

## Wnt/ $\beta$ -Catenin Signaling and Small Molecule Inhibitors

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**Abstract:** Wnt/ $\beta$ -catenin signaling is a branch of a functional network that dates back to the first metazoans and it is involved in a broad range of biological systems including stem cells, embryonic development and adult organs. Deregulation of components involved in Wnt/ $\beta$ -catenin signaling has been implicated in a wide spectrum of diseases including a number of cancers and degenerative diseases. The key mediator of Wnt signaling,  $\beta$ -catenin, serves several cellular functions. It functions in a dynamic mode at multiple cellular locations, including the plasma membrane, where  $\beta$ -catenin contributes to the stabilization of intercellular adhesive complexes, the cytoplasm where  $\beta$ -catenin levels are regulated and the nucleus where  $\beta$ -catenin is involved in transcriptional regulation and chromatin interactions. Central effectors of  $\beta$ -catenin levels are a family of cysteine-rich secreted glycoproteins, known as Wnt morphogens. Through the LRP5/6-Frizzled receptor complex, Wnts regulate the location and activity of the destruction complex and consequently intracellular  $\beta$ -catenin levels. However,  $\beta$ -catenin levels and their effects on transcriptional programs are also influenced by multiple other factors including hypoxia, inflammation, hepatocyte growth factor-mediated signaling, and the cell adhesion molecule E-cadherin. The broad implications of Wnt/ $\beta$ -catenin signaling in development, in the adult body and in disease render the pathway a prime target for pharmacological research and development. The intricate regulation of  $\beta$ -catenin at its various locations provides alternative points for therapeutic interventions.

**Keywords:**  $\beta$ -catenin, cancer, drug discovery, small molecule inhibitors, stem cells, Wnt.

### INTRODUCTION

Wnt/ $\beta$ -catenin signaling is a branch of an extensive functional network that developed around a class of proteins - called armadillo proteins - that dates back to the first anaerobic metazoans. Wnt/ $\beta$ -catenin signaling is involved in a broad range of biological systems, including stem cells biology, developmental biology, and adult organ systems.

The first detail of the Wnt/ $\beta$ -catenin network was reported in 1982 with the identification of the proto-oncogene *int-1* in mice [1]. Later its homolog in *Drosophila*, *Wingless*, was shown to be required for proper wing formation [2]. In 1989 injection of Wnt1 mRNA in *Xenopus* was shown to cause body axis duplication, and demonstrated the functional conservation of the pathway [3]. Since then, the functional importance of Wnt/ $\beta$ -catenin signaling has been shown in a plethora of developmental and organ systems including the cerebral cortex, the hippocampus, the eye, the lens, the spinal cord, limbs, bone, cartilage, somites, the neural crest, skin, teeth, the gut, the lungs, the heart, the pancreas, the liver, the kidneys, the mammary glands, the hematopoietic system and the reproductive system [4-7]. Deregulation of Wnt/ $\beta$ -catenin signaling is implicated in a wide spectrum of diseases including degenerative diseases, metabolic diseases and cancer [4], [8-11].

The key mediator of Wnt signaling, the armadillo protein  $\beta$ -catenin, is found in a dynamic mode at multiple subcellular localizations, including junctions where it contributes to stabilize cell-cell contacts, the cytoplasm where  $\beta$ -catenin levels are tightly controlled by protein stability regulating processes and the nucleus, where  $\beta$ -catenin is involved in transcriptional regulation and chromatin interactions. Central extracellular regulators of  $\beta$ -catenin levels are the Wnt morphogens. However, multiple other processes, including hepatocyte growth factor, prostaglandines, PKA (Protein Kinase A), E-cadherin, and hypoxia, can also influence  $\beta$ -catenin levels.

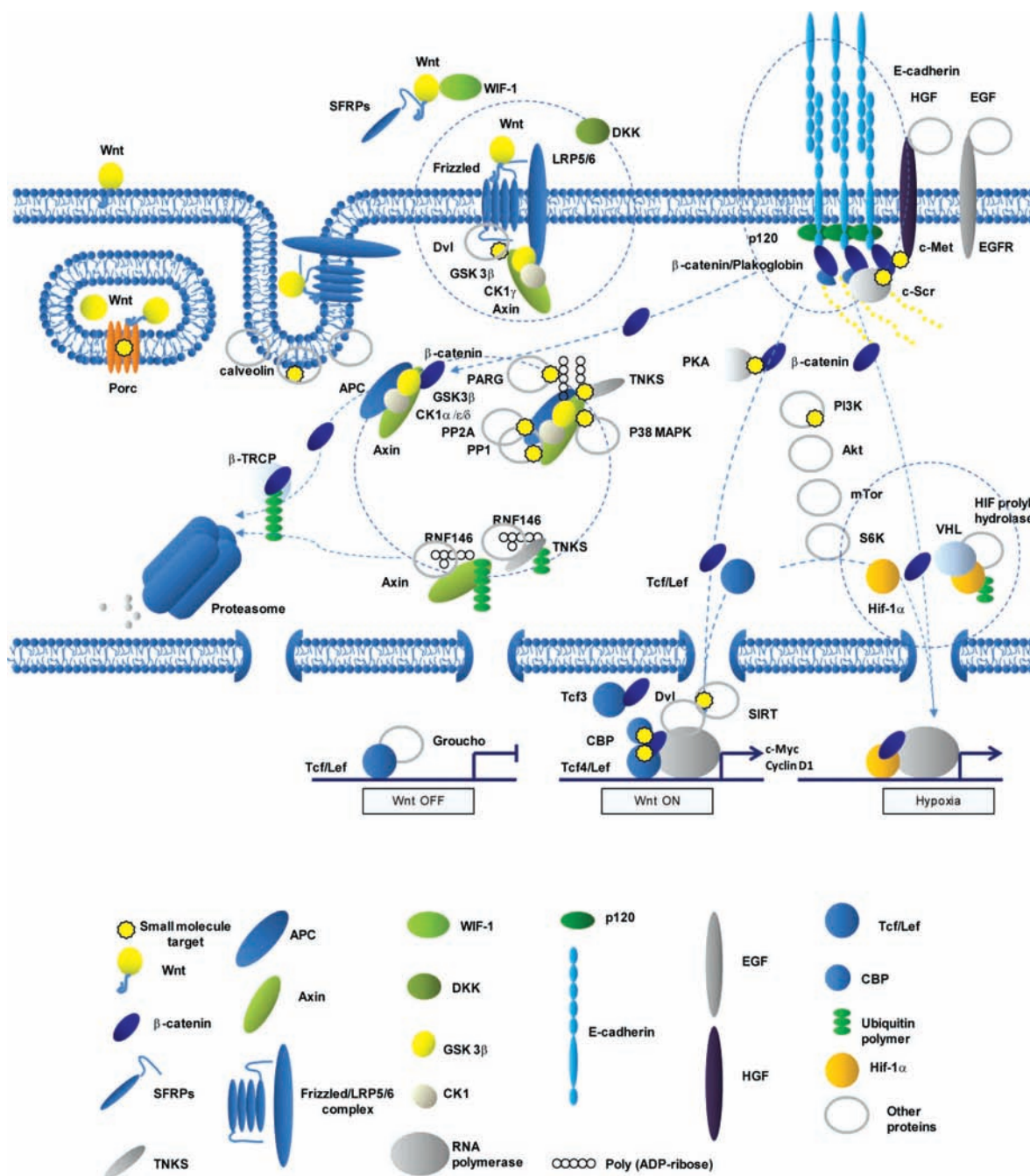
$\beta$ -catenin itself is a specialized member of the larger armadillo protein family that consists of three subfamilies: the p120 subfamily, the beta subfamily ( $\beta$ -catenin and plakoglobin) and the more distant alpha subfamily. The functional interplay between members of this protein family is not well understood, but an involvement of p120 and plakoglobin in Wnt/ $\beta$ -catenin signaling has been shown.

