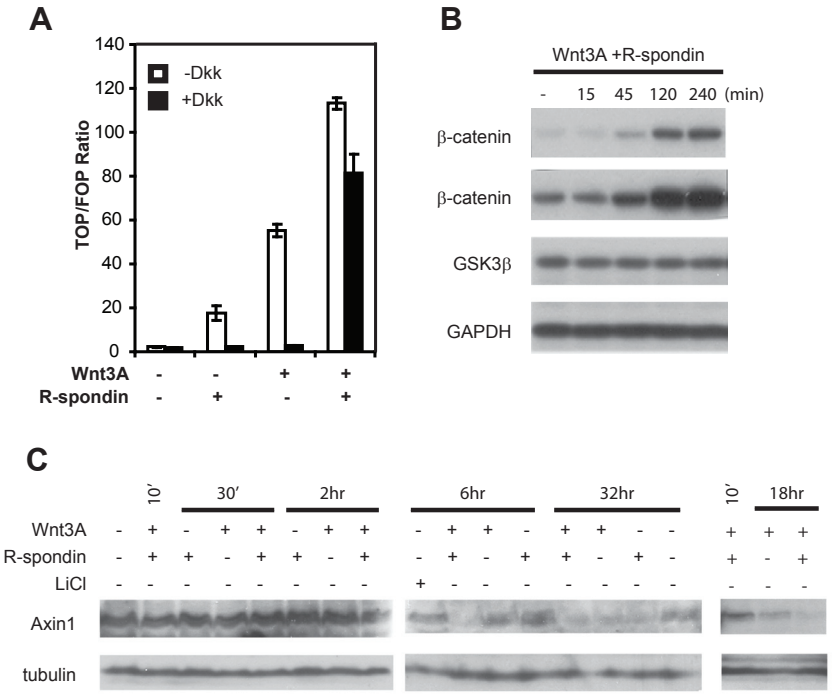

Figure 1. Wnt destabilized Axin1 protein degradation is not a direct mechanism for inactivation of β-catenin destruction complex in HEK293T cells.

(A) Wnt3A conditional medium and R-spondin activity and specificity. HEK293T cells were transfected with Dkk1 or control expression constructs together with TCF reporter gene construct and treat with control/Wnt3A-conditioanl medium and/or R-spondin 24hr after transfection. Treated cells were collected and analyzed at least 12hr after treatment. The error bars represent ± standard deviation. **(B)** Accumulation of cytosolic β-catenin over time upon Wnt3A conditional medium +R-spondin or control conditional medium treatment as indicated. GAPDH and GSK3β were used as the loading control. **(C)** Degradation of Axin1 protein upon Wnt stimulation. Axin1 protein expression level over time upon Wnt treatment as indicated. Tubulin was used as the loading control.

could not degraded by proteasome but could still interacts with APC, GSK3, Axin1 and other components of the complex, therefore Wnt pathway is constitutively activated in the cells. Both Axin1 and Axin2 were found form the complex with β-catenin and other known destruction complex components, like APC, GSK3, CKI and WTX protein in Ls174T cells as expected (Table1). Compare with HEK293T which only induce the Wnt target gene transcription in the present of Wnt ligands, MASS SPEC profile (Table1), it is very interestingly to see some of the known components of the dedicated complexes are only found in the Wnt activated condition. For example, compare to relatively equal amount of the Axin1 peptides we identified in the MASS SPEC, WTX, CKIα and β-catenin protein peptides were hardly detectable in the Axin1 immunoprecipitated complex in resting cells but dramatically increased in response to Wnt stimulation. In contrast to those proteins, whose interaction with Axin1 was positively regulated

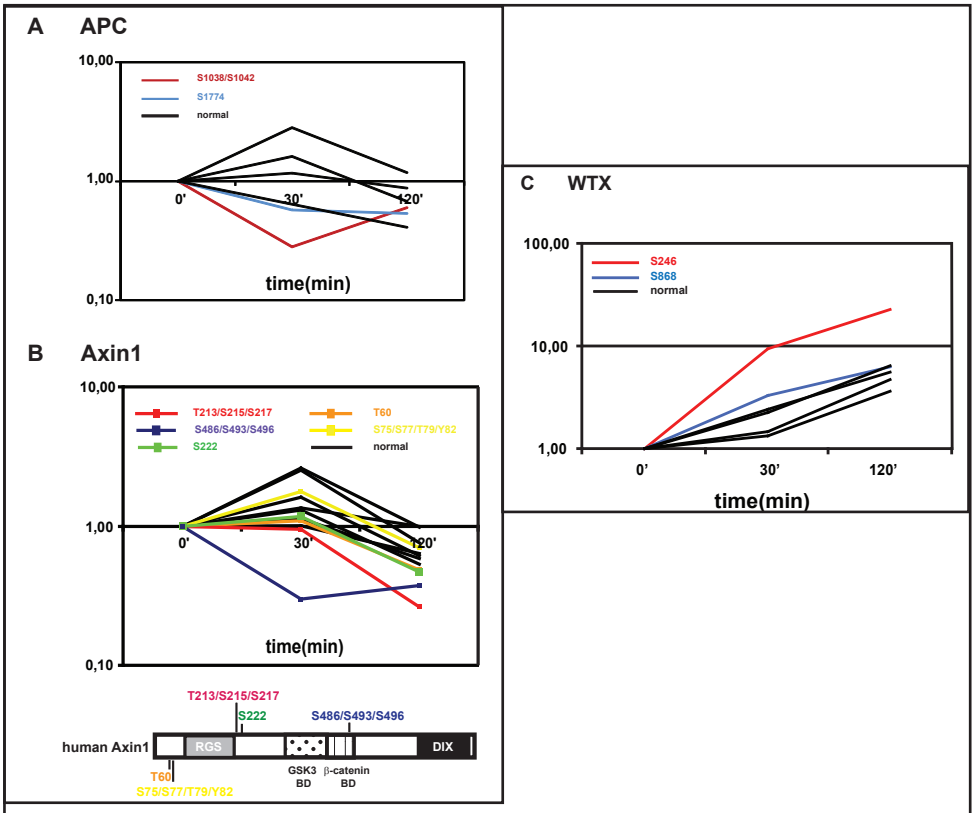

Figure 2. APC, Axin1 and WTX phosphorylation sites identified in Wnt induced HEK293T cells by Mass Spectrometry.

Phosphorylation sites (in colour) identified by MASS SPEC were compared with the normal peptides (in black) of indicated protein as shown in the figure. Y-axis: fold change (log10); X-axis: Wnt induced time (min) (A) APC phosphoryaltion sites. S1038/S1042 (in red) and S17742 (in blue) were identified as two promising phosphorylation sites of APC. Both sites, especially S1038/S1042 was dephosphorylated at 30 minutes post Wnt stimulation. (B) Axin1 phosphorylation sites. Upper panel showing the change of dedicated phosphorylated residue corresponding with Wnt induction. The bottom panel indicates the location of those phosphorylation residues on Axin1 protein. S213/S215/S217 (in red), S222 (in green), S486/S493/S496 (in blue) and T60 (in orange) are dephosphorylated in response to Wnt treatment. S222 residue is only detected in double phosphor-peptide. S75/S77/T79/Y82 phosphor-residue (in yellow) phosphorylation state is considered as no obvious change within 2 hours post Wnt activation. (C) WTX phosphorylation sites. Two novel phosphorylation sites of WTX, S246 (in red) and S868 (in blue) were identified. S246 residue phosphorylation is significantly increased upon Wnt activation.

by Wnt signalling, APC and GSK3 interaction with Axin1 was unaffected upon Wnt stimulation. These observations are very different from what has been proposed by other groups, implicating dissociation of GSK3 and APC from the complex as the mechanism of Wnt mediated inactivation of the β-catenin destruction complex. In contrast, our results indicate that all the core components of the β-catenin

Table 1 Proteins identified from Axin 1/2 HEK293T cells and Ls174T cells MASS SPEC.

Identified Protein	293T cells	Ls174T cells	
	Axin1 IP	Axin1 IP	Axin2 IP
Axin1	+	+	+
Axin2	-	+	+
APC	+	+	+
GSK3α	+	+	-
GSK3β	+	+	+
β-catenin	+	+	+
CKIα	+	+	?
WTX	+	+	+
DLG1	+	+	+
PPP2A	+	-	-
HAX1(HCLS1 associated protein X-1)	+	-	-
RanBP2 (RAN binding protein 2)	+	-	-
USP9X (ubiquitin specific protease 9, X-linked isoform 4)	+	-	-
Dishevelled 2	+	-	-
CKIε	+	-	-
PPP1CA (serine/threonine-protein phosphatase PP1-alpha catalytic subunit)	+	+	-
ZNF217 (zinc finger protein 217)	+	+	-
ZNF318 (zinc finger protein 318)	+	-	-
CDKL5 (uncharacterized protein CDKL5)	+	-	-
FLJ46026 (cDNA FLJ46026 fis,clone SPLEN 2024571)	+	-	-
cDNA FLJ43273 fis, clone KIDNE2005543	+	-	-
PTPN6 (Isoform 3 of Tyrosine-protein phosphatase non-receptor type 6)	+	-	-
RAB2B (Cdna FLJ14824 fis, clone OVARC1000771, moderately similar to RAS-Related protein RAB-2)	+	-	-
PHF6 (Isoform1 of PHD finger protein 6)	+	-	-
YTHDC1 (Isoform 1 of YTH domain-containing protein1)	+	-	-
YTHDC2 (YTH domain containing 2)	+	-	-
ARHGAP21 (Rho-GTPase activating protein 10)	+	+	-
PDK1L1 (Isoform1 of polycystic kidney disease 1-like protein)	+	-	-
ANKRD33 (ankyrin repeat domain 33)	+	-	-
CKIδ	-	+	-
CKIIα	-	+	-
MAP3K1	-	+	?
CASK1 (calcium/calmodulin-dependent serine protein kinase (MAGUK family))	-	+	-
GNB2L1(Lung cancer oncogene 7)	+	+	?
UBE1(ubiquitin-activating enzyme E1)	+	+	-
PSMD2 (26S proteasome non-ATPase regulatory subunit 2)	+	-	+
PSMC6 (26S protease regulatory subunit S10B)	+	-	-
PSMC6 (26S protease regulatory subunit 7)	+	-	-
UBR5 (E3 ubiquitin-protein ligase EDD1)	+	+	-
PGK1 (phosphoglycerate kinase 1)	+	-	+
AKAP8 (A kinase anchor protein 8)	+	-	+
FAM83G hypothetical protein LOC644815	-	-	+
KIAA0515 hypthetical protein LOC84726	-	+	+

Table 1. Proteins that identified in Axin 1/2 HEK293T cells and Ls174T cells MASS SPEC.
Identified proteins name is present in the left column; right bottom columns represent detection of the protein in dedicated Axin immunoprecipitation complexes: "+"= detected; "-"=not detected; "?"=not sure, due to Axin2 immunoprecipitated complexes were not included in that MASS SPEC analysis. Highlighted proteins represent amount of indicated protein peptides was found not obvious change (green), increase (yellow) or decrease (pink) respectively in HEK293T cells Axin1 immunoprecipitation complexes upon Wnt induction.

destruction complex remain intact in the Axin complex upon Wnt stimulation (for more detail please refers to Chapter 4).

In addition to the known interactors of Axin within the destruction complexes, we also found a list of interesting binding partners of Axin in both Ls174T cells and HEK293T cells, such as a MAP kinase, MAP3K1 that we identified in our Axin1 immunoprecipitation from Ls174T cells (Table 1 and Chapter 3). Our further analyses show that, in addition to its important kinase function in Axin/JNK pathway, MAP3K1 ubiquitin E3 ligase function is also critical for its regulation of canonical Wnt signalling (Chapter 3). Another interesting candidate we identified in our list of Axin1 interactors from HEK293T cells was a SUMO ligase, RAN binding protein 2(RanBP2) (Table 1). SUMOylazation of Axin1 on its C-terminal C6 motif (KVEKVD) was reported to be important for its protein stability by protecting Axin from ubiquitination[57]. RanBP2 is the only SUMO ligase we found in our endogenous Axin1 immunoprecipitation under physiological conditions. Interestingly, we found that less RanBP2 co-immunoprecipitated with Axin1 complexes in response to Wnt stimulation (Table 1). This observation suggests that RanBP2 might be a SUMO ligase of Axin1 and potentially play an important role in Axin1 protein stabilization. Further function studies on RanBP2 and other candidates will provide a better understanding of the Axin1-β-catenin destruction complex regulation.

Change is phosphorylation of Axin1, APC and WTX in response to Wnt stimulation.

Phosphorlation of Ser/Thr/Tyr amino acid residues of protein are a common modification that could regulate and effect protein activity, translocation or stability. Using this un-biassed proteomics approach, we identified potential phosphorylation sites of known components ithe destruction complex. APC and Axin1 were previously reported to be dephosphorylated in Wnt activated cells and this dephosphorylation was suggested to leads to their dissociation from GSK3 or/and degradation by proteasome. We identified several known and novel phosphorylation sites of both APC (Figure 2A) and Axin1 (Figure 2B) that were dephosphorylated in response to Wnt stimulation. While the S1038/S1042 phosphorylation site of APC was also previously identified by another group, we found S1774 as a novel APC phosphorylation site. The importance and potential regulatory role of these three phosphorylation sites in APC remains to be determined. Among the Axin1 phosphorylation sites we identified, S486/S493/S496 which is located in the β-catenin binding region was previously described as a GSK3 phoshorylation site and important for β-catenin binding[214]. Aside from the novel S222 residue, which we found to be phosphorylated in Axin1, the other phosphorylation sites we found were previously identified by other groups. However, the role of these residues and their phosphorylation on Axin1 function remains unknown.

Interestingly, the change in phosphorylation of WTX, a protein identified a few years ago as a negative regulator required for proper degradation of β-catenin, occurs in

the opposite direction as that observed for APC and Axin1 in that Wnt stimulation leads to phosphorylation of WTX (Figure 2C). We detected a dramatic increase in phosphorylation of WTX on two novel phosphorylation sites, Ser246 and Ser868 when Wnt3A+ R-spondin was added to HEK293T cells (Figure 2C). As a newly identified component in the complex, how WTX function is regulated in the Wnt pathway is not well understood. Our finding provides a possible important regulatory step of WTX protein by Wnt signalling and a possible mechanism for improper function of the destruction complex.

Materials and Methods

Antibodies

GSK3β (cell signaling), β-catenin (BD transduction), GAPDH (Abcam), tubulin (Santa Cruz).

Cell culture

HEK293T and Ls174T cells were maintained in RPMI 1640 (Invitrogen) supplemented with 5% fetal calf serum (FCS). Wnt3A producing-L-cells (kind gift from R. Nusse) or control-L-cells were cultured according to the manufacturer's instructions (ATCC).

Immunoprecipitation

At 80% confluency, HEK293T cells were treated with conditional medium or 500ng/ml R-spondin as indicated. Ls174T cells and HEK293T cells were washed and collected with cold PBS and then lysed in cold lysis buffer containing 150mM NaCl, 30mM Tris(pH 7.5), 1mM EDTA, 1% TritonX-100, 10% Glycerol, 0.1mM PMSF, 0.5mM DTT, protease inhibitor cocktail tablets (EDTA-free)(Roche) and phosphatase inhibitor cocktail tablets (Roche). After clarification by centrifugation (14,000rpm for 30 minutes at 4°C), the cellular lysates were pre-cleared with IgG-agarose beads (Sigma) for at least 6 hours at 4°C. Immunoprecipitation of endogenous complexes were carried out by incubating the cellular lysates with anti-Axin1 antibody or mouse IgG immobilized on Protein G PLUS-Agarose beads (Santa Cruz biotechnology) at 4°C overnight. Immunocomplexes were washed with cold lysis buffer six times, and resuspended in 2xSDS sample buffer. The immunoprecipitated material was subjected to SDS-PAGE and Mass Spectrometry analysis or western blot analysis.

Fractionation

HEK293T cells were stimulated with Wnt3A conditioned medium or control conditioned medium or combination with R-spondin as indicated and then washed and collected in ice cold PBS. The cell pellets were then resuspended in hypotonic lysis buffer containing 10mM KCl, 10mM Tris pH7.5 and 2mM EDTA with supply of protease inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets. Cell suspensions were incubate on ice for 30 minutes with multiple douncings in between. Lysis of the cells was controlled by checking under the microscope. Nuclear proteins including the unlysed cells were pelleted by spinning at

2000rpm 2 minutes, 4°C centrifugation. The supernatant which contained both cytoplasm and membrane proteins was then centrifuged at 14,000rpm for 30 minutes at 4°C. The supernatant was collected and resuspended in the 2xSDS sample buffer for further Western blotting analysis.

Reporter Gene Assays

Tcf reporter constructs were both modified to contain 10 optimized and 10 mutated Tcf-binding sites as previously described. cDNA of Dkk1(kind gift from C. Niehrs), mutated β-catenin (S33A) (kind gift from K. Kinzler), Wnt1 and Wnt3a (both kind gifts from R. Nusse) were subjected to PCR amplification and then cloned into pcDNA4TO. 140ng Dkk1 plasmid or control empty expression vector pCDNA4TO and 100ng reporter plasmid (TOP10 or FOP10) together with 10ng internal control plasmid (pCMV-Rennilla) were transfected into 40% confluency 24-well plate seeded HEK293T cells by using polyethylenimine (polysciences). 16hrs after TOPFlash transfection, the cells were treated with condition medium or together with 500ng/ml R-spondin and the measurements were performed 12-16 hours later. All experiments were performed in duplicate or triplicate and repeated at least twice.

In-gel digestion

Gels were sliced into smaller bands of all four pull downs (i.e. IgG and Axin1, Axin2 and subjected to in-gel digestion as described earlier[226]. Protein reduction and alkylation was performed with DTT (60°C, 1 hours) and iodoacetamide (dark, RT, 30 minutes), respectively. Digestion was performed with trypsin over night at 37°C. Peptides were extracted with 10% FA.