The regulation of the presence and stability of  $\beta$ -catenin and functionally convergent armadillo proteins - in particular p120 - at the various cellular localizations as well as their shuffling within the cell provides alternative intervention points for therapeutic reagents. The broad implications of Wnt/ $\beta$ -catenin signaling in development, the adult body and in disease renders it a prime target for pharmacological research and development. A short overview map for canonical Wnt signaling is presented on Fig. (1).

The armadillo protein  $\beta$ -catenin is the central denominator of Wnt/ $\beta$ -catenin (canonical Wnt) signaling. The levels of  $\beta$ -catenin at different subcellular localizations are regulated by a variety of processes including site-specific phosphorylation of  $\beta$ -catenin. In particular, the control of the turnover of cytoplasmic  $\beta$ -catenin by the destruction complex and the control of the destruction complex by the Wnt signalosome have been studied extensively. Other important mechanisms regulating subcellular  $\beta$ -catenin thresholds are those controlling its mobilization from adherens junctions and its translocation to the nucleus. One of the central end points of the Wnt/ $\beta$ -catenin signaling pathway is the regulation of transcription through the binding of  $\beta$ -catenin to members of the Tcf-1/lymphoid enhancer factors (Lef-1, 3, 4) family of transcription factors in the nucleus [12-14].

The structure of  $\beta$ -catenin can be divided into three domains: the N-terminal domain, the armadillo domain consisting of 12 armadillo repeats, and the C-terminal domain [15]. Through predominantly positively charged armadillo (Arm) repeats,  $\beta$ -catenin is a member of an expanded and evolutionary ancient protein family that includes plakoglobin ( $\gamma$ -catenin), APC (adenomatous polyposis coli), p120 and other proteins [16-18]. Local charge alterations of  $\beta$ -catenin through phosphorylation at a multitude of positions have been suggested to regulate its affinity to specific protein partners. This includes C-terminal phosphorylation that attenuates the bind-

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**Fig. (1).** Simplified schematic representation of drug targets (yellow stars) in Wnt/ $\beta$ -catenin-mediated signaling. Four key aspects that regulate  $\beta$ -catenin-mediated signaling are highlighted: the destruction complex, the Wnt/ $\beta$ -catenin signalosome, cadherin junctions, and the hypoxia sensing system Hif-1 $\alpha$  (hypoxia induced factor 1 $\alpha$ ). Proteins that directly interact with Wnt/ $\beta$ -catenin are marked as colored structures, other proteins are marked as circles.

ing of  $\beta$ -catenin to the cadherin adhesion complex and N-terminal phosphorylation that regulates its degradation in the proteasome. Furthermore, phosphorylation regulates the association of  $\beta$ -catenin with Tcf/Lef during transcriptional regulation [19].

In this review we will first describe the alterations of  $\beta$ -catenin in the destruction complex and the proteins that are involved in this process. We will then focus on the Wnt signalosome that recruits components of the destruction complex thus inactivating it. Subsequently, we will summarize how the signalosome is removed from the cell surface by endocytosis. Next we will describe the cellular pool of  $\beta$ -catenin at cell junctions and the mobilization of  $\beta$ -catenin from this pool. Then we will focus on the implications of  $\beta$ -catenin

in transcription control. Finally, we will summarize some of the pathways that influence Wnt/ $\beta$ -catenin signaling. Several proteins in the Wnt/ $\beta$ -catenin pathway that are implicated in other cellular processes will be briefly described.

**The  $\beta$ -Catenin Destruction Complex and its Proteins**

In the absence of an active Wnt signalosome, cytoplasmic  $\beta$ -catenin associates with the destruction complex. The main known structural components of the destruction complex are APC and Axin. To this structural core, the casein kinases CK1 $\alpha$ ,  $\delta$ , and  $\epsilon$  (to be referred to collectively as CK1) and GSK3 (glycogen synthase kinase 3) are recruited [20].

In the degradation complex the processing of  $\beta$ -catenin is considered as a phosphorylation-dependent flux along the Axin scaffold protein which is regulated by a stepwise series of phosphorylations triggered by the kinases CK1 and GSK3 [15, 20]. A current model proposes that  $\beta$ -catenin initially binds to Axin. The priming kinases CK1 phosphorylate  $\beta$ -catenin at Ser45 [20], which enables a subsequent phosphorylation by GSK3 at Ser33, Ser37 and Ser41 [21, 22]. Subsequent phosphorylation of APC by CK1 $\epsilon$  and GSK3, leads to an increased affinity between APC and  $\beta$ -catenin [23] triggering a transfer of  $\beta$ -catenin from Axin to APC, while Axin is able to bind the next  $\beta$ -catenin molecule. Finally, APC exposes the N-terminally phosphorylated  $\beta$ -catenin to  $\beta$ -TrCP ( $\beta$ -transducin-repeat-containing protein) [24], the ubiquitin ligase responsible for ubiquitinating  $\beta$ -catenin leading to its degradation in the proteasome [25]. N-terminal phosphorylation of  $\beta$ -catenin is not only required for its degradation, but also responsible for attenuating its effect on transcription [20, 26, 27].

### Axin

In diverse human cancers Axin mutations are associated with increased levels of  $\beta$ -catenin [28-30]. Mutations in the genes encoding Axin and Axin2 are found in 11% of cases of colorectal cancers and in hepatocellular carcinomas [31-33]. Furthermore, mutations in the Axin gene are observed in 12% of cases of medulloblastomas, in 35% of cases of adenoid cystic carcinomas and in 20% of cases of oral squamous cell carcinomas [34].

In the destruction complex, Axin serves as a coordinating scaffold for the kinases GSK3 and CK1, for the structural protein APC and Dishevelled (Dvl/Dsh) as well as for  $\beta$ -catenin [35]. Early mathematical kinetic modeling for Wnt/ $\beta$ -catenin signaling suggested that Axin levels may be the rate-limiting factor for the degradation of  $\beta$ -catenin. These models were based on the assumption that Axin concentrations are about three orders of magnitude lower than the concentration of other degradation complex components known at the time of the study [36]. Since Axin is considered to be a rate limiting protein in the destruction complex, strategies involving an alteration of Axin protein levels are considered to be promising in drug discovery [36-40].

There are two Axin proteins in humans - Axin1 (826aa, 92kDa predicted in humans), and Axin2 (also called conductin or Axil, 840aa, 93kDa). Each of the Axin genes encodes two isoforms, a and b, which differ by splicing variants [34]. Axin and Axin2 have redundant functions in Wnt/ $\beta$ -catenin signaling, both binding to various proteins of the  $\beta$ -catenin degradation complex [41, 42]. The transcription of Axin2 is a central target of Wnt/ $\beta$ -catenin signaling, whereby Axin2 forms a negative feedback loop in the pathway [43]. Hence, Axin2 expression is upregulated by Wnt/ $\beta$ -catenin signaling while Axin contributes centrally to the degradation of  $\beta$ -catenin [44].

Axin contains a number of domains including a RGS (Regulators of G protein signaling) domain (aa 121 to 247) and a DIX domain (Dishevelled/Axin homologous domain) (aa 716 - 900). The DIX domain is responsible for Axin homodimerization and the formation of heterodimers with Dishevelled [45, 46]. In this process, the residues 757-820 of the Axin DIX domain bind to the homologous DIX domain of Dishevelled [46-48]. In addition to heterodimerization through the DIX domain, Axin was shown to have two further domains - D and I - that can mediate homodimerization [49]. The RGS domain of Axin interferes with the  $\alpha$ -subunits of G-proteins (guanine nucleotide-binding proteins) [50, 51]. G $\alpha$  proteins were reported to disrupt interactions between Axin and GSK3 [52, 53] and in *Drosophila* it was shown that the  $\alpha$ -subunit of G $\alpha$  physically binds to Axin and recruits it to the plasma membrane [54]. Axin uses aa 437-506 to interact directly with the Armadillo repeats 2-3 of  $\beta$ -catenin [55]. The same repeats were also shown to interact with the armadillo protein plakoglobin [56]. Other binding areas in the Axin protein are amino acids 89-216 for APC, 507-712 for

Axam, 353-437 for GSK3, 530-712/757-820 for Dishevelled, 217-352/508-712 for CK1 and 353-437 for Diversin [35]. Recently it was also shown by X-ray analysis that the N-terminal domain of Axin (1-80 aa) is responsible for binding to Tankyrase [57]. Interactions with Axin promote dimerization of Tankyrase. Additional Axin-interacting proteins include MEKK1 (MAP kinase kinase kinase), MEKK4 (MAP kinase kinase kinase 4), CK1 $\alpha/\epsilon$ , I-mfa (inhibitor of myogenic basic helix-loop-helix transcription factors), Axam (Axin associating protein), PP2A (Protein phosphatase 2), Smad3 (Mothers against decapentaplegic 3), LRP5/6 (Low-density lipoprotein receptor-related proteins 5/6), MEKK4, Ccd1 (Coiled-coil-DIX1) and PIAS (Protein inhibitor of activated STAT) [35].