NanoLC–MS/MS

The extracted peptides were subjected to nanoscale liquid chromatography tandem MS (nano LC–MS/MS) analysis performed on an Agilent 1100 HPLC system (Agilent technologies) connected to an LTQ Linear Ion Trap Mass Spectrometer combined with either an Orbitrap (ThermoFisher, Waltham, MA, USA). The nanoLC was equipped with a 20 mm 100 m i.d. Aqua C18 trap column (Phenomenex, Torrance, CA, USA) and a 400 mm 50 m i.d. Reprosil C18 analytical column (Dr Maisch, Ammerbuch-Entringen, Germany)204. Trapping was performed at a flow of 5 l/minutes for 10 minutes and the fractions were eluted using a 60-minutes *(60-120 minutes)* linear gradient from 0 to 40% solvent B (0.1 M acetic acid in 80% ACN (v/v), in which solvent A was 0.1 M acetic acid). The analytical flow rate was 100 nl/minutes and the column effluent was directly introduced into the ESI source of the MS using a standard coated fused silica emitter (New Objective, Woburn, MA, USA) (o.d. 360 m, tip i.d. 10 m) biased to 1.7 kV. The mass spectrometer was operated in positive ion mode and in data-dependent mode to automatically switch between MS and MSMS. The three most intense ions in the survey scan were fragmented in the linear ion trap using collisional-induced dissociation. The target ion setting was 5e5 for the Orbitrap, with a maximum fill time of 250ms. Fragment ion spectra were acquired in the LTQ with an AGC value of 3e4 and a max injection time of 500 ms.

Protein identification

Raw MS data were converted to peak lists using Bioworks Browser software, version 3.1.1. Spectra

were searched against the International Protein Index (IPI) human database version 3.36 (69012 sequences; 29002682 residues) using Mascot software version 2.2.0 (www.matrixscience.com), with trypsin set as enzyme. The database search was made with the following parameters set to consider a peptide tolerance of 10 p.p.m., a fragment tolerance of 0.9 Da, allowing two missed cleavages, carbamidomethyl (C) as fixed modification and oxidation (M) and phosphorylation (S,T,Y) as variable modification.

Supplementary Data

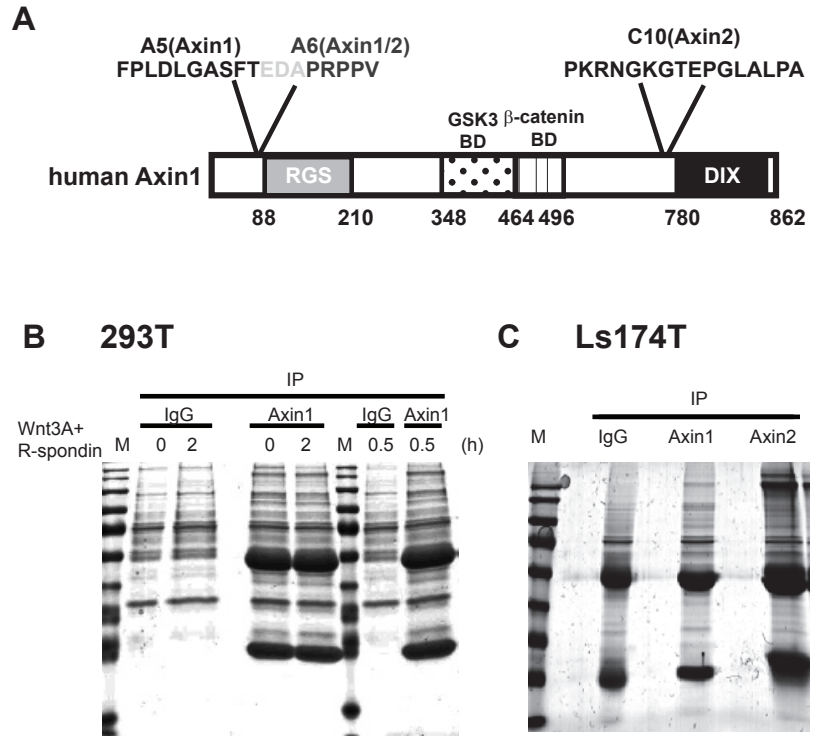

Figure S1. Epitope location and specificity of Axin antibodies.

(A) Axin1 and 2 protein structure and their antibody specificity and epitopes location. Axin1 specific (A5), Axin1/2 (A6) specific antibodies are locates at N-terminal of RGS domain; Axin2 specific antibody (C10) is located at N-terminal of the DIX domain. GSK3 and β-catenin binding regions are indicated with dot box and lined box. Coomassie Blue stained gel of Axin complexes immunoprecipitated from HEK293T **(B)** and Ls174T cells **(C)**. Whole gels were cut out, digested by trypsin and extracted peptides were subjected to nanoscale liquid chromatography tandem MS (nanoLC–MS/MS) analysis.

Figure S2. Protein peptide coverage (%) and respective protein score of Axin1, Axin2, APC, GSK3 (α,β), CKIα, β-catenin, RanBP2, DLG1 and CASK1.

Amino acid sequences of dedicated protein peptide detected in the Axin immunoprecipitate is given highlighted in grey. Best coverage of each protein from different MASS SPEC experiments was shown here.

Axin1 (From Ls174T MASS SPEC)
protein coverage: 65%; proten score: 3731

MNIQEQGFPLDLGASFTEDAPRPPVPGEEGELVSTDPRPASYSFCSGKGVGIKGETSTATPRRSDLDLGYEPEGS
ASPTPPYLKWAESLHSLLDDQDGISLFRTFLKQEGCADLLDFWFACTGFRKLEPCDSNEEKRLKLARAIYRKYILD
NNGIVSRQTKPATKSFIKGCIMKQLIDPAMFDQAQTEIQATMEENTYPSFLKSDIYLEYTRTGSESPKVCSDQSSG
SGTGKGISGYLPTLNEDEEWKCDQDMDEDDGRDAAPPGRLPQKLLLETAAPRVSSSRRYSEGREFRYGSWREP
VNPYYVNAGYALAPATSANDSEQQSLSSDADTLSLTDSSVDGIPPYRIRKQHRREMQESVQVNGRVPLPHIPRT
YRVPKEVRVEPQKFAEELIHRLEAVQRTREAEEKLEERLKRVRMEEEGEDGDPSSGPPGPCHKLPPAPAWHHFPP
RCVDMGCAGLRDAHEENPESILDEHVQRVLRTPGRQSPGPGHRSPDSGHVAKMPVALGGAASGHGKHVPKS
GAKLDAAGLHHHRHVHHHVHHSTARPKEQVEAEATRRAQSSFAWGLEPHSHGARSRGYSESVGAAPNASDG
LAHSGKVGVACKRNAKKAESGKSASTEVPGASEDAEKNQKIMQWIIEGEKEISRHRRTGHGSSGTRKPQPHENS
RPLSLEHPWAGPQLRTSVQPSHLFIQDPTMPPHPAPNPLTQLEEARRRLEEEEKRASRAPSKQRYVQEVMRRGR
ACVRPACAPVLHVVPAVSDMELSETETRSQRKVGGGSAQPCDSIVVAYYFCGEPIPYRTLVRGRAVTLGQFKELL
TKKGSYRYYFKKVSDEFDCGVVFEEVREDEAVLPVFEEKIIGKVEKVD

Axin1 (From 293T MASS SPEC)
protein coverage: 62%; proten score: 3582

MNIQEQGFPLDLGASFTEDAPRPPVPGEEGELVSTDPRPASYSFCSGKGVGIKGETSTATPRRSDLDLGYEPEGS
ASPTPPYLKWAESLHSLLDDQDGISLFRTFLKQEGCADLLDFWFACTGFRKLEPCDSNEEKRLKLARAIYRKYILD
NNGIVSRQTKPATKSFIKGCIMKQLIDPAMFDQAQTEIQATMEENTYPSFLKSDIYLEYTRTGSESPKVCSDQSSG
SGTGKGISGYLPTLNEDEEWKCDQDMDEDDGRDAAPPGRLPQKLLLETAAPRVSSSRRYSEGREFRYGSWREP
VNPYYVNAGYALAPATSANDSEQQSLSSDADTLSLTDSSVDGIPPYRIRKQHRREMQESVQVNGRVPLPHIPRT
YRVPKEVRVEPQKFAEELIHRLEAVQRTREAEEKLEERLKRVRMEEEGEDGDPSSGPPGPCHKLPPAPAWHHFPP
RCVDMGCAGLRDAHEENPESILDEHVQRVLRTPGRQSPGPGHRSPDSGHVAKMPVALGGAASGHGKHVPKS
GAKLDAAGLHHHRHVHHHVHHSTARPKEQVEAEATRRAQSSFAWGLEPHSHGARSRGYSESVGAAPNASDG
LAHSGKVGVACKRNAKKAESGKSASTEVPGASEDAEKNQKIMQWIIEGEKEISRHRRTGHGSSGTRKPQPHENS
RPLSLEHPWAGPQLRTSVQPSHLFIQDPTMPPHPAPNPLTQLEEARRRLEEEEKRASRAPSKQRYVQEVMRRGR
ACVRPACAPVLHVVPAVSDMELSETETRSQRKVGGGSAQPCDSIVVAYYFCGEPIPYRTLVRGRAVTLGQFKELL
TKKGSYRYYFKKVSDEFDCGVVFEEVREDEAVLPVFEEKIIGKVEKVD

Axin2 (From Ls174T MASS SPEC)
protein coverage: 47%; protein score: 2069

MSSAMLVTCLPDPSSSFREDAPRPPVPGEEGETPPCQPGVGKGQVTKPMPVSSNTRRNEDGLGEPEGRASPDS
PLTRWTKSLHSLLGDQDGAYLFRTFLEREKCVDTLDFWFACNGFRQMNLKDTKTLRVAKAIYKRYIENNSIVSKQ
LKPATKTYIRDGIKKQQIDSIMFDQAQTEIQSVMEENAYQMFLTSDIYLEYVRSGGENTAYMSNGGLGSLKVVCG
YLPTLNEEEEWTCADFKCKLSPTVVGLSSKTLRATASVRSTETVDSGYRSFKRSDPVNPYHIGSGYVFAPATSAND
SEISSDALTDDSMSMTDSSVDGIPPYRVGSKKQLQREMHRSVKANGQVSLPHFPRTHRLPKEMTPVEPATFAAE
LISRLEKLKLELESRHSLEERLQQIREDEEREGSELTLNSREGAPTQHPLSLLPSGSYEEDPQTILDDHLSRVLKTPGC
QSPGVGRYSPRSRSPDHHHHHHSQYHSLLPPGGKLPPAAASPGACPLLGGKGFVTKQTTKHVHHHYIHHHAV
PKTKEEIEAEATQRVHCFCPGGSEYYCYSKCKSHSKAPETMPSEQFGGSRGSTLPKRNGKGTEPGLALPAREGGA
PGGAGALQLPREEGDRSQDVWQWMLESERQSKPKPHSAQSTKKAYPLESARSSPGERASRHHLWGGNSGHP
RTTPRAHLFTQDPAMPPLTPPNTLAQLEEACRRLAEVSKPPKQRCCVASQQRDRNHSATVQTGATPFSNPSLAP
EDHKEPKKLAGVHALQASELVVTYFFCGEEIPYRRMLKAQSLTLGHFKEQLSKKGNYRYYFKKASDEFACGAVFE
EIWEDETVLPMYEGRILGKVERID

APC

protein coverage: 30%; proten score: 4942

MAAASYDQLLKQVEALKMENSNLRQELEDNSNHLTKLETEASNMKEVLKQLQGSIEDEAMASSGQIDLLERLKE
LNLDSSNFPGVKLRSKMSLRSYGSREGSVSSRSGECSPVPMGSFPRRGFVNGSRESTGYLEELEKERSLLLADLDK
EEKEKDWYYAQLQNLTKRIDSLPLTENFSLQTDMTRRQLEYEARQIRVAMEEQLGTCQDMEKRAQRRIARIQQIE
KDILRIRQLLQSQATEAERSSQNKHETGSHDAERQNEGQGVGEINMATSGNGQGSTTRMDHETASVLSSSSTH
SAPRRLTSHLGTKVEMVYSLLSMLGTHDKDDMSRTLLAMSSSQDSCISMRQSGCLPLLIQLLHGNDKDSVLLGN
SRGSKEARARASAALHNIIHSQPDDKRGRREIRVLHLLEQIRAYCETCWEWQEAHEPGMDQDKNPMPAPVEHQ
ICPAVCVLMKLSFDEEHRHAMNELGGLQAIAELLQVDCEMYGLTNDHYSITLRRYAGMALTNLTFGDVANKAT
LCSMKGCMRALVAQLKSESEDLQQVIASVLRNLSWRADVNSKKTLREVGSVKALMECALEVKKESTLKSVLSAL
WNLSAHCTENKADICAVDGALAFLVGTLTYRSQTNTLAIIESGGGILRNVSSLIATNEDHRQILRENNCLQTLLQH
LKSHSLTIVSNACGTLWNLSARNPKDQEALWDMGAVSMLKNLIHSKHKMIAMGSAAALRNLMANRPAKYKDA
NIMSPGSSLPSLHVRKQKALEAELDAQHLSETFDNIDNLSPKASHRSKQRHKQSLYGDYVFDTNRHDDNRSDN
FNTGNMTVLSPYLNTTVLPSSSSSRGSLDSSRSEKDRSLERERGIGLGNYHPATENPGTSSKRGLQISTTAAQIAKV
MEEVSAIHTSQEDRSSGSTTELHCVTDERNALRRSSAAHTHSNTYNFTKSENSNRTCSMPYAKLEYKRSSNDSLN
SVSSSDGYGKRGQMKPSIESYSEDDESKFCSYGQYPADLAHKIHSANHMDDNDGELDTPINYSLKYSDEQLNS
GRQSPSQNERWARPKHIIEDEIKQSEQRQSRNQSTTYPVYTESTDDKHLKFQPHFGQQECVSPYRSRGANGSET
NRVGSNHGINQNVSQSLCQEDDYEDDKPTNYSERYSEEEQHEEEERPTNYSIKYNEEKRHVDQPIDYSLKYATDI
PSSQKQSFSFSKSSSGQSSKTEHMSSSSENTSTPSSNAKRQNQLHPSSAQSRSGQPQKAATCKVSSINQETIQTY
CVEDTPICFSRCSSLSSLSSAEDEIGCNQTTQEADSANTLQIAEIKEKIGTRSAEDPVSEVPAVSQHPRTKSSRLQGS
SLSSESARHKAVEFSSGAKSPSKSGAQTPKSPPEHYVQETPLMFSRCTSVSSLDSFESRSIASSVQSEPCSGMVSGI
ISPSDLPDSPGQTMPPSRSKTPPPPPQTAQTKREVPKNKAPTAEKRESGPKQAAVNAAVQRVQVLPDADTLLHF
ATESTPDGFSCSSSLSALSLDEPFIQKDVELRIMPPVQENDNGNETESEQPKESNENQEKEAEKTIDSEKDLLDDS
DDDDIEILEECIISAMPTKSSRKAKKPAQTASKLPPPVARKPSQLPVYKLLPSQNRLQPQKHVSFTPGDDMPRVYC
VEGTPINFSTATSLSDLTIESPPNELAAGEGVRGGAQSGEFEKRDTIPTEGRSTDEAQGGKTSSVTIPELDDNKAEE
GDILAECINSAMPKGKSHKPFRVKKIMDQVQQASASSSAPNKNQLDGKKKKPTSPVKPIPQNTEYRTRVRKNAD
SKNNLNAERVFSDNKDSKKQNLKNNSKVFNDKLPNNEDRVRGSFAFDSPHHYTPIEGTPYCFSRNDSLSSLDFD
DDDVDLSREKAELRKAKENKESEAKVTSHTELTSNQQSANKTQAIAKQPINRGQPKPILQKQSTFPQSSKDIPDR
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β-catenin

protein coverage: 26%; proten score: 1090

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VIRGLNTIPLFVQLLYSPIENIQRVAAGVLCELAQDKEAAEAIEAEGATAPLTELLHSRNEGVATYAAAVLFRMSED
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WTX(FAM123B)
protein coverage: 7%; proten score: 251
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GATEKAVAEKFPSMPKPKKGLKGFFSSIRRHRKSKVTGAEQSEPGAKGPERVRARPHEHVSSAPQVPCFEETFQA
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GSK3α
protein coverage: 22%; proten score:559
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GSK3β
protein coverage: 57%; proten score: 1205
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LIFGATDYTSSIDVWSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPTREQIREMNPNYTEFKFPQIKAHPWTK
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DLG1 (Discs large homolog 1)
protein coverage: 9%; proten score: 398
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SPIKPTEAVLPSPPTVPVIPVLPVPAENTVILPTIPQANPPPVLVNTDSLETPTYVNGTDADYEYEEITLERGNSGLG
FSIAGGTDNPHIGDDSSIFITKIITGGAAAQDGRLRVNDCILRVNEVDVRDVTHSKAVEALKEAGSIVRLYVKRRKP
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ESSYRGQEEYVLSYEPVNQQEVNYTRPVIILGPMKDRINDDLISEFPDKFGSCVPHTTRPKRDYEVDGRDYHFVTS
REQMEKDIQEHKFIEAGQYNNHLYGTSVQSVREVAEKGKHCILDVSGNAIKRLQIAQLYPISIFIKPKSMENIMEM
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RanBP2 (RAN binding protein 2)
protein coverage: 5%; proten score: 243

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YQPLCLPLPVCKQLCTERQKSWWDAVCTLIHRKAVPGNVAKLRLLVQHEINTLRAQEKHGLQPALLVHWAE
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ANHVITKTMELKPLNVSNNALVWTASDYADGEAKVEQLAVRFKTKEVADCFKKTFEECQQNLMKLQKGHVS
LAAELSKETNPVVFFDVCADGEPLGRITMELFSNIVPRTAENFRALCTGEKGFGFKNSIFHRVIPDFVCQGGDIT
KHDGTGGQSIYGDKFEDENFDVKHTGPGLLSMANQGQNTNNSQFVITLKKAEHLDFKHVVFGFVKDGMDT
VKKIESFGSPKGSVCRRITITECGQI

CASK1 (calcium/calmodulin-dependent serine protein kinase isoform 1)
protein coverage: 4%; proten score: 127

MADDDVLFEDVYELCEVIGKGPFSVVRRCINRETGQQFAVKIVDVAKFTSSPGLSTEDLKREASICHMLKHPHI
VELLETYSSDGMLYMVFEFMDGADLCFEIVKRADAGFVYSEAVASHYMRQILEALRYCHDNNIIHRDVKPHC
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YGTKERLFEGIIKGKYKMNPRQWSHISESAKDLVRRMLMLDPAERITVYEALNHPWLKERDRYAYKIHLPETVE
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LDFLHSVFQDQHLHTLLDLYDKINTKSSPQIRNPPSDAVQRAKEVLEEISCYPENNDAKELKRILTQPHFMALL
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PAFKRKTLVLLGAHGVGRRHIKNTLITKHPDRFAYPIPHTTRPPKKDEENGKNYYFVSHDQMMQDISNNEYLE
YGSHEDAMYGTKLETIRKIHEQGLIAILDVEPQALKVLRTAEFAPFVVFIAAPTITPGLNEDESLQRLQKESDILQ
RTYAHYFDLTIINNEIDETIRHLEEAVELVCTAPQWVPVSWVY

Chapter 6

The kinase TNIK is an essential activator of Wnt target genes

Adopted from:

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Life is just a series of trying to make up your mind
-T. Fuller-

Abstract

Wnt signalling maintains the undifferentiated state of intestinal crypt/progenitor cells through the TCF4/β-catenin activating transcriptional complex. In colorectal cancer activating mutations in Wnt pathway components lead to inappropriate activation of the TCF4/β-catenin transcriptional program and tumourigenesis. The mechanisms by which TCF4/β-catenin activate key target genes are not well understood. Using a proteomics approach, we identified Tnik, a member of the germinal centre kinase family as a Tcf4 interactor in the proliferative crypts of mouse small intestine. Tnik is recruited to promoters of Wnt target genes in mouse crypts and in Ls174T colorectal cancer cells in a β-catenin-dependent manner. Depletion of TNIK and expression of TNIK kinase mutants abrogated TCF-LEF transcription, highlighting the essential role of the kinase activity in Wnt target gene activation. In vitro binding and kinase assays demonstrate that TNIK directly binds both TCF4 and β-catenin and phosphorylates TCF4. siRNA depletion of TNIK followed by expression array analysis demonstrated that TNIK is an essential, specific activator of Wnt transcriptional program. This kinase may present an attrative candidate for drug targeting in clorectal cancer.