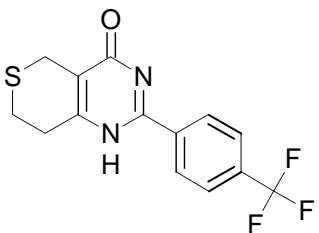
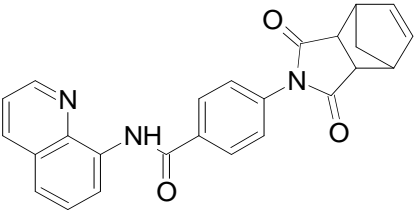
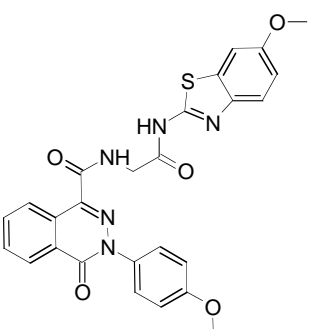
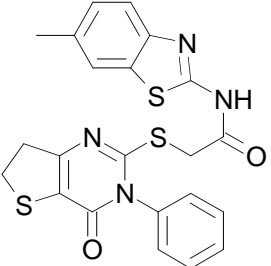
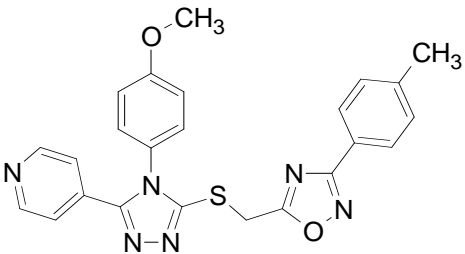
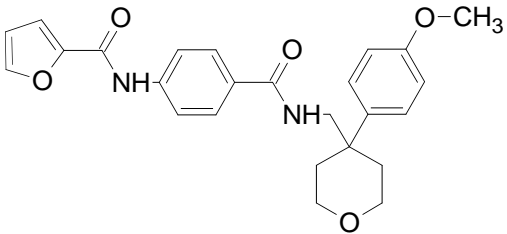
Axin thresholds and stability are regulated by different components of the Wnt/ $\beta$ -catenin pathway. Axin is stabilized by GSK3-mediated phosphorylation at Ser330, Thr341 and Ser343 [35, 58]. In mice, GSK3-mediated phosphorylation of amino acids Thr609 and Ser614 of Axin has been shown to be required for its activity [59]. Axin phosphorylation by GSK3 and CK1 also leads to increased affinity for  $\beta$ -catenin and enhances the phosphorylation and degradation of  $\beta$ -catenin [59-61]. Since the armadillo domain of  $\beta$ -catenin is positively charged in the area that mediates Axin interactions, phosphorylation of Axin can enhance the interaction, while a subsequent N-terminal phosphorylation of  $\beta$ -catenin adds a negative charge that presumably triggers its dissociation from the phosphorylated Axin [20].

Axin can be dephosphorylated by the serine/threonine phosphatases PP1 (Protein Phosphatase 1) and PP2C (Protein Phosphatase 2C) [60, 62] and the Ser/Thr phosphatase PP2A, which binds to aa 508-712 and aa 298-506 of Axin [35, 45, 55, 63-65]. PP2A was also reported to dephosphorylate APC [66]. PP1 acts on Ser residues of Axin to reverse CK1 $\alpha$ -mediated phosphorylation. Hence, inhibition of PP1 can lead to an increased phosphorylation of Axin followed by an enhancement of  $\beta$ -catenin degradation [60]. Phosphatases therefore may be a targetable interference point of Wnt/ $\beta$ -catenin signaling. For instance the phosphatase inhibitor okadaic acid (Table 1) reverses LiCl (inhibitor of GSK3) induced activation of Wnt/ $\beta$ -catenin signaling [58], and the PP1 inhibitor tautomycin (Table 1) was shown to reduce Wnt/ $\beta$ -catenin signaling [60].

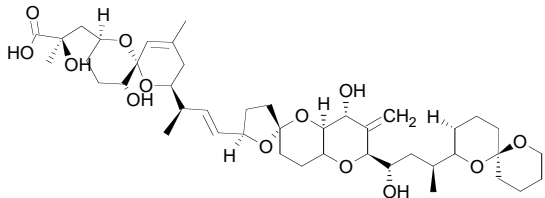
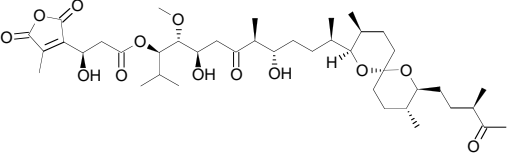
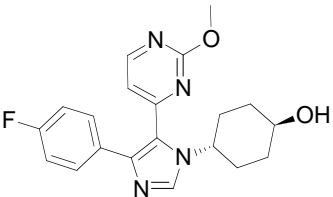
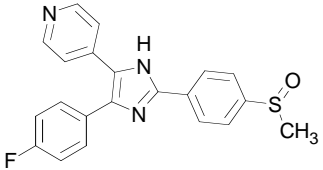
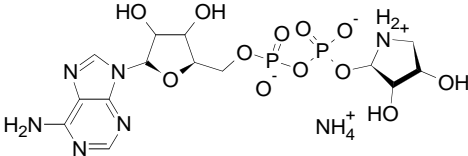
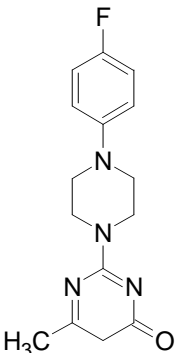
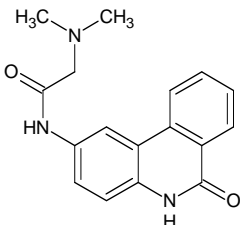
Axin protein stability and turnover in the cell is centrally regulated by poly(ADP-ribosylation), followed by ubiquitination and protein degradation in the proteasome. Axin ubiquitination is induced by the E3 ubiquitin ligases RNF146 (RING finger protein 146) that recognizes poly(ADP-ribosyl)ate tails at the protein that are added dynamically by the PARP (Poly (ADP-ribose) polymerase) proteins Tankyrase1 and Tankyrase2 [67, 68]. In contrast, SUMOylation was shown to prevent Axin polyubiquitination and thus to stabilize Axin. SUMOylation of Axin occurs at residues K951 and K954 in the C-terminal KVEKVD sequence and is implemented by E3 ligases of the PIAS family [69]. SUMOylation does not only regulate Axin stability, but also its subcellular localization [69]. Axam downregulates Wnt/ $\beta$ -catenin signaling [70] by binding to residues 507-712 of Axin and deSUMOylates the protein [35, 69-71]. Interestingly, Axam is also involved in deSUMOylation of Tcf-4 [72]. Another Axin2-interacting protein that has shown to regulate the stability of Axin, is the arginine methyltransferase PRMT1. PRMT1 directly interacts with Axin and methylates Arg378, resulting in a stability increase of Axin and leading to a reduction of Wnt/ $\beta$ -catenin signaling [73].

Three nuclear localization signal sequences (NLS) are found in the Axin proteins at positions 443-558, 474-483 and 537-547. Because Axin lacking NLS fails to regulate cytoplasmic levels of  $\beta$ -catenin it has been suggested that Axin may serve as a shuttle for  $\beta$ -catenin between the cytoplasm and the nucleus [74]. Interestingly, it has also been shown that Axin may act as a molecular shuttle to export  $\beta$ -catenin from the nucleus [74], and that this function may require Axin oligomerization into larger aggregates [74].

Table 1. Small Molecules, which Downregulate Wnt/ $\beta$ -Catenin Signaling

Structure	Compound	Target	Reference
	XAV939	Tankyrases1, 2	[37]
	IWR1	Tankyrases1, 2	[38]
	IWP-1	Porcupine	[38]
	IWP-2	Porcupine	[38]
	JW74	Tankyrases1, 2	[39]
	JW55	Tankyrases1, 2	[40]

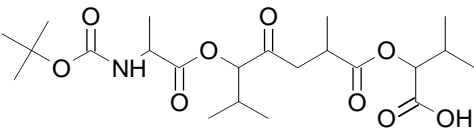
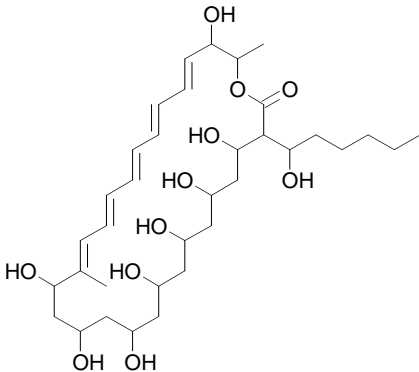
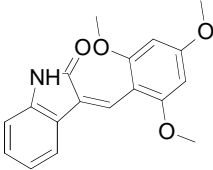
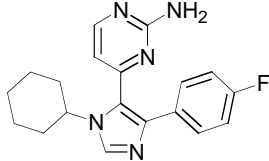
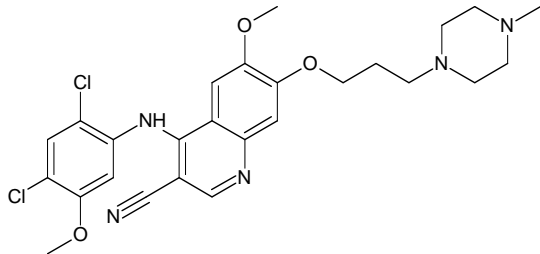
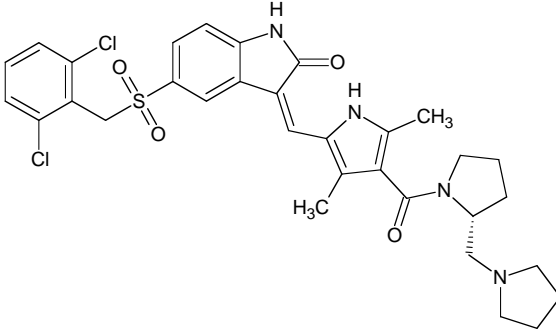
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Structure	Compound	Target	Reference
	Okadaic acid	PP2A phosphatase	[58]
	Tautomycin	PP1 phosphatase	[60]
	SB239063	p38 MAPK	[122, 137]
	SB203580	p38 MAPK	[122, 137]
	ADP-HPD	PARG	[148]
	2-[4-(4-fluorophenyl)piperazin-1-yl]-6-methylpyrimidin-4(3H)-one	Tankyrases1, 2	[165]
	PJ34	Tankyrases1, 2	[170]

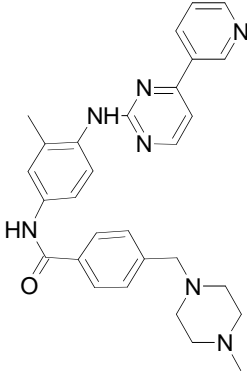
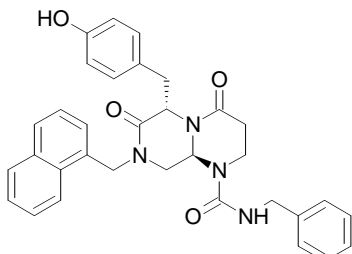
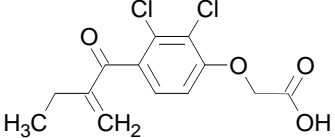
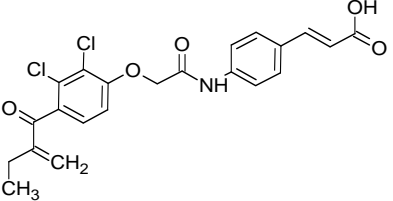
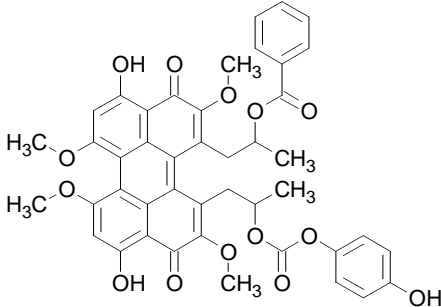
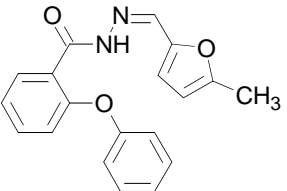
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Structure	Compound	Target	Reference
	Niclosamide	Downregulates Dvl-2, triggers LRP6 degradation	[253, 254, 255]
	Cambinol	SIRT1	[278]
	Sulindac	PDZ domain of Dishevelled	[284, 479]
	3289-8625	Dishevelled	[286]
	Scaffold A for series of analogs	Dishevelled	[288]
	Scaffold B for series of analogs	Dishevelled	[288]
	J01-017a	Dishevelled	[288]