Introduction

When Wnt signals engage their receptors, a complex series of biochemical events is set in motion, leading to stabilization of the key signalling molecule β-catenin in the cytoplasm. Upon transfer to the nucleus, stabilized β-catenin forms a complex with DNA-binding TCF/LEF transcription factors and serves as a transcriptional co-activator, thus switching on Wnt target genes[98, 99]. A primary function of canonical Wnt signalling in adult mammals involves the maintenance of stem and progenitor cells in the intestinal epithelium[2].

Mutational activation of the TCF4/β-catenin transcriptional program can lead to various types of cancer, most notably of the intestine. In these tumours, loss of the Wnt pathway inhibitors APC and Axin2[138, 202] or activating point mutations in β-cateni[169, 185] lead to the stabilization of β-catenin. The constitutive presence of TCF4/β-catenin complexes locks the Wnt transcriptional program in the "on" state[215, 216], leading to transformation in the gut epithelium. At present, the mechanisms by which the β-catenin/TCF4 complex activates expression of key target genes are incompletely understood. Roles have been proposed for a number of co-activators including CBP/p300[109, 112], Brg1[107] and Pygopus/Bcl9 complexes[116, 217]. Identification of the complete repertoire of nuclear TCF4/β-catenin co-activator complex components will be critical as potential drug targets to inhibit the aberrantly activated Wnt transcriptional program in colorectal cancer.

In this study, using a proteomics approach, we identified Traf2 and Nck-interacting

kinase (Tnik) as a novel protein interacting with Tcf4 in the mouse intestinal crypt. Tnik is a member of germinal center kinases (GCKs) possessing an N-terminal kinase domain, and can specifically activate the c-Jun N-terminal kinase (JKN) pathway similar to many GCKs[218]. Additionally, Tnik has also been reported as an effector of Rap2, to regulate actin cytoskeleton by disrupting F-actin structure, and subsequently inhibits cell spreading[218, 219]. However, a potential role for Tnik in transcriptional regulation has never been documented.

Here, we report that TNIK is a critical component of the transcriptional regulatory complex in the Wnt signalling pathway. TNIK is localized in the nuclei of Wnt active intestinal crypts and is recruited to promoters of Wnt target genes in mouse crypts and in colorectal cancer cells in a β-catenin dependent manner. TNIK interacts directly with both β-catenin and TCF4 and phosphorylates TCF4 leading to TCF/LEF driven transcriptional activation of Wnt target genes; Exogenous expression of TNIK kinase mutants abrogate TCF/LEF driven transcription, while siRNA depletion of TNIK followed by expression array analysis demonstrates the critical role of TNIK as an essential and specific activator of Wnt target genes.

Results and Discussion

Tnik Co-immunoprecipitates with Tcf4 in the mouse small intestinal crypt

As an unbiased approach towards the identification of components of the endogenous TCF4 complex in murine small intestinal crypts and villi, we applied the combination of affinity purification and mass spectrometry (MS). We first developed a fractionation method to separate proliferative crypt epithelium from differentiated villus epithelium. The quality of the fractionation was assessed by western blot analysis of known proteins differentially expressed between the two compartments. The TCF4/β-catenin target genes/crypt markers c-Myc[220], EPHB2 and EPHB3[201] were strongly enriched in the crypt fraction, while the villus marker Keratin 20[221] was present exclusively in the purified villus fraction (Figure 1A). TCF4 was detected in both crypt and villus fractions, as was tubulin.

To identify potential Tcf4 co-regulators in a physiological *in vivo* setting, we immunopurified Tcf4 from crypt and villus fractions using a TCF4 antibody as well as a non-immune IgG as control. The immunoprecipitates were subjected to SDS-PAGE and silver staining (Figure 1B), followed by mass spectrometric identification. Tcf4 and β-catenin were readily identified in the crypt fraction while Tcf4, but not β-catenin, was found in the villus fraction (Figure S1A and B). None of these proteins were observed in the IgG control. This implied that the approach allowed us to specifically isolate Wnt-activated Tcf4 complexes from crypts and inactive Tcf4 from villi. Amongst the proteins specifically co-precipitating with Tcf4/β-catenin from the crypt fraction, the serine/threonine kinase Tnik stood out in that a large portion of the protein sequence

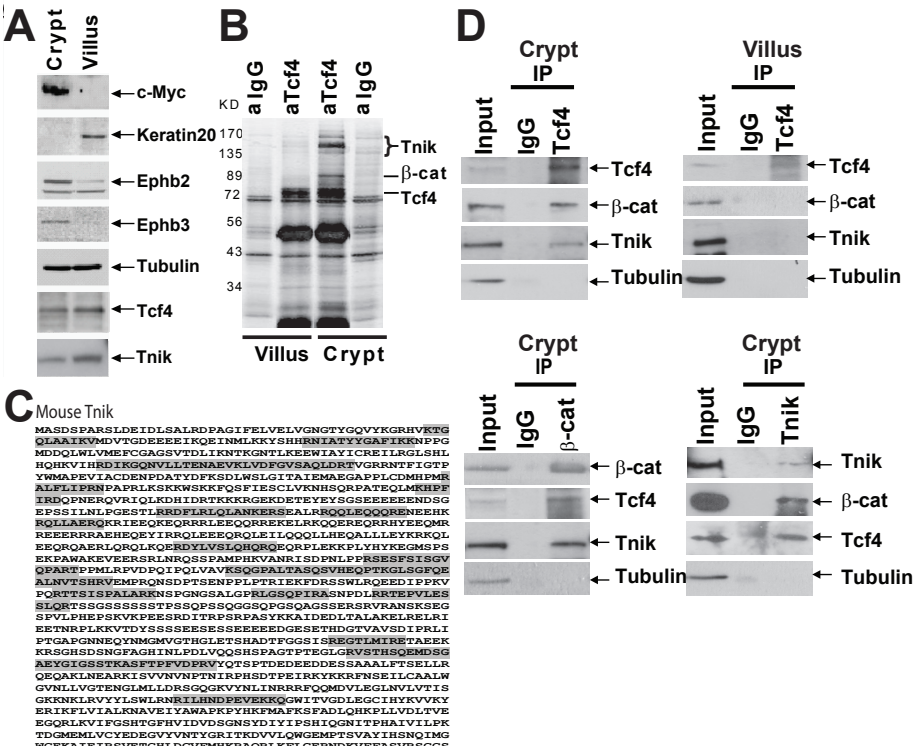

Figure 1. Tnik co-immunoprecipitates with Tcf4 in the mouse small intestinal crypt.

(A) Biochemical purification of crypt and villus fractions from mouse small intestine used in Mass Spec experiment. Western blot analysis using antibodies directed against genes expressed specifically in the crypt (c-Myc, Ephb2 and Ephb3), villus (Keratin 20), as well as Tubulin as control were used to assess the quality of the purified fractions. **(B)** Silverstained gel of Tcf4 containing complexes immunoprecipitated from purified crypt and villus fractions using antibody directed against Tcf4. The Tcf4-interacting proteins are indicated by arrows and were identified by mass spectrometry (see Material and Methods for details). **(C)** Peptide coverage of Tnik in MS experiment. Amino acid sequences of Tnik peptides detected in the Tcf4 immunoprecipitate from crypt lysates are high-lighted in grey. **(D)** Cell lysates from purified crypt and villus fractions were immunoprecipitated with antibodies directed against endogenous Tcf4, β-catenin and Tnik as indicated and analyzed by Western blotting with the indicated antibodies.

was covered by the MS-identified peptides (Figure 1C and Figure S1C). To confirm the interaction, we immunoprecipitated Tcf4 from crypt and villus fractions and probed for its association with endogenous Tnik by western blotting using a Tnik antibody. Despite the presence of Tnik protein in both fractions (Figure 1A), Tcf4 interacted with Tnik specifically in the crypt, but not the differentiated villus fraction (Figure 1D top panels). Immunoprecipitation (IP) of β-catenin from crypts followed by western blot analysis also detected Tnik (Figure 1D bottom left). The same endogenous interactions were confirmed in reverse, using the Tnik antibody for IP (Figure 1D bottom right). These results demonstrated that Tnik specifically interacts with the Tcf4/

β-catenin complex in murine small intestinal crypts.

Nuclear localization of Tnik in Wnt activated intestinal crypt

Tnik has been previously reported to localize in the cytoplasm and interact with and phosphorylate cytoskeletal structures[218]. The presence of Tnik in the Tcf4 transcriptional complex suggested nuclear localization of Tnik. We next examined the expression of TNIK in mouse and human intestinal tissue using immunohistochemistry (IHC). Tnik was detected in both mouse small intestinal and colonic epithelia (Figure 2A-D). Nuclear localization of Tnik was detected specifically in Wnt activated intestinal crypt (Figure 2B), consolidating our mass spectrometry findings on the specific interaction of Tnik with Tcf4 in mouse intestinal crypt but not villus. Using a mouse monoclonal antibody against the intermediate part of human TNIK, we further confirmed the nuclear localization of human TNIK in normal colonic epithelia as well as colorectal cancer tissues (Figure 2E-H). These results demonstrate that in addition to the reported cytoplasmic expression, Tnik is strongly enriched in the cell nuclei of Wnt activated intestinal crypts.

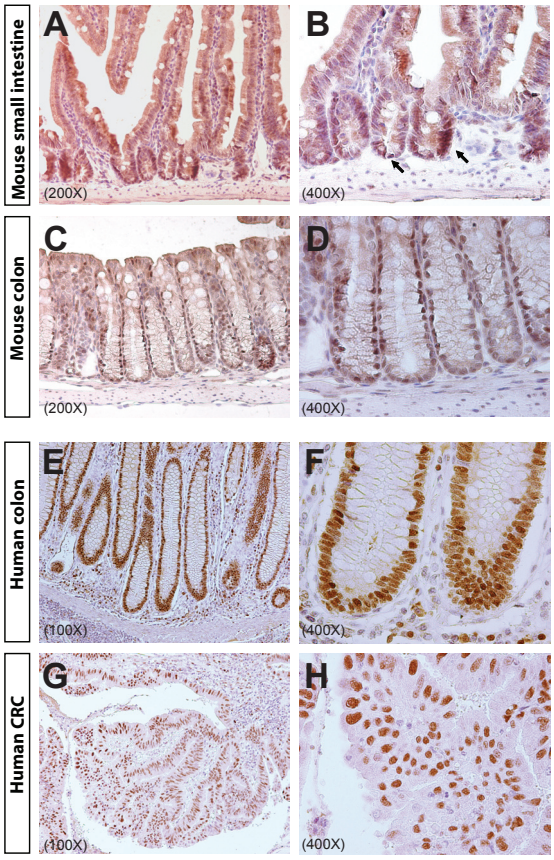

Figure 2. Nuclear localization of Tnik in Wnt activated intestinal crypt. Immunohistochemistry of Tnik in mouse small intestine **(A-B)**, mouse colon **(C-D)**, human normal colon **(E-F)** and human colorectal tissues **(G-H)**. **(B)** Nuclear localization of Tnik in mouse intestinal crypt is indicated by arrows. Corresponding magnification is shown on the bottom left of figures.

Tnik is recruited to the proximal promoters of Wnt target genes in mouse small intestinal crypts *in vivo*

We have previously determined TCF4 binding sites by genome-wide chromatin IP (ChIP) in human colon cancer cells[222]. To test whether Tnik is associated with such Tcf4/

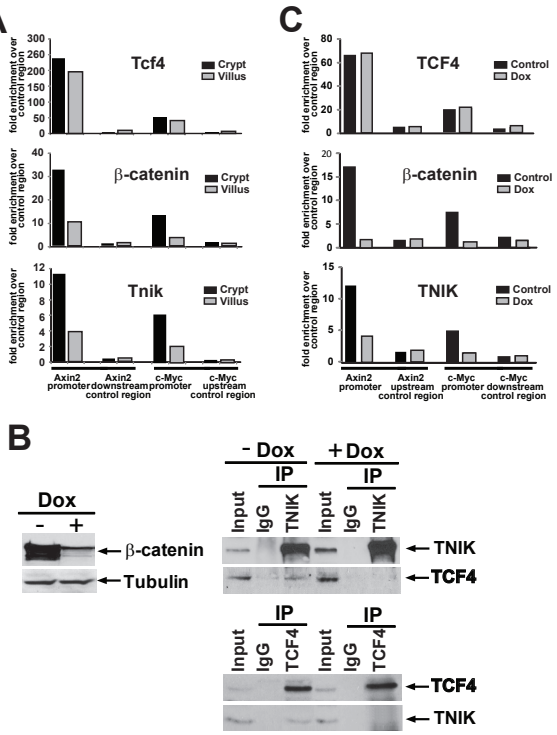

Figure 3. Tnik is recruited to Wnt target genes in mouse crypts and CRC cells in β-catenin-dependent manner.

(A) Purified crypt and villus fractions from mouse small intestine were subjected to chromatin immunoprecipitation using antibodies directed against Tcf4, β-catenin and Tnik. Formaldehyde cross-linked chromatin was immunoprecipitated with the specified antibodies followed by qPCR using primers specific for the Axin2 and c-Myc proximal promoters as well as Axin2 downstream and c-Myc upstream control regions as indicated. Results are presented as relative enrichment over the non-bound downstream SP5 control region and are representative of three independent experiments. **(B)** TNIK interaction with TCF4 is mediated by β-catenin. Western blot analysis of β-catenin depletion in Ls174T cells expressing doxycycline(Dox) inducible β-catenin shRNA (left panel). Immunoprecipitated TNIK-protein complexes from untreated or doxycycline-treated cells were resolved by SDS-PAGE followed by western blotting using antibodies directed against TNIK and TCF4 (top right panel), and the result was further confirmed by the reverse IP (bottom right panel). **(C)** Chromatin immunoprecipitation experiments in Ls174T cells uninduced or induced with Dox using antibodies specific for TCF4, β-catenin, and TNIK. The immunoprecipitated DNA was analyzed by qPCR using primer pairs specific for the Axin2 and c-Myc promoters, and Axin2 upstream and c-Myc downstream control regions. Results are presented as relative enrichment over the non-bound second exon of the myoglobin gene and are representative of three independent experiments.

β-catenin response elements *in vivo,* we performed ChIP on purified intestinal crypt and villus fractions with antibodies specific for Tcf4, β-catenin and Tnik. Quantitative PCR (qPCR) analysis of the immunoprecipitated material was performed for homologous elements in two murine intestinal Wnt target genes, the Axin2 and c-Myc proximal promoters and up/downstream control regions (Figure 3A). As expected, Tcf4

was bound specifically to the target gene promoters in both the crypt and villus, while β-catenin was enriched on the target promoters specifically in the crypt-proliferative compartment (Figure 3A). While not enriched on up/downstream control regions, Tnik was specifically recruited to the promoters of the two Wnt targets genes in the crypt, but not the villus fraction (Figure 3A).

TNIK is recruited to Wnt target genes in a β-catenin dependent manner

To examine whether the Tcf4-Tnik association was mediated by β-catenin, we used a stable transfectant of the human colon cancer cell line Ls174T which expresses a shRNA targeting β-catenin in response to doxycycline treatment[223]. Of note, Ls174T cells harbour a stabilizing oncogenic mutation in β-catenin, resulting in the constitutive presence of β-catenin/TCF4 nuclear complexes. In the transfected cells, the β-catenin protein was strongly reduced 72 hours post-doxycycline treatment (Figure 3B left panel). We immunoprecipitated TNIK from the Ls174T cells with and without doxycycline treatment and probed for the presence of TCF4. TCF4 bound TNIK in these cells demonstrating conservation of the TCF4-TNIK association across mammalian species (Figure 3B right top panel). However, depletion of β-catenin resulted in loss of the TNIK/TCF4 interaction, implying that β-catenin serves as a bridge. Conversely, immunoprecipitation of TCF4 in β-catenin depleted cells resulted in loss of TCF4-TNIK interaction (Figure 3B bottom right panel). Using the same system, we examined the recruitment of TNIK to the promoters of TCF4 target genes Axin2 and c-Myc in vivo in the presence or absence of β-catenin (Figure 3C). TCF4 was bound to the Axin2 and c-Myc proximal promoters regardless of β-catenin status. As expected, β-catenin disappeared from the target gene promoters upon β-catenin knockdown. Importantly, while specifically present on the Axin2 and c-Myc promoters, TNIK enrichment over these targets was decreased upon β-catenin depletion (Figure 3C). Thus, TNIK recruitment to TCF4 target genes Axin2 and c-Myc occurs in a β-catenin dependent manner.