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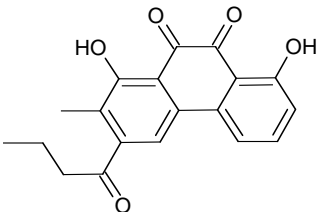
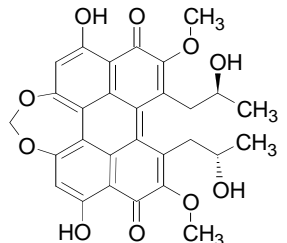
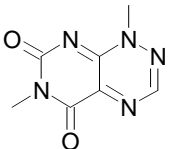
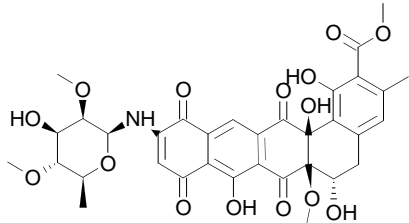
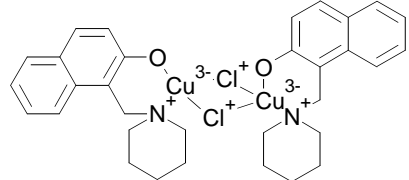
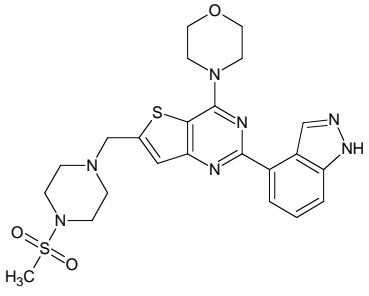
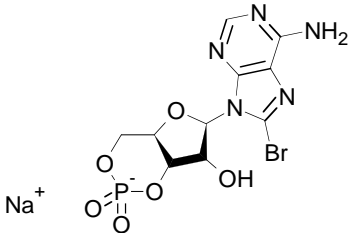
Structure	Compound	Target	Reference
	NSC668036	Dishevelled	[289, 441]
	Filipin	Caveolin-mediated endocytosis	[291]
	IC261	CK1 $\epsilon/\delta$	[308]
	PF670462	CK1 $\delta$ and CK1 $\epsilon$	[308]
	Bosutinib	Src kinase	[356, 357]
	PHA665752	c-Met	[379, 386]

(Table 1) Contd.....

Structure	Compound	Target	Reference
	Imatinib	Different tyrosine kinases	[385]
	ICG-001	CREB binding protein (CBP)	[434, 441]
	Ethacrynic acid	Lef-1	[437, 439]
	Ethacrynic acid derivative	Lef-1	[439]
	PKF115-584	$\beta$ -catenin	[440, 441, 443]
	PNU-74654	$\beta$ -catenin	[441, 443]



(Table 1) Contd.....

Structure	Compound	Target	Reference
	PKF118-744	$\beta$ -catenin	[440, 441, 443]
	CGP049090	$\beta$ -catenin	[440, 441, 443]
	PKF118-310	$\beta$ -catenin	[440, 441, 443]
	ZTM000990	$\beta$ -catenin	[440, 441, 443]
	BC21	$\beta$ -catenin	[444]
	GDC-0941	PI3K	[449]
	Rp-8-Br-cAMP	PKA	[461]

Finally, Axin as well as several other components of the degradation complex (GSK3,  $\beta$ -catenin, Tankyrase and APC) may colocalize to centrosomes and mitotic spindles [75-78], where Axin modulates the distribution of Axin associated-proteins such as PLK1 (Serine/threonine-protein kinase, also known as polo-like kinase 1) and GSK3, thereby modulating the mitotic process [79].

The structural protein Axin participates not only in Wnt/ $\beta$ -catenin signaling but also in TGF $\beta$  (Transforming growth factor beta) signaling and MAPK (Mitogen-activated protein-kinase)-mediated signaling [35]. An overexpression of Axin has been reported to lead to an activation of MAP kinase (Mitogen-activated protein kinases) and the c-Jun N-terminal kinase JNK. In TGF $\beta$  signaling, Axin assists in TGF $\beta$ -mediated activation of Smad3 [80]. Smad3 in turn can activate  $\beta$ -catenin signaling through a direct interaction with  $\beta$ -catenin whereby Smad3 protects  $\beta$ -catenin from ubiquitination and degradation [81].

### APC

APC is the largest structural core protein of the destruction complex (2843 amino acids, 312 kDa). The APC protein has several functional domains including an oligomerization domain (responsible for homodimerization), seven armadillo repeats and three  $\beta$ -catenin binding repeats of 15 amino acids [82, 83]. The  $\beta$ -catenin binding repeats were proposed to bind  $\beta$ -catenin and assist in its positioning to the binding sites of the kinases in the destruction complex. In addition, APC has seven 20 aa repeats that are involved in release of  $\beta$ -catenin after its phosphorylation [15, 24]. In most cases, oncogenic mutations in the gene encoding APC are caused by a truncation of the  $\beta$ -catenin binding region [84, 85]. However, APC mutations that do not affect  $\beta$ -catenin binding may also be cancerogenic e.g. if they lead to a reduction of Axin/APC binding and thus to destabilization of the destruction complex [9, 86].

APC can be phosphorylated by CK1 $\epsilon$  at Ser1279 and Ser1392 [87]. Phosphorylated APC outcompetes Axin from forming a complex with  $\beta$ -catenin and it has been suggested that the synchronized coordination between Axin,  $\beta$ -catenin and APC phosphorylation is important for a stepwise processing of  $\beta$ -catenin in the degradation complex [20, 88].

Similar to Axin, APC was found to act as a nuclear shuttling protein and has been implied in nuclear  $\beta$ -catenin import as well as export [89, 90, 91]. APC has two nuclear localization signals (NLS), which use the importin  $\alpha/\beta$ -system to shuttle APC into the nucleus [92]. It was shown that phosphorylation of APC at Ser2054 (C-terminal of the second NLS) negatively regulates APC transport to the nucleus [92]. Curiously, APC which lacks the NLS can still enter the nucleus [93] and it was reported that B56 $\alpha$ , the catalytic subunit of PP2A, facilitates the nuclear transport of APC [93]. Nuclear APC was found to negatively regulate  $\beta$ -catenin-mediated transcription [94].

Among other cytoplasmic proteins that interact with APC are plakoglobin ( $\gamma$ -catenin) [95], tubulin [96], EB1 (microtubule-associated protein of the RP/EB family) [97] and hDLG (human disks large homolog 1) [94, 98]. In the nucleus APC has been shown to interact with DNA polymerase  $\beta$ , proliferating cell nuclear antigen (PCNA), the protein tyrosine phosphatase (PTP-BL) [94], the transcription factor activator protein AP-2 $\alpha$  and the nuclear export factor Xpo1 (Exportin 1) [94].

### GSK3

Glycogen synthase kinase-3 (GSK3) was initially identified as a serine/threonine protein kinase, which phosphorylates glycogen synthase in rabbit skeletal muscles leading to an inhibition of glycogen synthesis [99]. In humans there are two isoforms, GSK3 $\alpha$  (483 aa, 51kDa) and GSK3 $\beta$  (433 aa, 47kDa), that are encoded by different genes. The two isoforms have high amino acid sequence identity (97%) in the catalytic domain, but are less conserved otherwise. GSK3 $\beta$  has two splicing isoforms, one containing a 13

aa insertion (GSK3 $\beta$ 2) [100]. Although mutations in GSK3 are usually not associated with cancers, downregulation of GSK3 has been observed in hepatocellular carcinoma, squamous cell carcinoma and prostate cancer [101-103]. However, GSK3 was also suggested as anti-cancer biotarget [104]. GSK3 is involved in a large number of cellular processes [104-107]. A knockout of GSK3 $\beta$  in mice leads to embryonic lethality and is not compensated by GSK3 $\alpha$  [108]. Although GSK3 recognition sequences can be found in almost half of all human proteins, a recent overview provides a list of 77 validated substrates of GSK3 [109]. These substrates can be clustered into several functional subsets: inflammation, cellular proliferation, structural rearrangements and glucose metabolism. Importantly, GSK3 appears to be involved in decision points between maintaining stem cell properties, and triggering differentiation. Inhibition of GSK3 together with inhibiting FGF-MAPK (FGF - Fibroblast Growth Factor) signaling enables long-term propagation of embryonal stem cells in mice [110]. Furthermore, deletion of both GSK3 $\alpha$  and GSK3 $\beta$  in the brain increases self-renewal of neuronal progenitor cells, while neurogenesis is downregulated [111].

In the context of Wnt/ $\beta$ -catenin signaling, the GSK3 $\alpha$  and GSK3 $\beta$  isoforms were shown to be fully redundant [112] and thus will be referred to herein collectively as GSK3. However, in other cellular processes GSK3 $\alpha$  and GSK3 $\beta$  may not fully compensate each others functions [113, 114].

An involvement of GSK3 in Wnt/ $\beta$ -catenin signaling was first shown in *Xenopus laevis* embryos, where a mutated GSK3 $\beta$  induced a ventral axis duplication indicative of overactive canonical Wnt signaling [21, 115]. In contrast, active GSK3 was shown to negatively regulate Wnt/ $\beta$ -catenin signaling through an N-terminal phosphorylation of  $\beta$ -catenin in the destruction complex [116-118]. Interestingly, plakoglobin can also undergo GSK3-dependent phosphorylation and proteasomal degradation [119-120]. In the case of  $\beta$ -catenin, GSK3 requires a priming kinase that acts on a 4-5 amino acid C-terminal to a GSK3 phosphorylation site. Phosphorylated amino acids of the priming site bind to the catalytic pocket in GSK3 $\beta$ , formed by the amino acids Arg96, Arg180 and Lys205 and facilitate further phosphorylation through GSK3 [121].

The kinase activity of GSK3 can be attenuated by a phosphorylation of Ser21/Ser9 (GSK3 $\alpha$ /GSK3 $\beta$ ) through different kinases: protein kinase A (PKA), Akt/PKB (protein kinase B), PKC (protein Kinase C), p90 ribosomal S6 kinase/MAPK-activating protein (p90RSK/MAPKAP) and p70 ribosomal S6 kinase (p70S6K) [107]. p38 MAPK (p38 mitogen-activated protein kinases) can selectively reduce the kinase activity of GSK3 $\beta$ , but not GSK3 $\alpha$  through a phosphorylation of Thr390, which can lead to reduced  $\beta$ -catenin degradation [122]. In contrast, autophosphorylation of GSK3 $\alpha$ /GSK3 $\beta$  at Tyr279 or Tyr216 respectively can enhance the activity of GSK3 [107, 123].

There are multiple further protein/protein interactions that can modulate GSK3 activity. FRAT1 (frequently rearranged in advanced T-cell) and FRAT2, members of the GSK-3-binding protein family, compete with Axin for GSK3 binding and hence inhibit the activity of GSK3 in the context of Axin [124-126] whereby the Axin binding site on the GSK3 protein overlaps with the binding site for FRAT. Also, Dishevelled can interact with FRAT1, recruiting it to a ternary complex between Dvl, Axin and GSK3. This complex leads to an inhibition of GSK3 and consequently to a stabilization of  $\beta$ -catenin and an activation of Wnt/ $\beta$ -catenin signaling [125]. FRAT1 overexpression is associated with tumorigenesis [127-130]. Interestingly, FRAT1 is considered to be one of the links between  $\beta$ -catenin dependent (canonical) and  $\beta$ -catenin independent (non-canonical) Wnt signaling through its activation of JNK and AP-1(activator protein 1) [131].

In addition to a direct involvement in regulating  $\beta$ -catenin stability by phosphorylation, GSK3 has also a plethora of indirect

implications on Wnt/ $\beta$ -catenin signaling, predominantly synergizing with its function in antagonizing Wnt/ $\beta$ -catenin signaling. In particular GSK3 has both an effect on the transcriptional regulation of  $\beta$ -catenin, and on central  $\beta$ -catenin target genes. The oncogene c-Myc is among the primary target genes that are upregulated by  $\beta$ -catenin/Lef. GSK3 phosphorylates the Thr58 residue of c-Myc leading to a reduction of its half-life [109]. Importantly, GSK3 phosphorylates Ser residues in the oxygen-dependent degradation domain of the transcription factor Hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) that links hypoxia to  $\beta$ -catenin-mediated signaling. An inhibition of GSK3 promotes HIF-1 $\alpha$  stability while an upregulation of GSK3 has an opposite effect [132]. Another interesting substrate of GSK3 with implications on  $\beta$ -catenin-mediated signaling is the zinc-finger transcription factor Snail, which represses the transcription of E-cadherin. An inhibition of GSK3 leads to an upregulation of Snail followed by a down-regulation of E-cadherin which could lead to a cytoplasmic mobilization of  $\beta$ -catenin [133]. A more detailed description of GSK3 and GSK3 inhibitors is given in [104].