The Kinase activity of TNIK is required for TCF/LEF transcriptional activation

The specific association of TNIK with β-catenin/TCF4 in crypts and Ls174T cells was suggestive of a co-activator function for TNIK. To test this, we depleted TNIK from Ls174T cells using transient siRNA transfection and examined the effect on transcriptional activity of a Tcf reporter TOPFlash (Figure 4A). Removal of TNIK by siRNA resulted in specific suppression of TOPFlash activity but not of the mutant FOPFlash control. Similar suppression of TOPFlash activity was observed upon TNIK depletion in another colorectal cancer cell line, SW480 (Figure S2A). Conversely, over-expression of wild type (WT) Flag-TNIK resulted in a dosage dependent specific increase in TOPFlash activity (Figure 4C right panel). The N-terminal kinase domain of TNIK is highly conserved among serine/threonine protein kinases (e.g. Ste20 family and protein kinase A) and contains several characterized subdomains that fold into a catalytic core structure[188] (Figure 4B). To examine the role of the kinase activity of TNIK

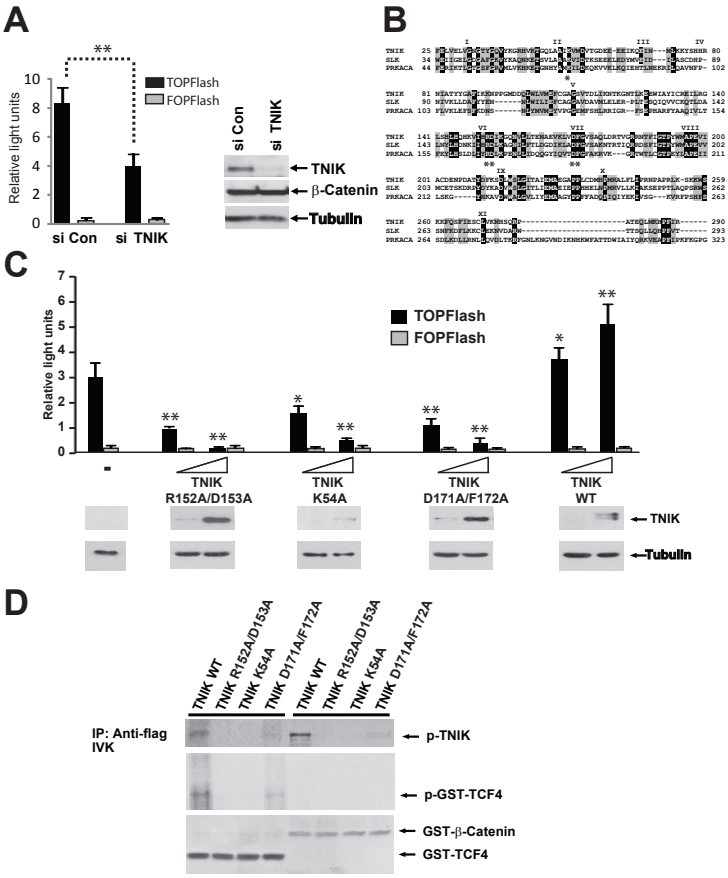

Figure 4. TNIK kinase activity is required for TCF/LEF mediated transcription.

(A) siRNA mediated depletion of TNIK reduces β-catenin /TCF driven transcription in Ls174T CRCs. Activity of the TOPFlash (black bars) and FOPFlash (grey bars) 96 hours post siRNA transfection is shown. Error bars represent standard deviation from three independent experiments. Expression of TNIK, β-catenin, and control Tubulin was analyzed by western blotting after depletion of TNIK (left panel). **(B)** Amino acid alignment of the N-terminal kinase domain of human TNIK with Ser/Thr kinases SLK and PRKACA. Identical amino acids are shown in black and conserved amino acids are highlighted in grey. Asterisks indicate the conserved amino acids mutated to generate TNIK mutants used in C. **(C)** Expression of TNIK kinase mutants (second, third and fourth panels) abrogates β-catenin /TCF driven transcription while over expression of WT TNIK (last panel) specifically increases β-catenin /TCF driven transcription in Ls174T CRCs. Black bars indicate TOPFlash activity while grey bars indicate FOPFlash activity 24 hours post transfection of expression vectors. Error bars represent standard deviation from three independent experiments. Western blot analysis indicates expression of Flag-tagged WT and mutant TNIK proteins using M2 Flag antisera (bottom panel). * *p-value*<0.05; ** *p-value*<0.01. **(D)** Phosphorylation of TCF4 by TNIK is demonstrated by *in vitro* kinase assay using immunoprecipitated Flag-tagged wild type and mutant TNIK and GST-TCF4 and GST-β-catenin as substrate. WT TNIK is able to phosphorylate TCF4 but not β-catenin, whereas the phosphorylation is largely suppressed in all the TNIK kinase mutants. Auto-phosphorylation of WT TNIK is also detected. GST-tagged protein input is shown at the bottom.

in TCF/LEF mediated transcriptional activation, we generated dominant negative TNIK kinase mutants TNIK R152A/D153A, TNIK K54A, and TNIK D171A/F172A, harbouring mutations in the distinct conserved subdomains IV, II, and VII respectively, essential to catalytic activity. Expression of increasing amounts of dominant negative TNIK kinase mutants in Ls174T colorectal cancer cells was examined by western blotting (Figure 4C bottom panels). As previously reported, WT TNIK migrates as two bands comprising phosphorylated and unphosphorylated forms (Figure 4C right bottom)[219]. All TNIK kinase mutants lacked the lower mobility phosphorylated form of WT TNIK, consistent with their inability to autophosphorylate. As shown in Figure 4C, expression of all TNIK kinase mutants abolished Tcf-reporter TOPFlash transcription activity in a dose dependent manner. The results were further confirmed in another colorectal cancer cell line DLD1 (Figure S2B). Thus, while depletion of TNIK by siRNA compromises TOPFlash activity, exogenous expression of kinase mutant TNIK results in a dominant negative effect on TOPFlash reporter activity.

We next tested whether TNIK can phosphorylate TCF4 or β-catenin using in vitro kinase assay. We immunoprecipitated wild type or kinase mutant TNIK from 293T cells and performed in vitro kinase assays using GST-TCF4 and GST- β-catenin as substrate as shown in Figure 4D. As reported previously, wild type TNIK was capable of autophosphorylation[218], while the kinase mutants failed to autophosphorylate (Figure 4D). Importantly, wild type TNIK and not the kinase mutants specifically phosphorylated GST-TCF4, identifying TCF4 as a substrate for TNIK kinase activity (Figure 4D). Taken together, these results demonstrate that the kinase activity of TNIK is essential to its role as a TCF4/ β-catenin co-activator.

TNIK interacts directly with both TCF4 and β-catenin.

To confirm the role of TNIK in regulation of the Wnt target gene expression, we employed the HEK293T cell line in which the Wnt pathway is present, yet not mutationally activated. We examined the interaction between TNIK and TCF4 in HEK293T cells treated with either control or Wnt3A conditioned media for 8 hours. TNIK was immunoprecipitated from HEK293T lysates and probed for interaction with TCF4 and β-catenin by Western blotting (Figure 5A top panel). While absent from the TNIK complex in the absence of Wnt signalling, both TCF4 and β-catenin specifically associated with TNIK in response to Wnt treatment. Conversely, immunoprecipitation of TCF4 from HEK293T lysates (either untreated or treated with Wnt) demonstrated a Wnt-induced interaction with TNIK (Figure 5A bottom panel). As expected, the interaction between β-catenin and TCF4 was Wnt-dependent. Next, we examined the effect of siRNA mediated knock-down of TNIK on TCF/β-catenin mediated TOP/FOP transcriptional activation in the presence and absence of Wnt. Significant depletion of TNIK was accomplished at 72 hours post siRNA treatment (Figure 5B top panel). Removal of TNIK resulted in specific suppression of Wnt-dependent TOPFlash activity (Figure 5B bottom panel). We concluded that TNIK is required for optimal TCF4/ β-catenin transcriptional activation in response to Wnt.

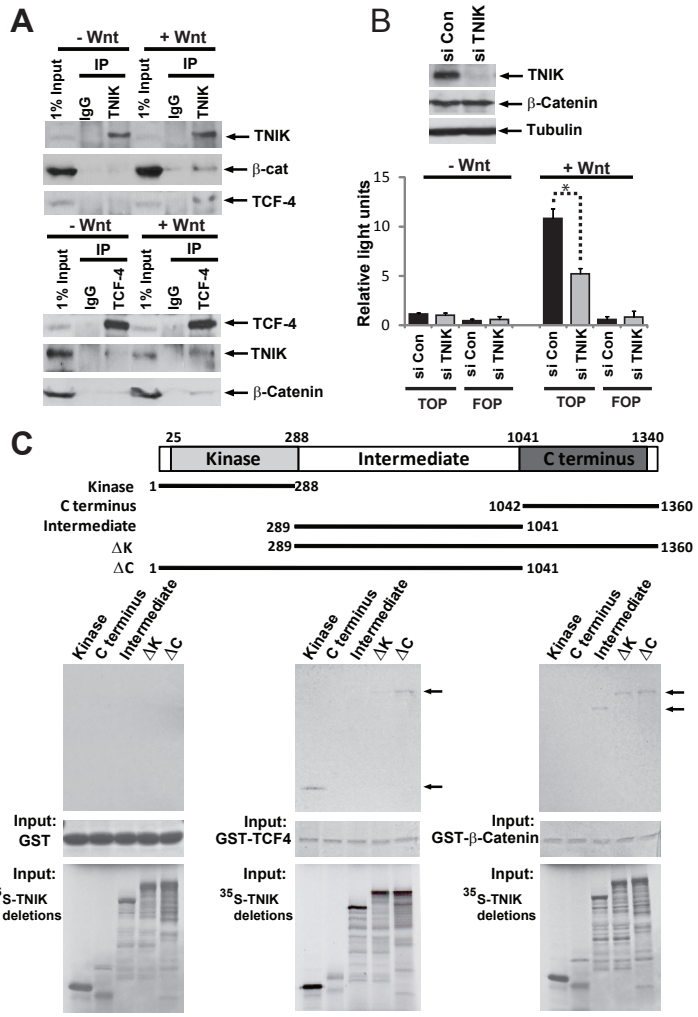

Figure 5. TNIK is interacting with TCF4 in β-catenin dependent manner. (A) TNIK interacts with TCF4 and β-catenin upon Wnt stimulation in HEK293T cells. Lysates from cells stimulated with either Wnt3A or control conditioned media as indicated for 8 hours were used to immunoprecipitate TCF4 (bottom panel) or TNIK (top panel) and their associated proteins. Immunoprecipitated protein complexes were resolved by SDS-PAGE followed by western blotting using antibodies directed against TNIK, β-catenin and TCF4 as indicated. **(B)** siRNA mediated depletion of TNIK reduces β-catenin /TCF driven transcription in HEK293T cells. Expression of TNIK and control Tubulin was analyzed by western blotting after depletion of TNIK (top panel). Activity of the TOPFlash and FOPFlash 12 hrs post Wnt stimulation is shown (bottom panel). Error bars represent standard deviation from three independent experiments. * *p-value=0.006*. **(C)** Schematic summary of different TNIK deletion mutants (top panel). All five TNIK deletion mutants were in vitro translated with S^{35} labelled methionine, and were incubated with GST-TCF4 or GST-β-catenin to examine the direct interaction. GST protein alone was used as control (bottom left panel). Only TNIK kinase and ΔC domains bind to GST-TCF4 (bottom middle panel, arrows), whereas TNIK intermediate, ΔK and ΔC domains bind to GST-β-catenin (bottom right panel, arrows).

We next probed whether TNIK can directly contact TCF4/β-catenin using in vitro binding assays. We generated in vitro translated radiolabeled TNIK deletion mutants (shown in Figure 5C) and probed their direct binding to GST-fused TCF4 and β-catenin immobilize on glutathione beads. The TNIK kinase domain and the kinase domain-containing C bound TCF4 (Figure 5C middle panel) but not to control GST beads (Figure 5C left panel) while the K TNIK lacking the kinase domain was incapable of binding TCF4 (Figure 5C middle panel). Thus, the TNIK kinase domain directly contacts TCF4. β-catenin also displayed direct binding to TNIK. The TNIK Intermediate domain and deletion mutants C and K, which contain the Intermediate region bound β-catenin, pointing to this region of TNIK as the β-catenin interaction interface (Figure 5C right panel). Thus, different domains of TNIK directly contact both TCF4 and β-catenin.

TNIK is an essential and specific activator of Wnt target genes in HEK293T cells

To determine the role for TNIK in the control of endogenous Wnt target gene expression, we studied the effect of TNIK depletion in HEK293T cell line stimulated with Wnt3A by microarray analysis. We first characterized the Wnt target gene program of HEK293T cells by inducing the cells with Wnt3A-conditioned medium followed by microarray expression analysis at three early time points, i.e. 4 hours, 7 hours and 9 hours. This resulted in 172 overlapping probes representing 144 unique genes (Figure 6A and Table S1) selected against 1.5 fold increase at either 7 or 9 hour time points of Wnt induction. While some of the Wnt-induced genes were previously described as Wnt target genes, such as TCF7[224], CCND1[225] and AXIN2[52-54] in other cell/tissue types (http://www.stanford.edu/~rnusse/Wntwindow.html)), the large majority were unique to the HEK293 kidney cells. These include various transcription factors such as BACH2, ID1, YY1, ETS2 and RUNX3. To increase the likelihood of examining direct transcriptional effects, we then focused on the 4 and 7 hour time points to examine Wnt induced expression profile changes upon TNIK depletion.

By comparing the expression pattern of Wnt induction in TNIK depleted cells to the Wnt activated genes with greater than 1.5 fold increase at 7 hour Wnt induction, depletion of TNIK by siRNA resulted in a consistent opposite pattern in both 4 hour and 7 hour time points (Figure S3A). The negative association between the overlapping genes of 7 hours Wnt induction in the presence or absence of TNIK (+7hr Wnt and si TNIK +7hr Wnt) was statistically significant (Pearson correlation coefficient, -0.508; $p < 0.001$; Figure S3B), in which genes up-regulated upon Wnt induction were down-regulated after TNIK suppression and vice versa. These results suggest an essential role for TNIK in the regulation of Wnt target gene expression.

To understand if TNIK is essential to Wnt pathway regulation, we filtered out 315 probes, representing 304 unique genes (Table S2, Figure 6B) with 1.5 fold variation in Wnt induced TNIK depleted cells (si TNIK +7hr Wnt). Surprisingly, 91.4% (288 out of 315) of the probes were down-regulated at 7 hour post Wnt induction upon TNIK depletion, demonstrating that TNIK serves preferentially as an activator in the Wnt

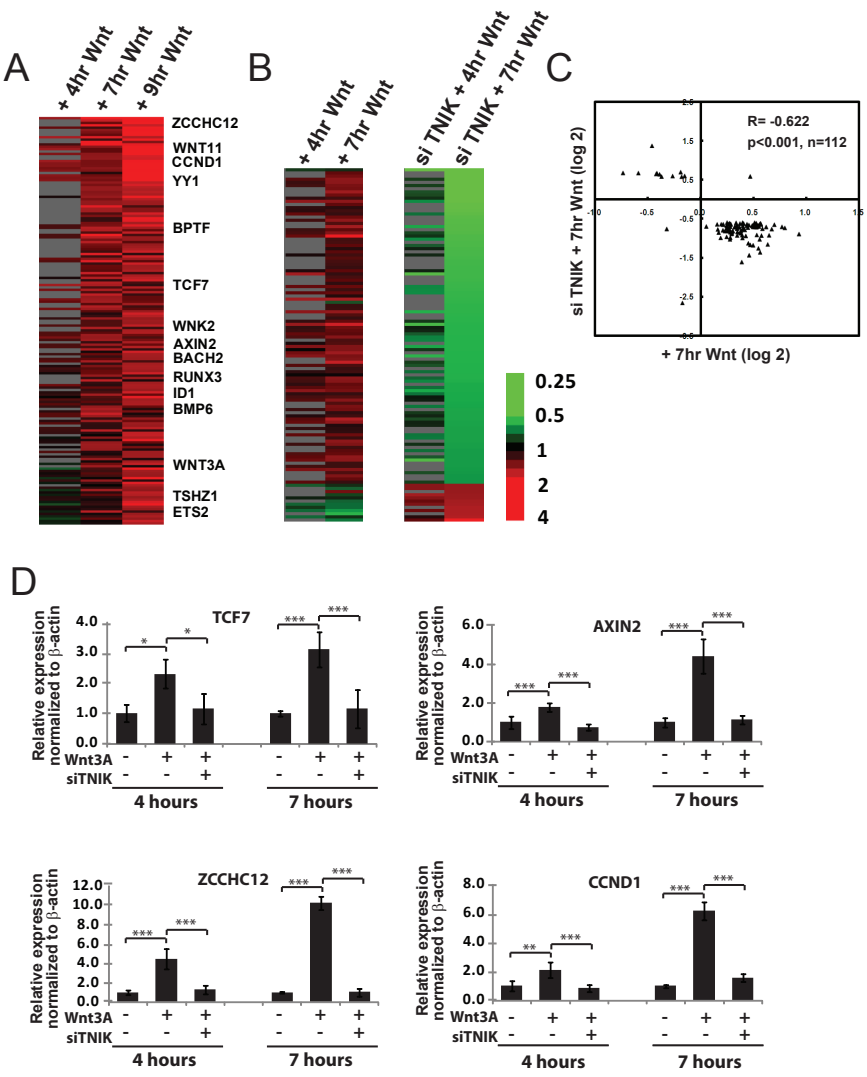

Figure 6. TNIK is an essential activator and specifically regulates Wnt target genes in HEK293T cells.

(A) Expression pattern of 172 Wnt induced genes selected against overlapping probes with 1.5 fold change in either 7 or 9 hour Wnt induction. Representative Wnt induced genes were listed. **(B)** Differential expression pattern of genes after TNIK suppression in HEK293T cells upon 4 and 7 hours Wnt induction with greater than 1.5 fold variation at 7 hour time point. The corresponding expression pattern upon Wnt induction without TNIK suppression is shown on the left. Green, down-regulated after TNIK suppression; Red, upregulated after TNIK suppression; Grey, missing data. **(C)** Significant negative correlation of the overlapping probes between 7 hours WNT induction and TNIK suppression selected by 1.5 fold change after TNIK suppression. X-axis: fold change (log2) after 7 hours Wnt induction; Y-axis: fold change (log2) after TNIK suppression. **(D)** Quantitative RT-PCR validation of four selected candidate genes. * *p-value*<0.05; ** *p-value*<0.01; *** *p-value*<0.001.

signalling pathway. In addition, 98% of the observed overlapping genes down-regulated upon TNIK depletion were up-regulated after 7 hour Wnt induction (Pearson correlation coefficient, -0.622; $p < 0.001$, Figure 6C) indicating that TNIK is an essential component in the Wnt signalling pathway. The results were reproduced by independent biological replicates with good correlation (Pearson correlation coefficient, 0.654; $p < 0.001$, Figure S4). To validate the microarray data, we chose four representative genes, *AXIN2*, *TCF7*, *CCND1* and *ZCCHC12* for quantitative RT-PCR validation. All the selected genes were upregulated in response to Wnt stimulation at 4 and 7 hours. Expression of selected target genes was abrogated to basal levels upon TNIK depletion, highlighting the essential role played by TNIK in activation of Wnt target genes (Figure 6D). In summary, our data demonstrate that TNIK serves as an essential, specific activator in regulating the Wnt target gene signature.

Nuclear Wnt signalling requires the concerted action of numerous co-activating transcriptional complexes, many of which are enzymes such as CBP[112], p300[109], MLL[108, 116]. However, most of the co-activating enzymes implicated in TCF gene activation are pleiotropic factors involved in additional cellular processes and transcriptional pathways, and thus are not specific to regulation of Wnt signalling. In fact, despite the passage of a decade after the discovery of TCF4 and β-catenin as the molecular effectors of the Wnt signal, few transcriptional activators unique to Wnt transcriptional regulation have been found. Here we report the identification of TNIK, a member of the STE20 germinal center kinase family[218], as a TCF4-interacting protein in the crypt proliferative compartment of the mouse small intestine as well as in human colorectal cancer cells. TNIK is specifically recruited in a β-catenin dependent manner to the Wnt target genes Axin2 and c-Myc. Expression of dominant negative TNIK kinase mutants abrogate TCF\LEF reporter activity highlighting the essential role of TNIK kinase activity in TCF4/β-catenin transcription regulation. Distinct regions of TNIK directly contact TCF4 and β-catenin and TNIK specifically phosphorylates TCF4. Examination of the function of TNIK by siRNA mediated knock-down followed by gene expression array analysis identifies TNIK as an essential and specific co-activator of the Wnt target gene transcription program as over 90% of genes down-regulated in response to TNIK depletion were Wnt target genes.

Mammalian TNIK has three close homologues: NIK/MAP4K4 (NCK interacting kinase), MINK (misshapen/NIK-related kinase) and NRK (NIK related kinase). While not conserved within the intermediate region, these NIK family members all share very high homology within the conserved N-terminal catalytic kinase domain as well as the C-terminal regulatory domain. In this study, our mass spectrometry data specifically identified TNIK, and not the other homologous NIK family members, as a TCF4 interactor in the proliferative mouse intestinal crypt. The specific TNIK-TCF4 interaction despite the expression of both MINK and NIK in the crypt compartment (Vries R.G. et al, unpublished expression array data), is suggestive of a unique role for TNIK in regulating the Wnt signaling pathway. Our current data reveals that TNIK binds to β-catenin via the unconserved intermediate domain, postulating

that TNIK regulates the Wnt pathway by interacting with β-catenin, resulting in phosphorylation of TCF4 and transcription activation. The specificity of TNIK for the Wnt pathway therefore likely lies in the intermediate region, which is not conserved among the different NIK family members. Further study of the homologues on the binding and phosphorlyation of β-catenin and TCF4 will help to clarify the mechanism of control of TCF/LEF/β-catenin transcription regulation. Given the interaction with β-catenin, it will also be of interest to determine whether TNIK serves as a general β-catenin/TCF activator and interacts with, phosphorylates, and regulates the activities of other TCF/LEF members in complex with β-catenin.
Subsequent study to probe the role for TNIK in the multitude of biological phenomena controlled by Wnt signalling will be important. The current data imply TNIK as a potential target for the generation of small molecule inhibitors to specifically block the Wnt pathway in disease states such as colorectal cancer.

Materials and Methods

Biochemical fractionation of mouse crypt and villus.

The small intestine of four 6-12 week old BALB C mice sacrificed by CO_2 chambers and cervical dislocation was removed, flushed with ice-cold PBS, and cut open longitudinally to expose crypts and villi. Small intestine was cut into small 1-2cm pieces and crypt and villus fractions were isolated using incubations in a mild PBS-EDTA/EGTA chelation solution combined with vigorous shaking followed by further purification using 70m (for villi) and 40m (for crypts) nylon cell strainer (Falcoln). Briefly, intestine pieces were washed several times in cold $PBS^{+/+}$ (Ca^{++}/Mg^{++}) and incubated in PBS^{o}EDTA EGTA (no Ca^{++}/Mg^{++} + 1mM EDTA + 1mM EGTA) 10min on rotator. Buffer was decanted followed by addition of fresh PBS^{o} and vigorous shaking approximately 10-15 times. Incubation in PBS^{o}EDTA-EGTA, decanting and shaking in PBS^{o} was repeated. Fractions were put through a 70 m strainer. Whole villus structures remain on top of cell strainer and are collected while the flow through (F/T) discarded. Incubation, shaking and separation through cell strainer was repeated and fractions 2-5 collected containing pure villus structures. From fraction 5, the F/T of the cell strainer containing intact crypts was collected and further purified by passage through a 40 m cell strainer. This was repeated until the mesenchyme was stripped of epithelial cells. Typically, fractions 2-4 contained pure villi while fractions 6-8 F/T contained pure crypts. Purified Crypts and villi were washed twice and kept in $PBS^{+/+}$ for up to one hour before further use.

DNA constructs

Details of cloning procedures are available upon request. Briefly, using a PCR-based strategy a sequence encoding the FLAG-epitope was added at the beginning of the TCF4, β-catenin and TNIK coding sequences and cloned into pcDNA3.1 (Invitrogen). For expression of GST-fusion proteins, the coding sequences of TCF4 and β-catenin were cloned in pGEX6P-1. For in vitro transcription/translation of TNIK deletions, indicated regions of TNIK coding sequence were cloned in pcDNA3.1

(Invitrogen).

Immunoprecipitation

Total cellular extracts from Ls174T cells, HEK293T cells or primary mouse crypt or villus material were prepared in PLB buffer (1 %Triton X-100, 2 mM EDTA, 1 mM DTT, 5 % glycerol in PBS) supplemented with protease inhibitors (PIs) (Complete, Roche Molecular Biochemicals). Cellular lysates were pre-cleared with IgG-agarose beads (Sigma) for 6 h at 4 °C. Immunoprecipitations of endogenous complexes from mouse crypt and villus, Ls174T and HEK293T cells were carried out overnight at 4 °C with anti-TCF4 (Santa Cruz), anti β-catenin (BD transductions), or anti-TNIK (Santa Cruz) (2 µg/mL) in combination with a 50 % protein G-Agarose slurry (Sigma). Immunoprecipitated material was washed three times in PLB supplemented with PIs. Bound proteins were subjected to SDS-PAGE and Western blot analysis or Mass Spectrometry Analysis.

Mass spectrometry analysis

In-gel digestion: Gel bands putatively containing TCF4, β-catenin and TNIK were sliced-out from the gels of all four pull downs (i.e. IgG villus, TCF4 villus, IgG crypt, TCF4 cryo), Figure 1B, and subjected to in-gel digestion as described previously[226]. Protein reduction and alkylation was performed with DTT (60°C, 1h) and Iodoacetamide (dark, RT, 30min), respectively. Digestion was performed with trypsin over night at 37°C. Peptides were extracted with 10%FA.

NanoLC-MS/MS: The extracted peptides were subjected to nanoscale liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) analysis, performed on an Agilent 1100 HPLC system (Agilent technologies) connected to an LTQ Linear Ion Trap Mass Spectrometer combined with either an Orbitrap (ThermoFisher, Waltham, MA) or an Fourier Transform Ion Cyclotron Resonance cell (ThermoFisher, Waltham, MA). The nanoLC was equipped with a 20mm x 100µm i.d. Aqua C18 trap column (Phenomenex, Torrance, CA) and a 400mm x 50µm i.d. Reprosil C18 analytical column (Dr Maisch, Ammerbuch-Entringen, Germany)[227]. Trapping was performed at a flow of 5 µl/min for 10 min and the fractions were eluted using a 45 min linear gradient from 0 to 40% solvent B (0.1M acetic acid in 80 %ACN (v/v), in which solvent A was 0.1M acetic acid), 40 to 100 % solvent B in 2 min and 100 % B for 2.5 min. The analytical flow rate was 100 nl/min and the column effluent was directly introduced into the ESI source of the MS using a standard coated fused silica emitter (New Objective, Woburn, MA, USA) (o.d. 360µm, tip i.d. 10µm) biased to 1.7kV. The mass spectrometer was operated in positive ion mode and in data-dependent mode to automatically switch between MS and MSMS. The three most intense ions in the survey scan were fragmented in the linear ion trap using collisional induced dissociation. The target ion setting was 5e5 for the Orbitrap, with a maximum fill-time of 250ms and 1e6 for the FTICR, with a maximum fill-time of 250ms. Fragment ion spectra were acquired in the LTQ with an AGC value of 3e4 and a max injection time of 500ms.

Protein Identification: Raw MS data were converted to peak lists using Bioworks Browser software, version 3.1.1. Spectra were searched against the IPI (International Protein Index) Human database version 3.36 (69012 sequences; 29002682 residues) or against the IPI mouse database version 3.36 (51326 sequences; 23682061 residues) using Mascot software version 2.2.0 (www.matrixscience.com), with trypsin set as enzyme. The database search was made with the following parameters set to consider a peptide t lerance of ± 15ppm, a fragment tolerance of

± 0.9Da, allowing 3 missed cleavages, Carbamidomethyl (C) as fixed modification, Oxidation (M).

Immunohistochemistry

Tissues were fixed in 10% formalin, paraffin embedded, and sectioned. Antibodies rabbit anti-Tnik (1:1000, Santa Cruz) and mouse anti-Tnik, clone 3D4 (1:500, Abnova) were used. Peroxidase conjugated secondary antibodies used were mouse or rabbit EnVision+ (DAKO).

In vitro binding assay

^{35}S-labeled reticulocyte expressed TNIK deletion mutants were generated using the TNT quick coupled transcription translation kit (Promega). Bacterially expressed GST, GST-TCF4 and GST-β-catenin were immobilized and purified on Glutathione beads for 2h at 4°C followed by extensive washes with HEMG buffer (25mm HEPES-KOH (pH7.6), 0.1mm EDTA, 12.5mm$MgCl_2$, 10% glycerol, 1mm DTT, 0.01% Nonidet P-40 (NP-40), protease and phosphatase inhibitors) containing 0.5-0.1M KCl. Beads were incubated for 2h with ^{35}S-labeled reticulocyte expressed TNIK deletion mutants, washed extensively with HEMG buffer containing 0.1M KCl and analyzed by SDS-PAGE followed by autoradiography.

In vitro kinase assay

Immunoprecipitated wild type or mutant Flag-TNIK was incubated with purified GST-TCF4 and GST-β-catenin fusion proteins. Phosphorylation reactions were performed in 30μl kinase buffer (20mM Hepes (pH 7.5), 20mM $MgCl_2$, 0.1mM orthovanadate, and 2mM dithiothreitol) supplemented with 20μM ATP and 5μCi of [γ-^{32}P]ATP at 30°C for 30min. Reactions were stopped by the addition of 4x Laemmli sample buffer and resolved by SDS-PAGE followed by autoradiography.

RNA interference

Pre-designed Dharmacon siRNA pools targeting transcripts of the human TNIK(L-004542-00) and non-target control siRNA pool(D-001810-10-20) were used to knock down TNIK in Ls174T colorectal cancer and HEK293T cells. siRNA was delivered into Ls174T, SW480 and HEK 293T cells using the siLentFect Lipid Reagent (BioRad). siRNA (15nM) was used to transfect 2X10^5 cells, and protein levels were examined by western blot analysis 72h after transfection.

Transient transfection and luciferase assays

Ls174T CRC and HEK 293T cells were seeded at a density of 2 x 10^5 cells/12-well plate. siRNA targeting the human TNIK gene or control non-targeting siRNA was delivered the next day with siLentFect Lipid Reagent (BioRad) according to the manufacturer's instructions. After 72h, cells were transfected with plasmid constructs pGL-TOP and pGL-FOP optimal and mutated TCF luciferase-reporter constructs as described previously208. The cells were lysed after 24h using luciferase lysis buffer (Promega Corp.), and luciferase activities were measured using the Dual-Luciferase reporter assay system. Transfection efficiency was normalized using the cotransfected *Renilla* luciferase activity as an internal control. The data shown are of two independent triplicate experiments as the mean ± S.D. of the ratio between the TOP/FOP and *Renilla* reporters.

Chromatin immunoprecipitation

Ls174T CRC cells or purified mouse crypt and villus fractions were fixed by adding formaldehyde to a final concentration of 1 % for 30 min at room temperature. Glycine was added to a final concentration of 125mM to quench the reaction. Cells were washed twice with buffer B (0.25 % Triton-X 100, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6) and buffer C (150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6). Cells were resuspended in 0.3 % SDS, 1 % Triton-X 100, 0.15M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6) and sheared using a BioRuptor sonicator (Cosmo Bio Co., Ltd) with twelve 30-sec pulses at the maximum setting. 5g of the indicated antibody TCF4 (Santa Cruz), β-catenin (BD Transduction), TNIK (Santa Cruz) was incubated with the sheared cross-linked chromatin and BSA blocked protein-G beads overnight at 4°C. Approximately 20 million cells were used per IP. IPs were washed twice with each buffer1 (0.1% SDS, 0.1% deoxycholate, 1%Triton-X 100, 150mM NaCl, 1mM EDTA, 0.5mM EGTA, 20mM Hepes pH7.6), buffer2 (0.1%SDS, 0.1%deoxycholate, 1%Triton-X100, 0.5M NaCl, 1mM EDTA, 0.5 mM EGTA, 20 mM Hepes pH7.6), buffer3 (250 mM LiCl, 0.5% deoxycholate, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6), buffer4 (1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.6). Immunoprecipitated complexes were eluted in 1% SDS, 0.1 M $NaHCO_3$ for 20 min, and incubated overnight at 65 °C for removal of crosslinks in presence of 200 mM NaCl. DNA was phenol: chloroform extracted, chloroform: isoamylalcohol extracted, ethanol precipitated. Input and immunoprecipitated DNA were subjected to Sybergreen qPCR with primers overlapping the promoters, upstream/downstream regulatory regions of mouse and human Axin2 and c-Myc genes.

Quantitative RT-PCR (qRT-PCR)

ChIP experiments were analyzed by qPCR in an iCycler iQ real-time PCR detection system (BioRad) using iQ Sybergreen Supermix (BioRad). ChIP values were normalized as a percentage of input, and presented as fold enrichment over the percentage of input immunoprecipitated over the second exon of the myoglobin gene (human samples) and the Sp5 downstream region (mouse samples) as control. The following primer pairs were used for examining recruitment to the human Axin2 promoter: 5'GTTACCTGGAAGACGAAGG3'; 5'TCT GGA GGC GTT CAG TTG3', human Axin2 upstream control region: 5'GCC AGA GTC AAG CCA GTA GT3'; 5'TAG CCT AAT GTG GAG TGG AT3', mouse Axin2 promoter: 5'CCA CCA AGA CCT ACA TAC3'; 5'CCA CTC CTC ACA TAT TCC3', mouse Axin2 downstream control region: 5'CTA CCG TGT TAC TCT GAC TAT TCT TC3'; 5'GCG GCT CAG TGG CTA AGG3', human c-Myc promoter: 5'ACT CAG TCT GGG TGG AAG G3'; 5'GGA ATG ATA GAG GCA TAA GGA GTA TC3', human c-Myc downstream control region: 5'CAG CAA GAT AGC AGA GGA AG3'; 5'TGG AGA TGG ATT GAA TGA AGG3', mouse c-Myc promoter: 5'CTC ACT GGA ACT TAC AAT CTG3'; 5'CAA CGC CCA AAG GAA ATC3', mouse c-Myc upstream control region:5'ACT CAA GAA CTG GTA GAC3'; 5'CTA AGT GGG TAA GGT AGG3', second exon of human Myoglobin gene: 5'AAG TTT GAC AAG TTC AAG GAC C3'; 5'TGG CAC CAT GCT TCT TTA AGT C3', mouse Sp5 downstream control region: 5'TGG AGT TAC AGG CAG TGG TG3'; 5'ATT GAT TTC TTT AGG TTG GGT GGA G3'.

Site-directed mutagenesis

TNIK kinase mutants TNIK R152A/D153A, TNIK K54A, and TNIK D171A/F172A were generated using the Quick Change Site-Directed Mutagenesis kit (Stratagene). Wild type Full length Flag tagged

TNIK expression vector was used as a template for PCR based mutagenesis using the following primers: TNIK R152A/D153A For 5'CCA GCA TAA AGT GAT TCA TGC AGC TAT TAA AGG GCA AAA TGT CTT G3', TNIK R152A/D153A Rev 5'CAA GAC ATT TTG CCC TTT AAT AGC TGC ATG AAT CAC TTT ATG CTG G3', TNIK K54A For 5'GCC AGC TTG CAG CCA TCG CGG TTA TGG ATG TCA CAG G3', TNIK K54A Rev 5'CCTGTGACATCCATAACCGCGATGGCTGCAAGCTGGC3', TNIK D171A/F172A For 5'GCA GAA GTT AAA CTA GTG GCC GCT GGA GTC AGT GCT CAG C3', TNIK D171A/F172A Rev 5'GCT GAG CAC TGA CTC CAG CGG CCA CTA GTT TAA CTT CTG C3'.

Microarray analysis

Total RNA was extracted from HEK293T cells after siRNA transfection (siTNIK or siNon target control) and induced with Wnt3A expressing conditioned medium (Wnt3A) or control medium (CM) for 4, 7 or 9 h using RNeasy Mini Kit (Qiagen) according to ATCC instruction. 2µg of RNA from each sample was labelled by Quick Amp Labelling Kit, two colour (Agilent) and corresponding pairs of samples were hybridized to 4X44K Whole Human Genome dual colour Microarrays (Agilent, G4112F). Six pair of samples: si-control+Wnt3A 4h / si-control+CM 4h (+4hr Wnt); si-control+Wnt3A 7h / si-control+CM 7h (+7hr Wnt); si-control+Wnt3A 9h / si-control+CM 9h (+9hr Wnt); si-TNIK+Wnt3A 4h / si-control+Wnt3A 4h (siTNIK+4hr Wnt); si-TNIK+Wnt3A 7h / si-control+Wnt3A 7h (siTNIK+7hr Wnt, Experiment1); si-TNIK+Wnt3A7h / si-control+Wnt3A 7h (siTNIK+7hr Wnt, Experiment2) were hybridized in two dye swap experiments resulting in twelve individual arrays. Microarray signal and background information were retrieved using Feature Extraction program (V.9.5.3, AgilentTechnologies). For each pair of experiment, fluorescent signals in either channel with greater than 2 times above the local background and showing the same trend of fold change in the corresponding dye swap experiments were considered as well measured and average was taken. Overlapping genes with greater than 1.5 fold variations in either +7hr Wnt or +9hr Wnt (144 unique genes) were selected as Wnt regulated genes in HEK293T cells, whereas genes with greater than 1.5 fold variation (304 unique genes) in siTNIK +7hr Wnt were selected as TNIK regulated genes. Array data is available at Gene Expression Omnibus under accession number GSE17623.

Statistical analysis

Comparison of differences between TOPFlash activities or qRT-PCR expression was performed using two-tailed student *t*-test (where equal variance between groups was assumed). To examine the correlation of expression fold change in corresponding microarray experiments, Pearson correlation was performed whereas R represent correlation coefficient and P value<0.05 was considered as significant.

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Supplementary Data

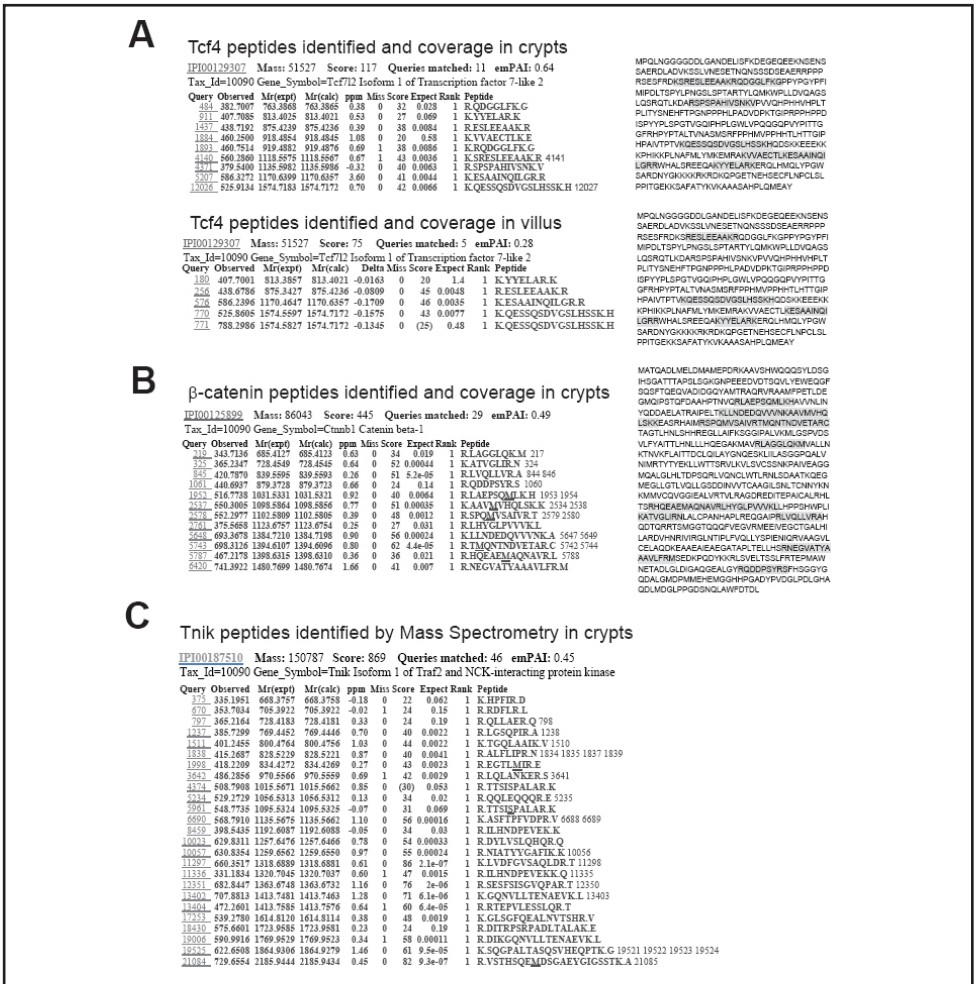

Figure S1. Peptides identified and coverage of TCF4, β-catenin and TNIK in Tcf4 complexes from mouse crypt and villus.

(A) Tcf4 in crypt (top) and villus (bottom). **(B)** β-catenin in crypt. **(C)** TNIK in crypt.

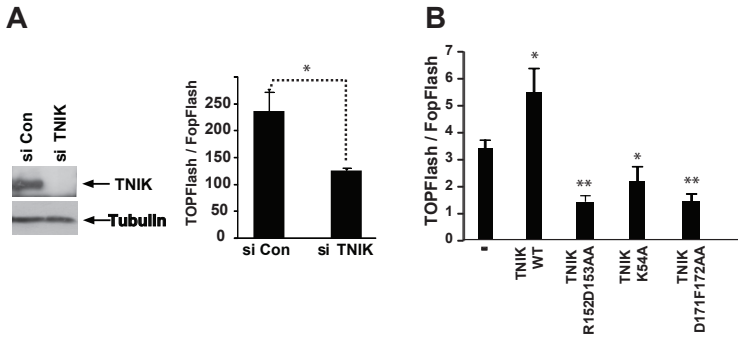

Figure S2. TNIK expression modulates TCF/LEF transcription regulation.

(A) Suppression of TOPFlash activity upon TNIK depletion in SW480 cells. **(B)** Over-expression of WT TNIK specifically increases β-catenin/TCF driven transcription in DLD1 CRC cells whereas expression of the three TNIK kinase mutants abrogates β-catenin/TCF driven transcription. Black bars indicate ratio of TOP/FOPFlash activity. * *p-value*<0.05; ** *p-value*<0.01.

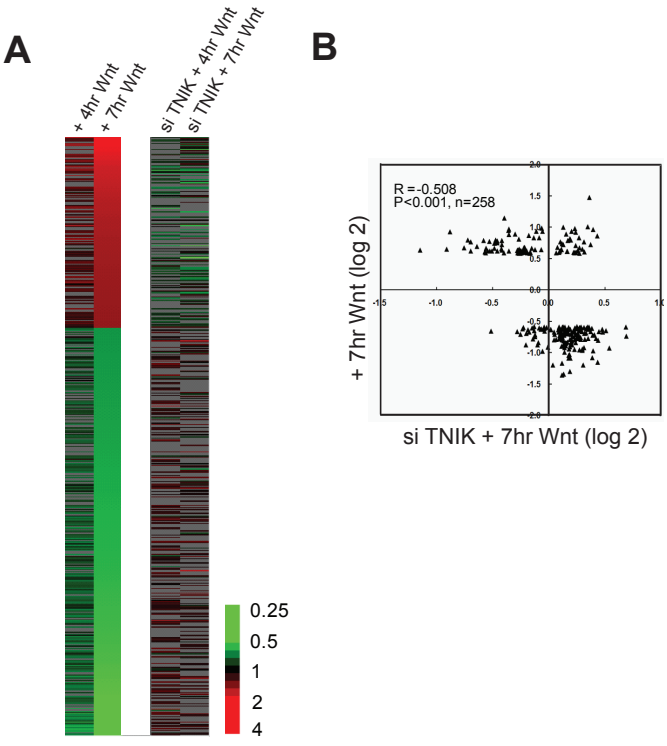

Figure S3. Comparison of expression profile changes between Wnt induction and TNIK depletion.

(A) Differential expression pattern of Wnt regulated genes in HEK293T cells upon 4 ans 7 hours Wnt induction with greater than 1.5 fold variation at 7 hour time point. The corresponding expression pattern after TNIK suppression is shown on the right. Red, up-regulated by Wnt; green, down-regulated by Wnt; Grey, missing data. **(B)** Significant negative correlation of the overlapping clones between 7 hours Wnt induction and TNIK suppression selected by 1.5 fold change after 7 hours Wnt induction. X-axis: fold change (log2) after TNIK suppression; Y-axis: fold change (log2) after 7 hours Wnt induction.

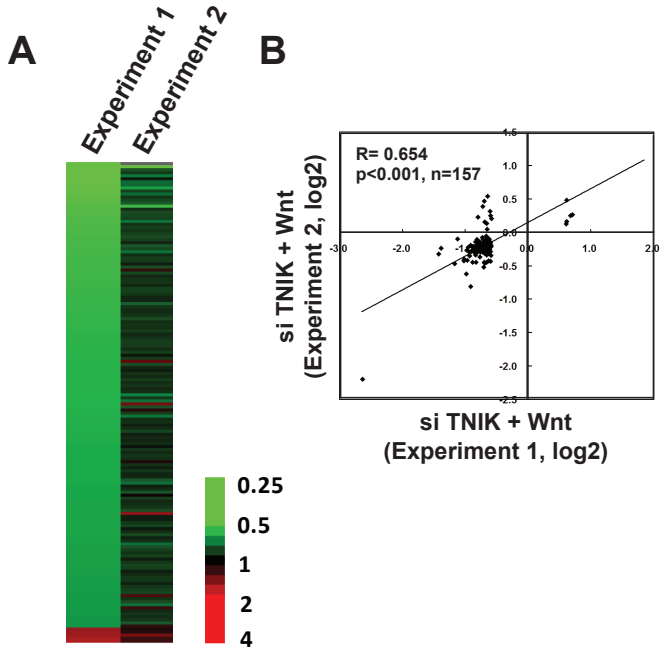

Figure S4. Comparison of independent biological replicates of si TNIK +7hr Wnt stimulation.

(A) Differential gene expression pattern after TNIK suppression in HEK293T cells with Wnt induction in two independent experiments with greater than 1.5 fold variation at 7 hour time point. **(B)** Pearson correlation of the 157 overlapping probes between the two biological replicates. Trendline representing the positive correlation is shown.

Supplementary Table 1 (Table S1) and Supplementary Table2(Table S2) are available at The EMBO Journal Online (http://www. embojournal.org).

Chapter 7

Summarizing Discussion

Since the discovery of the Wnt-1/Int-1 gene as a proto-oncogene in mammary gland tumors, intense investigation by numerous laboratories has clearly shown the essential role of the Wnt/β-catenin signaling pathway in embryonic development and adult tissue regeneration[2, 228, 229]. During Wnt/β-catenin signal transduction, there are at least three essential steps which occur after binding of Wnts to the co-receptors Frz/Fz and LRP5/6[2]: (1) phosphorylation of LRP5/6 receptor PPPSPxS motifs which leads to Axin1 membrane recruitment; (2) inhibition of β-catenin destruction complex which results in accumulation of cytoplasmic β-catenin; and (3) the recruitment of β-catenin to Tcf/Lef family-bound regulatory DNA regions leading to activation of the Wnt target gene transcription program. However, the dynamic processes and underlying molecular mechanisms under physiological conditions following Wnt stimulation remain elusive. The major focus of this thesis is to unravel the regulatory mechanisms of the Wnt/β-catenin signal cascade transduction, particularly the modulation of the β-catenin destruction complex and Tcf/Lef mediated target gene transcription.

In Wnt unstimulated cells, E-cadherin unbound cytoplasmic β-catenin protein is constantly eliminated by the active β-catenin destruction complex. The limiting factor Axin1 serves as a scaffold and mainly recruits APC, CKIα and GSK3α/β to form the destruction complex[2]. Within the complex, CKIα phosphorylates β-catenin on Ser45 priming β-catenin phosphorylation by GSK3α/β on Thr41, Ser37 and Ser33[8]. Ser37 and Ser33 phosphorylated β-catenin phosphorylation is then recognized by β-TrCP, an E3 ubiquitin ligase, which subsequently ubiquitinates β-catenin leading to its proteasomal degradation[8-10]. Upon Wnt conjugation to the Frz/Fz and LRP5/6 co-receptor, the LRP5/6 intercellular PPPSPxP motifs is phosphorylated through undefined mechanisms[14]. Phosphorylated LRP5/6 then recruits Axin1 to the membrane thereby dephosphorylating and destabilizing Axin1[37-39]. However how these events lead to inactivation of the β-catenin destruction complex is not well established yet. Several inactivation models have been proposed by numerous research groups including: (1) inactivation of GSK3α/β kinase activity[70, 77-79] ; (2) disassembly of the complex due to either Axin1 degradation or its membrane recruitment or dissociation of APC and GSK3α/β from the complex[13, 15, 17, 19, 66, 67, 69, 70]; and (3) dephosphorylation of phosphorylated β-catenin by phosphatases[86].

GSK3α and GSK3β, collectively termed GSK3, are a global constitutively active serine/theronine kinase involved in multiple biological signaling pathways[165]. GSK3 can be inactivated through PKB/Akt phopshorylation on its N-terminal serine residue, GSK3α on Ser21 and GSK3β on Ser9[75, 76]. Several growth factors such as insulin can activate PKB/Akt kinase activity through activation of PI3K. Similar with Wnt/β-catenin pathway, the PI3K/PKB pathway also participates in diverse biological processes and its aberrant activation also leads to many pathological changes. Previous studies have shown that the inhibitory serines from the two GSK3 proteins had no effect on Wnt

signaling, suggesting that these two pathways do not cross-talk at the level of GSK3[167, 230]. On the other hand, several other studies were conducted on the assumption that both PI3K/PKB and Wnt/β-catenin pathways share a single pool of GSK3 effector for their signal transduction (**Chapter 2 Supplementary Table1**). According to this premise, the PI3K/PKB signaling pathway could influence Wnt/β-catenin signal transduction through inactivation of GSK3 or by causing its dissociation from Axin1, leading to deregulated Wnt signaling. If PI3K/PKB pathway and Wnt/β-catenin shared the same pool of GSK3 protein, constitutive activation of PI3K/PKB pathway would also activate Tcf/Lef mediated transcription. Therefore, in **Chapter 2**, we first examined a series of cancer cell lines, which contain constitutive active PI3K/PKB pathway for their Wnt transcriptional activity. Using β-catenin/Wnt/Tcf (TOPFlash) reporters, we examined if Wnt is active in these cell lines. We found none of these PI3K active cell lines to be positive except one, DU4475, which also harboured an APC mutation[85]. Further analysis showed that, most of the tested cell lines can activate the TOPFlash when either treated with GSK3 inhibitor, LiCl or with Wnt3A conditioned medium, or when transfected with Wnts or β-catenin. More importantly, this activation is not influenced by the presence of PI3K or PKB inhibitors. This observation is further confirmed in an *in vivo* system, the nematode *C.elegans* and a physiological model HEK293T cells. Moreover, by taking advantage of the Axin specific antibodies we generated, we found that when GSK3β bound to Axin1, its respective serine residue phopshorylation state is no longer modulated by PI3K/PKB pathway activity. Noteworthy that GSK3α and GSK3β contribute equally in Wnt/β-catenin signaling regulation, and a single *GSK3α* or *GSK3β* gene allele is enough for normal Wnt/β-catenin transduction[231]. In addition, the amount of GSK3β that bound to Axin1 is also not affected by the PI3K/PKB activity. These results indicate that, Axin1-bound GSK3 is shielded from activated PKB. We provide for the first time, direct evidence for the presence of different endogenous pools of GSK3 in the cells, a hypothesis originally presented by Frame and Cohen[182]. Our results demonstrate that the two pathways do not necessarily cross-talk via sharing the same GSK3 effector molecule. Moreover, we found Axin1-bound GSK3β pool contains "Ser9 phosphorylated" GSK3β. It was shown previously that this serines phosphorylation event is not required for GSK3 kinase regulation in Wnt/β-catenin signaling[167, 182, 230, 231]. In the future it will be interesting to examine the kinase activity of Ser9- phosphorylated GSK3 and its role in the destruction complex.

Our studies clearly showed that PKB inactivation of GSK3 does not affect Wnt/β-catenin signal transduction. However, how Wnt inactivates GSK3 or more generally how Wnt inhibits the function of the β-catenin destruction complex and induces cytoplasmic β-catenin accumulation remains unresolved. To probe this question, we set out to identify potential novel components of the destruction complex in both its active state as well as in the presence of Wnt stimulation, which functionally inactivates the complex. Using an unbiased approach, we immunoprecipitated Axin complexes

from Wnt-inducible HEK293T cells and oncogenic β-catenin mutant expressing cell line Ls174T coupled with MASS SPEC analysis (**Addendum Chapter 5)**. With this approach, we successfully identified several interesting Axin interactors such as MAP3K1, RanBP2, HAX-1, Dlg-1 and CASK. **In Chapter 3**, we explored the role of one of these interactors, MAP3K1, a candidate we found in the Axin1 complex in Ls174T cells, in the canonical Wnt pathway. MAP3K1 was originally identified as an Axin interactor shown to be essential in the JNK signaling pathway (non-canonical Wnt signaling)[158-160]. We found that the interaction between Axin1 and MAP3K1 is modulated by canonical Wnt signaling; Wnt stimulation results in increased binding of MAP3K1 to Axin1. Moreover, using different point mutants as well as siRNA depletion of endogenous MAP3K1, we found that only the ubiquitin E3 ligase function of MAP3K1 is essential for its positive regulation of the Wnt/β-catenin pathway. The kinase activity of MAP3K1 shown previously to be critical for its role in the non-canonical Wnt pathway was dispensable for its regulation of the Wnt/β-catenin pathway. Our data suggests that MAP3K1 regulates canonical and non-canonical Wnt signaling via distinct functional domains. Moreover, depletion of MAP3K1 or expression of dominant negative MAP3K1 does not affect TCF-driven transcription in HCT116 CRC, which expresses mutated β-catenin, thereby placing MAP3K1 upstream of β-catenin as a positive regulator of Wnt signaling. Future studies are needed to identify the ubiquitin substrate of MAP3K1 within the destruction complex and to determine how this ubiquitination affects the function of the destruction complex.

Within the destruction complex, APC and Axin1 have been reported to be dephosphorylated and degraded upon Wnt induction. However, thus far, the dedicated ubiquitin ligase for neither protein has been identified. Given the Wnt-dependent presence of MAP3K1 in the Axin1 complex, it will be interesting to examine whether MAP3K1 is the dedicated E3 ubiquitin ligase for either APC or Axin1. Aside from its role in marking proteins for proteasomal degradation, ubiquitination has also been reported to control intracellular localization and activity of proteins. Therefore, MAP3K1-mediated ubiquitination may also potentially modulate the activity and localization of its substrate and thus regulate the function of the dedicated β-catenin destruction complex.

In parallel to the identification and functional characterization of novel components of the Axin-β-catenin destruction complex, we also examined the mechanism by which Wnt activation functionally inhibits the endogenous β-catenin destruction complex. Although biochemical studies probing the dynamic states of the endogenous β-catenin destruction complex in response to Wnt activation are largely lacking, several models have been proposed to describe how Wnt activation disables the destruction complex. These include degradation of Axin1 itself, sequestration of Axin1 at the membrane, and disruption of the interaction between Axin1 and/or APC and GSK3 or β-catenin, and inactivation of the kinases GSK3 and CK1. Most of these

proposed models assume a physical dissociation of the destruction complex as a consequence of Wnt stimulation. In **Chapter 4** we used a specific antibody against Axin1, the least abundant regulatory scaffold of the complex to determine, by immunoprecipitation, the Wnt-induced dynamic change in composition of the known components within the complex. First, as Axin1 was reported to become degraded in the presence of Wnt, we compared the kinetics of Axin1 protein degradation with the accumulation of cytoplasmic β-catenin upon Wnt induction in HEK293T cells. We found Axin1 protein level was stable for several hours after β-catenin was accumulated in the cytoplasm indicating inactivation of the destruction complex. Therefore, we concluded that Axin1 degradation is not the direct cause of Wnt mediated inhibition of the destruction complex which was also proposed by other groups[58-60]. Axin1 immunoprecipitation experiments demonstrated that the core components of the dedicated complex, APC, GSK3β and β-catenin remain correctly assembled in Wnt stimulated HEK293T cells. Thus, it is unlikely that dissociation of the core components of the complex is a direct mechanism for its Wnt inactivation. We also examined the state of phosphorylation of β-catenin in the Axin1 complex and found the pool of phosphorylated β-catenin bound by Axin1 to be increased in the Wnt activated conditions. This observation indicates that the two essential kinases GSK3 and CK1 remain functional within the complex after Wnt stimulation. Although β-catenin was still able to be phosphorylated in the destruction complex after Wnt stimulation, probing the potential ubiquitination of the phosphorylated β-catenin in the following step, we found that Wnt stimulation indeed blocks β-catenin ubiquitination. This blockage of ubiquitination we found was the consequence of dissociation of the dedicated E3 ubiquitin ligase, β-TrCP from the complex.

Our observations suggest that, Wnt stimulation prevents the ubiquitination of phosphorylated inactive β-catenin in the destruction complex, thereby "clogging" the destruction complex with phosphorylated β-catenin. As a result, the limitedly abundant Axin1 is saturated with phosphorylated β-catenin, leading to accumulation of newly synthesized β-catenin that shuttles into the nucleus to activate the Wnt transcriptional program. Our data also indicates that, the β-catenin destruction complex is required not only for β-catenin phosphorylation but also for its ubiquitination and degradation. This notion is further supported by our Axin1 MASS SPEC screen for novel interactors within the complex from Wnt induced HEK293T cells, in which we identified several proteasomal subunits co-immunoprecipitated in the Axin1 complex **(Addendum Chapter 5, Table 1**). Our data reveals an as yet unexplored step in processing of the Wnt signal. How Wnt induces the dissociation of β-TrCP from the other components of the complex, and which component of the destruction complex mediates the recruitment and binding of β-TrCP are immediate questions of interest, which further studies will determine.

Of interest, a recently identified negative regulator, WTX was reported to be essential

for β-catenin ubiquitination[87]. However, how WTX modulates β-catenin ubiquitination is not well undefined. From our MASS SPEC analysis of Axin1 interactors in the presence and absence of Wnt induction, we found not only that more WTX associated with Axin1, but we also observed an increase in phosphorylation of WTX upon Wnt induction. This data provides a starting point to study the role of WTX on β-catenin ubiquitination. Generation of specific WTX antibodies, both phosphor- and nonphosphor- specific antibodies for endogenous functional study of this protein will give us some clues.

Another possible target in functional inactivation of the destruction complex is through inactivation of APC and Axin1 protein function. The tumor suppressor APC gene has been detected in about 80% of sporadic colorectal polyps and carcinomas[120]. Most APC mutations in colorectal cancer are clustered in a certain region of the central domain, also called the Mutation Cluster Region (MCR)[121, 122]. This results in a protein that is truncated at its C-terminus, eliminating the Axin binding SAMP motifs and several β-catenin binding 20-aa repeats. This truncation in APC is commonly believed to abolish its binding to Axin1 and also weaken its binding to β-catenin, thereby causing β-catenin to escape phosphorylation and degradation by the proteasome. β-catenin then accumulates, translocates to the nucleus and together with Tcf/Lef family aberrantly activates Wnt target genes as an early event in tumorigenesis in the intestinal tract. However, there has been a lack of biochemical data probing the endogenous destruction complex to support this assumption. In **Chapter 4**, we also examined the endogenous composition of the known components in constitutively Wnt activated colon cancer cell (CRC) lines, Ls174T, DLD-1 and SW480. Surprisingly, we found that APC co-immunoprecipitated together with Axin1 not only in Ls174T cells which express wild-type APC and Ser45 point mutated β-catenin but also in DLD-1 and SW480 cells that express truncated APC. Moreover, β-catenin and GSK3 were also present in Axin1 complexes in these cell lines. When we further examined the β-catenin phosphorylation state in the Axin1 complex, we found again phosphorylated β-catenin in APC mutant DLD-1 and SW480 CRCs as in Wnt activated HEK293T cells. This further highlights first, that Wnt activation does not lead to disassembly of the dedicated destruction complex, and that the destruction complex is required for other critical steps in addition and subsequent to phosphorylation of β-catenin. Our results refute the currently accepted dogma regarding the mechanism of Wnt activation in APC-truncated CRCs; in DLD-1 and SW480 CRCs we found truncated APC was bound and present in the Axin1 complex, and GSK3β was also present and functional within the complex as shown by the presence of a large pool of phosphorylated β-catenin. These observations, in particular by conducting experiments using physiological endogenous conditions, raise the important question of how truncated APC results in accumulation and nuclear translocation of β-catenin. In other words, what is the essential role played by APC during the β-catenin elimination process? Our observations indicate that

APC protein function is most likely required post-β-catenin phosphorylation. APC was reported to act as a "ratchet" to remove phosphorylated β-catenin from Axin for ubiquitination and for making Axin available for further rounds of β-catenin phosphorylation[64, 65]. It will be critical to check whether the ubiquitination states of β-catenin in DLD-1 and SW480 cells are similar to Wnt induced HEK293T cells, and whether the E3 ubiquitin ligase β-TrCP is capable of interacting with the Axin1 complex in APC truncated CRCs. Interestingly, Su *et al* have recently shown that β-TrCP can only interact with β-catenin in the presence of ectopic expressed wild-type APC in those cell lines. Their studies further proposed that APC functions by protecting phosphorylated β-catenin from dephosphorylation by PP2A and allows its targeting to β-TrCP for ubiquitination[86]. However, endogenous interaction of these proteins in Wnt-inducible systems such as HEK293T has not been demonstrated. Moreover, how wild-type APC is inactivated by Wnt thereby prohibiting its function to protect β-catenin dephosphorylation and transferring phosphorylated β-catenin to β-TrCP also remains elusive. Similar to what has been reported by other groups, we also found dephosphorylation of both Axin1 and APC upon Wnt induction. Although dephosphorylation of these two proteins has been reported to cause their degradation or dissociation from the complex, dephosphorylation may also work by functionally inactivating these proteins. Further studies of these phosphorylation sites may provide some clues as to their function as negative regulators of the Wnt pathway. In addition, our observed increased binding of the ubiquitin E3 ligase, MAP3K1 to the destruction complex in Wnt activated cells also provides another possible mechanism of inactivation of Axin1 or APC leading to β-TrCP dissociation.

Axin1 is recruited to phosphorylate LRP5/6 immediately after Wnt engagement to its co-receptors. Moreover, this recruitment is also believed to bring CKI and GSK3 to the membrane to phosphorylate LRP5/6 and amplify the Wnt signal[15, 19]. Additionally, recruitment of Axin1 to LRP5/6 is also suggested to result in its dephosphorylaton and degradation[17, 60]. Our studies clearly show that the total amount of Axin1 remains stable for several hours after Wnt stimulation in HEK293T cells. This indicates that, degradation of Axin1 in response to Wnt may be a mechanism to amplify the effect of the Wnt signal rather than a primary event to inactivate the destruction complex causing cytoplasmic β-catenin accumulation. It will be important to examine whether upon Wnt stimulation, the compositionally intact destruction complex is recruited to the membrane together with Axin1, or if Wnt stimulation causes recruitment of only a part of the Axin1-GSK3 complex to the membrane. Future biochemical fractionation experiments will determine the change in cellular localization of Axin1 and other components of the complex in response to Wnt stimulation. Such studies will clarify if there is more than one Axin1 complex present in cells in the Wnt on and off states and can probe any potential change in their composition. Moreover, using cyclohexamide treatment, it will be possible to address whether de novo synthesis of β-catenin or dephosphorylation of phosphorylated β-catenin is the main cause and source of rapid

β-catenin accumulation upon Wnt induction as suggest by other group.

How Wnt activation leads to transcription of target genes is another important regulatory and final step in relaying of the Wnt signal. After β-catenin accumulation in the cytoplasm and nuclear localization, β-catenin is thought to complex with members of the Tcf/Lef family of sequence-specific transcription factors, displacing the Grouche/TLE, which in resting cells represses the transcription of Wnt target genes as a Tcf/Lef co-repressor[11]. β-catenin/Tcf/lef are thought to activate Wnt target gene transcription via recruitment of a number of other transcriptional co-activators to Tcf/Lef target genes to facilitate transcription[116]. These include chromatin associated factors (Pygopus, BCL9, members of the two classes of chromatin modulating complexes, ATP dependent chromatin remodelers (BRG1, ISWI) and enzymes that mediate covalent modifications on histone tails (p300, CBP, MLL) as well as others. However, the mechanism by which Tcf/β-catenin coordinate the activities of these co-activators to activate Wnt target genes is not well understood. In addition, given the significant consequences of constitutive Wnt signaling in colorectal cancer, it is important to identify the complete Tcf/β-catenin co-activator complex, not only for better understanding the mechanism of Wnt target gene activation but also to identify potential new targets for drug therapy. In **Chapter 6**, we set out to identify, using an unbiased MASS SPEC approach, potentially novel co-activators/co-repressors of Tcf4 from purified mouse small intestinal tissue. In the intestine, Wnt/β-catenin signaling is required for proliferation and differentiation of the cells. Wnt/β-catenin signaling is active in the crypt compartment and necessary for maintenance and proper function of progenitors and stem cells. However, as progenitors divide and move upwards in the crypt structure, they lose contact with the Wnt signal, which is expressed in a gradient starting at the bottom of the crypt. As a result they start to differentiate into the villus-specific cells. Wnt/β-catenin signaling is therefore inactivated in the differentiated villus compartment. We performed Tcf4 immunoprecipitation from purified mouse crypt and villus fractions followed by MASS SPEC analysis of the Tcf4 interacting proteins from the proliferative and differentiated compartment. We identified Tnik, a member of the germinal centre kinase family as a Tcf4 interactor specifically in the proliferative crypts of mouse small intestine.

We found that Tnik is recruited to promoters of Wnt target genes in mouse crypts and in Ls174T colorectal cancer cells in a β-catenin-dependent manner. Depletion of TNIK and expression of TNIK kinase mutants abrogated TCF–LEF transcription, highlighting the essential function of the kinase activity in Wnt target gene activation. We found that TNIK directly binds both TCF4 and β-catenin. However, *in vitro* kinase assays further showed that, Tcf4 but not β-catenin is the phosphorylation substrate of Tnik. We examined the function of this interaction on Wnt target gene expression using siRNA depletion of TNIK followed by expression array analysis. We found that TNIK is

an essential and specific activator of the Wnt transcriptional program, at least in the cell lines examined. Due to this specificity, this kinase may present an attractive candidate for drug targeting in colorectal cancer.

How β-catenin/Tcf coordinates the binding and function of the diverse enzymatic activities and cofactors that are required for Wnt target genes activation is not fully characterized. For example, it is not clear whether these identified co-activators are present in one large complex, or if the Tcf/β-catenin-coactivator complexes are different on distinct regulatory regions and target gene promoters. Furthermore, the role of Tnik in this process also remains to be elucidated. It will be interesting to examine the effect of Tcf4 phosphorylation by Tnik on its DNA binding, transcriptional activity, interaction with other cofactors, and cellular localization. Future studies will be critical to delineate these mechanisms.

In summary, the findings described in this thesis support a model for regulation of the β-catenin destruction complex and TCF/β-catenin target gene transcription as summarized in Figure1. In the absence of Wnt, Frizzled and LRP5/6 co-receptors together with the cytoplasmic effector protein Dvl are inactive, thereby the Axin1 scaffolded β-catenin destruction complex functions properly and eliminates cytoplasmic β-catenin (Figure 1A). In the nucleus, the Tcf/Lef transcription factor family members complexed with transcription co-repressor such as Grouche/TLE suppress Wnt target gene expression. Within the dedicated complex, β-catenin is spontaneously sequentially phosphorylated by CKIα and GSK3 kinases Phosphorylated β-catenin is then recognized and ubiquitinated by E3 ubiquitin ligase, β-TrCP within the complex. Our detection of several proteasomal subunits in the Axin1 complex by MASS SPEC suggests that degradation of ubiquitinated β-catenin by proteasome also occurs within the complex. However, further biochemical studies are required to confirm this degradation model. Moreover, the function of the protein Dvl within the dedicated complex (if only a single Axin1 complex exits in cells) still remains unclear.
Upon Wnts engagement to its receptors, LRP5/6 is phosphorylated and Dvl and Axin1 are recruited to the membrane (Figure 1B and 1C). Our observations lead to two possible inactivation models of the β-catenin destruction complex. In the first model, all components of the β-catenin destruction complex are recruited to the membrane together with Axin1 as part of the Axin1 complex (Figure 1B). This membrane recruitment results in further phosphorylation of LRP5/6 by CKI and GSK3 and also dephosphorylation of β-catenin or inhibiton of GSK3 phosphorylation on β-catenin by phosphorylated LRP5/6. According to the second model, there are two distinct Axin1 complexes present in Wnt activated cells, and only part of the destruction complex including Axin1, APC, GSK3, CKI and β-catenin is recruited to the membrane. Another pool of the Axin1 complex may remain in the cytoplasm and is saturated by phosphorylated and unubiquitinated β-catenin (Figure 1C). As a result, β-catenin is accumulated in the cytoplasm and translocated to the nucleus. In the

nucleus, the kinase Tnik complexes with β-catenin and Tcf co-activator complex to turn on the Wnt target genes transcriptipon program.

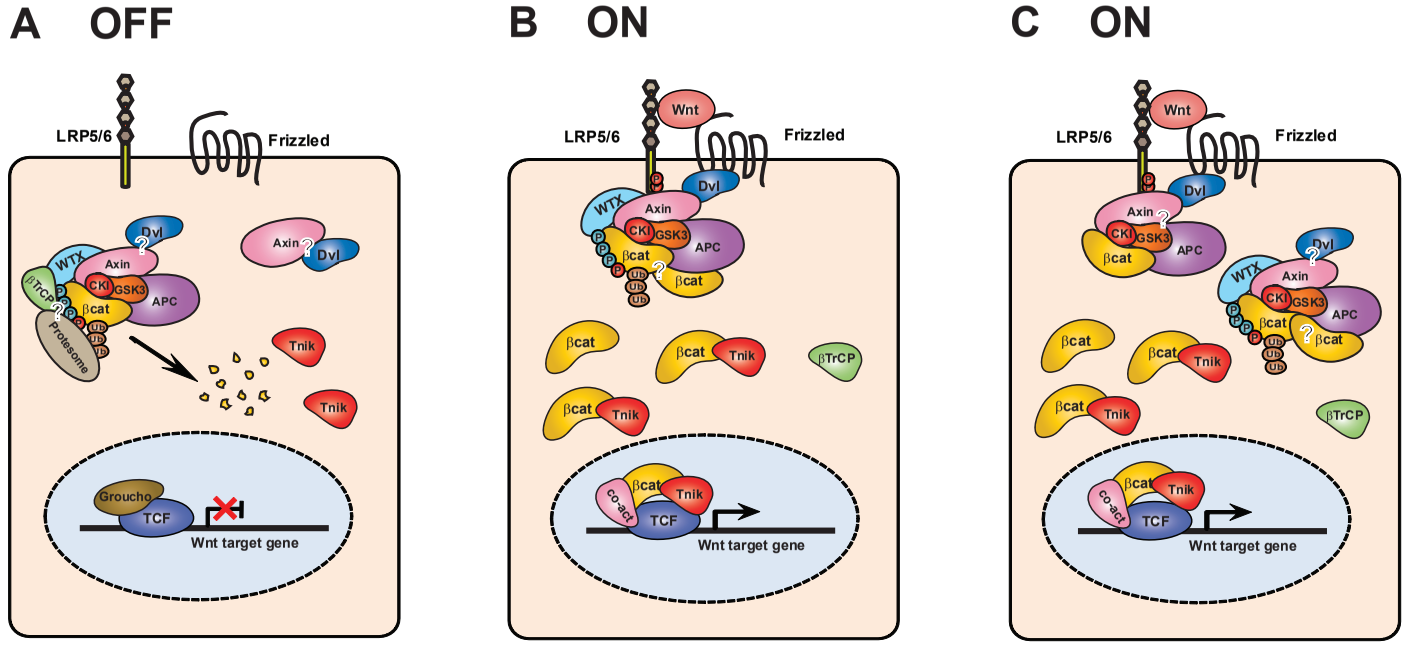

Figure1. Regulatory model of Wnt/β-catenin signaling

(A) Wnt OFF state: In resting cells, limited scaffold protein Axin1 recruits APC, GSK_3, CKIα, β-catenin, WTX and β-TrCP to form the β-catenin destruction complex. β-catenin is sequentially phosphorylated by CKIα (red) and GSK_3 (green) kinases and ubiquitinated by β-TrCP within the complex. Ubiquitinated β-catenin is recognized and degraded by the proteasome potentially within the destruction complex. The presence and function of Dvl within the dedicated complex or possible presence of another Axin1-Dvl complex remains elusive. **(B, C)** Wnt ON state: In the presence of Wnt ligand, either all of the β-catenin destruction complex is recruited to the membrane together with Axin1, and β-catenin is then dephosphorylated and activated **(B)** or only part of the destruction complex (a sub-complex) is recruited to the membrane together with Axin1 while a distinct Axin1 containing sub-complex remains in the cytoplasm**(C)**. In both cases, β-TrCP is no longer bound to Axin1 and β-catenin thereby saturates the limited destruction complex with phosphorylated β-catenin. The de novo synthesized β-catenin accumulates in the cytoplasm and translocates to the nucleus. In the nucleus, Tcf/Lef-β-catenin recruits specific transcription co-activator such as the kinase Tnik to Wnt target genes to activate transcription.

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Summary

The highly conserved Wnt/β-catenin signaling pathway controls diverse biological processes during embryonic development. In the adult, Wnts play important roles in maintaining homeostasis of adult-self renewing tissues such as the hemapoetic system, skin and the intestine. Genetic mutations which activate the Wnt/β-catenin cascade are often linked to unrestricted cell growth and malignancy. The aim of this thesis is to delineate and understand the mechanisms which modulate Wnt/β-catenin signaling under physiological and pathological conditions and to identify potential novel drug targets in the pathway for therapeutic purposes.

Modulation of cytoplasmic β-catenin protein levels is the critical regulatory step in Wnt/β-catenin signaling cascade. In cells devoid of a Wnt signal, cytosolic β-catenin levels are kept low due to constant degradation by the dedicated β-catenin destruction complex, which is mainly composed of the scaffold protein Axin, the tumor suppressor APC, and two global kinases CKIα and GSK3α/β. CKIα and GSK3α/β sequentially phopshorylate β-catenin, resulting in its recognition by the E3 ubiquin ligase β-TrCP, followed by its subsequent ubiquitination and proteasomal degradation.

GSK3α/β, the constitutively active serine/theronine kinase, which is a core component of the β-catenin destruction complex, has been shown to be inactivated upon phosphorylation by Protein Kinase B (PKB). PKB is a downstream effector of the Phosphatidyl Inositol 3-Kinase (PI3K) mediated pathway, which is activated by growth factors such as Insulin, Insulin-Like Growth Factor-1 (IGF-1) and Platelet-Derived Growth Factor (PDGF). Many studies have proposed the existence of crosstalk between the PI3K/PKB pathway and Wnt/β-catenin pathway through inactivation of GSK3α/β, especially in pathological conditions. In **Chapter 2**, we examined the potential presence of this crosstalk in a series of cancer cell lines that contain constitutively active PI3K/PKB signaling. From 24 breast cancer and 2 well established prostate cancer cell lines tested for their Wnt-mediated transcriptional activity, only one contained Wnt mediated transcriptional activity without Wnt stimulation. This cell line,DU4475, was also found to harbor an APC mutation, which resulted in active Wnt signaling. We found that Wnt-induced activation of TCF/LEF-driven transcription in the PI3K/PKB-active cell lines was not influenced by PI3K and PKB activities. These results were consistent both in an *in vivo* system, the nematode *C.elegans*, as well as in HEK293T cells, which respond to Wnt and Insulin induction. We immunoprecipitated Axin and found that the Axin-bound GSK3α/β pool is protected from PKB phosphorylation. Our results indicate that, there are two distinct pools of GSK3 α/β present in the cells. When GSK3α/β is bound is to Axin1, it is shielded from PKB. Therefore, the PI3K/PKB pathway cannot activate Wnt/β-catenin signaling through inactivation of GSK3.

We next examined the underlying molecular mechanisms of how Wnt-induction leads to the accumulation of cytoplasmic β-catenin, which results in activation of the Wnt transcriptional program. To this end, we performed a large scale Axin immunoprecipitation coupled to MASS SPEC analysis in Wnt-inducible HEK293T cells and oncogenic constitutively Wnt activated Ls174T cells (**Addendum Chapter 5**). This approach led to the successful identification of several interesting novel interactors of Axin.As well, we identified potential regulatory phosphorylation sites of Axin1, APC and WTX from Wnt time point induced HEK293T cells. In **Chapter 3**, we focus on characterizing and delineating the role of one of these Axin1 binding candidates, MAP kinase kinase kinase 1 (MAP3K1), in regulating the canonical Wnt signaling pathway. MAP3K1 was previously reported to interact with Axin and play an essential role in the non-canonical Axin/JNK pathway via its kinase domain. In our studies, we not only confirmed the interaction between these two proteins but found that their interaction is modulated by canonical Wnt signaling. Moreover, we showed that, MAP3K1 regulates the canonical and non-canoncial Wnt signaling pathways through distinct enzymatic activities; loss of function studies using different mutants of MAP3K1 together with siRNA mediated depletion of endogenous MAP3K1 illustrated that, MAP3K1 positively regulated Wnt/β-catenin transcription via its ubiquitin E3 ligase function. Importantly, the kinase activity of MAP3K1 shown to be important in Axin/JNK pathway is not required for this regulation. Future studies will be important to identify the ubiquitination target of MAP3K1 within the destruction complex and to determine how this ubiquitination leads to up-regulation of β-catenin levels in the cytoplasm.

In parallel to the identification and functional characterization of novel components of the Axin-β-catenin destruction complex, we also examined the mechanism by which Wnt activation functionally inhibits the endogenous β-catenin destruction complex. As biochemical studies probing the dynamic states of the β-catenin destruction complex in response to Wnt activation are largely lacking, in **Chapter 4** we used a specific antibody against Axin1, the least abundant regulatory scaffold of the complex to determine by immunoprecipitation, the Wnt-induced dynamic change in composition of known components within the complex. We found that the core components of the dedicated complex remain correctly assembled in both constitutive Wnt activated colon cancer cell (CRC) lines and Wnt stimulated HEK293T cells, which harbor a wild-type Wnt pathway. In HEK293T cells, Wnt stimulation resulted in increased binding of phosphorylated β-catenin to the Axin1 complex. In disagreement with the current dogma, β-catenin was efficiently phosphorylated by GSK3 upon Wnt stimulation in HEK293T as well as the CRC cell lines examined. Yet the next step, the ubiquitination of phosphorylated β-catenin, is blocked by Wnt stimulation. We found the block in β-catenin ubiquitination occurred due to the dissociation of the ubiquitin E3 ligase, β-TrCP from the complex in response to Wnt. Thus, inactivation of the destruction complex by Wnt stimulation is a consequence of its saturation by phosphorylated β-catenin. Our results suggest that the destruction complex is

required not only for phosphorylation but also ubiquitination of β-catenin. As Wnt stimulation inhibits interaction with β-TrCP, the Axin1-β-catenin destruction complex becomes saturated with the phosphorylated, non-ubiquitinated form of β-catenin and consequently inactivated. As a consequence β-catenin accumulates in the cytoplasm.

Accumulated cytosolic β-catenin translocates to the nucleus where it complexes with Tcf/Lef family member transcription factors to bind to the regulatory regions of and by recruiting a number of cofactors, activate the transcription of Wnt target genes. However, the mechanisms by which TCF4/β-catenin activate key target genes and the role and identity of other potential cofactors in this process are not very clear. In the intestine, Wnt/β-catenin signaling is essential for proliferation and differentiation of the cells. While Wnt/β-catenin signaling is activated in the crypt compartment and essential for progenitors and stem cell functions, it is inactivated in the differentiated villus compartment. In **Chapter 6**, we used a proteomics approach, to identified Tnik, a member of the germinal centre kinase family as a Tcf4 interactor in the proliferative crypts of mouse small intestine. Tnik is recruited to promoters of Wnt target genes in mouse crypts and in Ls174T colorectal cancer cells in a β-catenin-dependent manner. Depletion of TNIK and expression of TNIK kinase mutants abrogated TCF–LEF transcription, highlighting the essential function of the kinase activity in Wnt target gene activation. Wer found that TNIK directly binds both TCF4 and β-catenin and phosphorylates TCF4. siRNA depletion of TNIK followed by expression array analysis showed that TNIK is an essential and specific activator of the Wnt transcriptional program. Therefore, this kinase may present an attractive candidate for drug targeting in colorectal cancer.

Samenvatting

De sterk geconserveerde Wnt/β-catenine signalerings route reguleert diverse biologische processen tijdens de ontwikkeling van het embryo. In het volwassen organisme speelt Wnt signalering een belangrijke rol tijdens homeostase en zelf-vernieuwing van verscheidene weefsels; inclusief het bloed, de huid en de darm. Genetische mutaties die Wnt signalering activeren, resulteren vaak in ongelimiteerde celgroei en ziekten. Het doel van dit proefschrift is het ontrafelen en begrijpen van de mechanismen die Wnt/β-catenine signalering reguleren tijdens normale en pathologische condities, en indien mogelijk potentiele nieuwe drug targets in de route te identificeren voor therapeutische doeleinden.

Het controleren van de hoeveelheid cytoplasmatisch β-catenine is de essentiele stap in de regulatie van Wnt/β-catenine signalering. In afwezigheid van een Wnt ligand, wordt het niveau van β-catenine in het cytosol laag gehouden door constante degradatie door het β-catenine destructie complex,wat bestaat uit het "scaffold" eiwit Axin, de tumor suppressor APC en twee algemene kinases CKIα en GSK3α/β. CKIα and GSK3 α/β phopshoryleren na elkaar β-catenine, wat resulteert in de herkenning van β-catenine door het E3 ubiquitine ligase β-TrCP, vervolgens wordt β-catenine geubquitinileert en afgebroken door het proteasome.

Er is aangetoond dat GSK3α/β, een permanent active serine/theorine kinase en "core component" van het β-catenine destructie complex, wordt geinactiveerd door phosphorylatie door Protein Kinase B (PKB). PKB is een effector van de Phophatidyl Inositol 3-Kinase (PI3K) signalering, welke geactiveerd wordt door groeifactoren zoals Insulin, Insulin-Like Growth Factor-1 (IGF-1) en Platelet-Derived Growth Factor (PDGF).

Verscheidene studies suggereren dat er "crosstalk" tussen PI3K/PKB signalering en Wnt/β-catenine signalering plaatsvindt, met name in ziekteprocessen. In **hoofdstuk 2,** hebben wij onderzocht of deze crosstalk tussen PI3K/PKB signalering en Wnt/β-catenine signalering plaatsvindt in verscheidene cellijnen met een constitutioneel actieve PI3K/PKB signalering. Wij hebben 26 goed beschreven cellijnen, 24 borstkanker- en 2 prostaatkankercellijnen, getest op Wnt/β-catenine signalering, hiervan had maar één cellijn actieve Wnt/β-catenine signalering zonder stimulatie met Wnt. Deze cellijn, DU4475, heeft een mutatie in het APC gen,wat leidt tot een actieve Wnt/β-catenine signalering. Onze resultaten laten zien dat; Wnt-geinduceerde activatie van TCF/LEF gedreven transcriptie niet wordt beïnvloedt door PI3K and PKB activiteit. Deze resulaten zijn in overeenstemming met bevindingen in HEK293T cells, die reactief zijn voor Wnt and Insulin, en met bevindingen in vivo in de platworm *C.Elegans*. Doormiddel van immunoprecipitatie van Axin hebben wij

gevonden dat GSK3α/β wat gebonden is aan Axin, niet kan worden gephosphoryleerd. Onze resultaten tonen aan dat er twee afzonderlijke populaties van GSK3 in de cel aanwezig zijn. Als GSK3α/β gebonden is aan Axin1, wordt het afgeschermd van PKB, waardoor phophorylatie niet mogelijk is. Hierom is het onmogelijk dat PI3K/PKB signalering een activerende rol heeft in Wnt/β-catenine signalering door het inactiveren van GSK3.

Vervolgens hebben wij de moleculaire mechanismen onderzocht, welke een rol spelen in de accumulatie van cytoplasmatisch β-catenine, wat resulteert in de activatie van het Wnt-transciptie programma. Hiervoor hebben wij een grootschalige immunoprecipitatie van Axin in Wnt-gevoelige HEK293T cellen en LS174T cellen, met een een permanent actieve Wnt-signalering, gekoppeld aan een MASS SPEC analyse (**Addendum Hoofdstuk 5**). Deze aanpak heeft geleidt tot de identificatie van verscheidene nieuwe en interessante eiwitten die met Axin kunnen binden. Tevens hebben we in HEK293T cellen, op verschillende tijdspunten na Wnt-stimulatie, phophorylatie sites van Axin1, APC en WTX geïdentificeerd in, die mogelijk een rol spelen in de regulatie van deze proteïnen.

In **Hoofdstuk 3** onderzoeken wij een van deze Axin1 "interactors"; MAP kinase kinase kinase 1 (MAP3K1) en haar rol in de regulatie van Wnt/β-catenine signalering. Er is aangetoond dat MAP3K1 een interactie aangaat met Axin en een essentiele rol speelt in de "non-canonical" Axin/JNK pathway door middel van het kinase domein. In onze studies hebben wij niet alleen bevestigd dat Axin en MAP3K1 een interactie aangaan, maar ook dat deze interactie wordt beinvloedt door Wnt/β-catenine signalering. Daarnaast hebben wij aangetoond dat MAP3K1 "canonical" en "non-canonical" Wnt signalering reguleert door middel van verschillende enzymatische activiteiten. Door gebruik te maken van verschillende mutanten van MAP3K1 en siRNA gericht op endogeen MAP3K1 hebben wij aangetoond dat MAP3K1 een positieve regulator in van Wnt/β-catenine signalering via haar functie als ubiquitin E3 ligase. Van belang is het feit dat de kinase activiteit van MAP3K1, die essentieel is in de Axin/JNK pathway, geen rol van betekenis speelt in de Wnt/β-catenine signalering regulatie. Vervolg studies zullen moeten aantonen of en welk onderdeel van het destructie complex een target is voor ubiquitinilatie door MAP3K1 en hoe deze ubiquitinilatie leidt tot een verhoging van β-catenine hoeveelheid in het cytoplasma.

Parallel aan de identificatie en karakterisatie van nieuwe componenten van het Axin-β-catenine destructie complex, hebben wij ook het mechanisme onderzocht waardoor Wnt activatie het destructie complex remt. Goede biochemische studies die het dynamische gedrag van β-catenine in het destructie complex tijdens Wnt-stimulatie zijn vooralsnog afwezig. Daarom onderzoeken wij in **Hoofdstuk 4** door middel van immunoprecipitatie van Axin1, het minst aanwezige onderdeel van het destructie complex, hoe de compositie van het destructie complex verande door Wnt-

stimulatie. Onze bevindingen waren dat de interactie tussen de "core components" van het destructie complex intact blijven in zowel colon kanker cellijnen (CRC), met constitutioneel actieve Wnt/β-catenine signalering, als in Wnt gestimuleerde HEK293T cells. In HEK293T cells, leidde stimulatie met Wnt tot een verhoging van de binding van gephosphoryleerd β-catenine aan het destructie complex. In tegenstelling tot het tegenwoordige "Wnt dogma", werd β-catenine efficiënt gephosphoryleerd door GSK3 tijdens Wnt-stimulatie in HEK293T cellen, zo ook in de CRC lijnen. Echter de volgende stap; ubiquitinilatie van β-catenine wordt geremd door Wnt stimulatie. Deze stop van ubiquitinilatie van β-catenine werd veroorzaakt door de dissociatie van β-TrCP, een E3 ubiquitine ligase, als gevolg van Wnt-stimulatie. Dus de inhibitie van de destructie complex door Wnt stimulatie is een gevolg van de verzadiging van het complex met gephosphoryleerd β-catenine. Onze resultaten suggeren dus dat het destructie complex niet alleen essentieel is voor de phosphorylatie maar ook voor de ubiquitinilatie van β-catenine. En dat Wnt stimulatie de interactie tussen het destructie complex en β-TrCP inhibeert, waardoor het destructie complex verzadigd wordt met gephosphoryleerd, maar niet geubiquitnileerd β-catenine. Het gevolg van deze verzadiging is een opeenhoping β-catenine in het cytoplasma en activatie van Wnt-signalering.

Opeengehoopt β-catenine in het cytosol gaat naar de celkern, waar het complexen vormt met leden van de Tcf/Lef familie van transcriptie factoren. Deze β-catenine-Tcf/Lef complexen binden aan regulatoire elementen in het DNA en door het recruteren van verscheidene cofactoren, activeren ze Wnt-target genen. Echter, de mechanismen waarmee TCF4/β-catenine de belangrijkste "target" genen activeert en de rollen en identiteiten van cofactoren in dit procres zijn niet erg duidelijk. In de darm is Wnt/β-catenine signalering essentieel voor de proliferatie en differentiatie van het epitheel. In de darmcrypt is Wnt/β-catenine signalering actief en reguleert het essentiele processen in stam- en voorlopercellen, in de gedifferentieerde cellen op de villus is Wnt/β-catenine signalering niet actief. In **Hoofdstuk 6**, hebben wij door middel van proteomics, Tnik, een lid van de germinal centre kinase familie, geïdentificeerd als een interactor van Tcf4 in de crypten van de muis. In de crypten en is LS174T cellen wordt Tnik, onder invloed van β-catenine, gerecruteerd naar de promotoren van Wnt target genen. Wij hebben aangetoond dat de kinase activiteit van TNIK een essentiele rol speelt in de activatie van Wnt target genen, door depletie van TNIK door middel van siRNA en expressie van TNIK kinase mutanten verlaagde transcriptie TCF-LEF target genen. Onze resultaten tonen aan dat TNIK een directe interactie aangaat met zowel TCF4 en β-catenine en dat TCF4 gephophoryleerd wordt door TNIK. Microarray analyse na depletie van TNIK met siRNA toonde aan dat TNIK een essentiele en specifieke activator is van het Wnt-transcriptie programma. Hierdoor is, deze kinase een potentiele kandidaat voor medicijn ontwikkeling tegen (darm)kanker.

About the author

The author of this thesis was born in Kelang, Selangor, Malaysia on 27th of May 1978. She had a tough but cheerful childhood with a lot of close and nourishing contact with the nature, which built up her great interest in different kinds of organisms. In November 1996, she finished her pre-graduate education in a Chinese private secondary school, Hin Hua High School, in her lovely hometown. After passing the university entering exam, she started her biology study at Tsinghua University, Beijing, China in September 1997. In 2000, she had her first research experience in molecular biology and biochemistry under the supervision of Professor Zhi-Jie Chang, and has been in love with them since then. After obtaining her Bachelor of Science degree in the summer of 2001, she decided to pursue her three-year master's programme in Professor Chang's laboratory. In 2004 and, she worked as a secondary school teacher in her hometown right after her Master of Science degree while enjoying the great time with her family and friends. In the meanwhile, she also got a PhD student position offer in the Hubrecht Institute for Developmental Biology and Stem Cell Research, Utrecht, The Netherlands under Professor Hans Clevers. She started her PhD studies in January 2006 and the results of her research are presented in this thesis. Following her PhD defence on 28th September 2010, she is going to look for a postdoctoral position where she will learn to work on animal model in addition to biochemistry and molecular biology.

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