Numerous small molecular GSK3 inhibitors have been reported [134, 135]. Most GSK3 inhibitors target the ATP-binding site in the catalytic domain of the protein, which has 86% amino acid identity to the ATP-binding sites of CDK1 (cyclin-dependent kinase 1) and other kinases [107]. Hence, most of the published GSK3 inhibitors show low selectivity for GSK3. However, inhibitors that target the substrate binding site of GSK3 with increased specificity, are also reported [136].

There are also possibilities for increasing the activity of GSK3 by pharmacological intervention. Phosphorylation of GSK3 by p38 MAPK on Thr390 reduces the activity of the GSK3 kinase. Accordingly, small molecular inhibitors of p38 MAPK (SB203580 or SB239063, Table 1) can lead to increased GSK3 activity and in consequence reduced Wnt/ $\beta$ -catenin signaling [122, 137]. Interestingly, both compounds affect only GSK3 $\beta$ , but not GSK3 $\alpha$ , making the intervention isoform-specific. Several p38 MAPK inhibitors are in clinical trials including the anti-inflammatory drug PH-797804 and dilmapiomod [138, 139].

### Tankyrases

There are two Poly (ADP-ribose) polymerases (PARPs) that are implicated in Wnt/ $\beta$ -catenin signaling: Tankyrase 1 (PARP5a) and Tankyrase 2 (PARP5b) [140, 141]. To a large extent, Tankyrases 1 and 2 appear to have redundant functions.

Tankyrase 1 has four functional domains: the HPS domain (consisting of His, Pro and Ser repeats), Ankyrin domain (consists of 20 ankyrin repeats), a SAM (sterile alpha motif) domain and the catalytic PARP domain. In contrast to Tankyrase 1, Tankyrase 2 lacks the HPS domain [141]. The PARP domain catalyzes poly(ADP-ribosylation), the SAM and Ankyrin domains participate in the formation of protein-protein complexes with substrates, while the functions of the HPS remain obscure. One of the central properties of Tankyrases is their capability to form dynamic oligomers predominantly through their SAM domain, but presumably also assisted by the ankyrin domains, and to subsequently destabilize such oligomers through increasing, context depending poly(ADP-ribosylation) [142-146]. The ability of Tankyrase to form dynamic multimers has led to the suggestion that Tankyrase oligomers can regulate the assembly and disassembly of large polymerized structures in response to signals [145]. In the context of the destruction complex, poly(ADP-ribosylation) of Tankyrases, and possibly Axin appear to trigger deoligomerization due to an accumulation of negative charges and repulsive forces [145]. Through poly(ADP-ribosylation) Tankyrases also regulate a number of further protein complexes, including complexes involving IRAP (insulin-responsive amino peptidase), NuMa (nuclear mitotic apparatus protein 1), Mcl-1 (myeloid cell leukaemia 1), EBNA-1 (Epstein-Barr nuclear antigen1), TRF1 (telomeric repeat binding factor 1), TAB182 (Tankyrase 1 binding protein 182) and GRB14 (growth

factor receptor-bound protein 14). A recent overview of Tankyrase substrates is provided in [146].

Poly(ADP-ribosylation) is a catalytic reaction whereby nicotinamide adenine dinucleotide (NAD<sup>+</sup>) is used as a substrate to create multimeric side chains. At the first step, in the catalytic PARP domain, the nicotinamide part of NAD<sup>+</sup> interacts with the Gly1032 (human Tankyrase 2 amino acid sequence numbering) residue of Tankyrase. At the second step, ADP-ribose is transferred from NAD<sup>+</sup> to a glutamic acid residue of the target protein. Next, a further monomer is added to the polymer via the same mechanism using the hydroxyl group of the previous monomer. Poly(ADP-ribosylation) may include branching points in a process that is hitherto poorly understood. In the context of the destruction complex, poly(ADP-ribosylated) proteins appear to interact with the ubiquitin ligase RNF146 [37, 67, 68].

Poly(ADP-ribosylation) is a reversible process since poly(ADP-ribose) polymers may be removed by poly(ADP-ribose) glycohydrolase (PARG). It was recently suggested that in this process the Glu115 residue of PARG (*T. curvata*) replaces the ribose moiety from an ester followed by a replacement of Glu115 by a water molecule [147]. A PARG inhibitor, ADP-HPD was shown to decrease Tankyrase stability [148, 149].

The involvement of Tankyrase in attenuating the destruction complex was described [37]. In the process, Tankyrase poly(ADP-ribosylates) Axin. Poly(ADP-ribosylated) Axin is then recognized by the RNF146 ubiquitin ligase followed by ubiquitination and degradation [37, 67, 68]. The interaction of RNF146 with the poly(ADP-ribose) tail of Axin appears to be mediated through a recognition of the iso-ADP-ribose moiety (but not ADP-ribose) by the WWE domain of RNF146 [150]. In parallel, Tankyrase auto poly(ADP-ribosylation) also leads to RNF146-mediated ubiquitination and subsequent degradation [67, 68]. Furthermore, RNF146 is poly(ADP-ribosylated) and ubiquitinated [68]. The HECT-type ubiquitin E3 ligase HUWE1 associates to RNF146 and was suggested to participate in ubiquitin chain elongation [68]. A number of RNF146-interacting proteins were identified, including PARP1, PARP2 and three proteins involved in DNA-damage response [68]. Noteworthy, RNF146 prevents Tankyrase co-localization to centrosomes [68].

To date it remains unclear whether Tankyrase presence and stability in the degradation complex can be regulated by mechanisms other than poly(ADP-ribosylation), and whether such regulation might be dependent on components of Wnt/ $\beta$ -catenin signaling. Several kinases are known to be involved in Tankyrase phosphorylation: GSK3, PLK1 and MAPK. PLK1 complexes with Tankyrase1 both *in vivo* and *in vitro* and activates Tankyrase through phosphorylation [151]. Disruption of PLK1 decreases the stability of Tankyrase 1 and leads to a reduction of its PARP activity. Interestingly, phosphorylation of Tankyrase by PLK1 was also shown to affect mitotic spindle assembly (see below) and the regulation of telomeric ends [79]. PLK1 also mediates phosphorylation of Dishevelled2 [152]. MAPK has been shown to enhance the catalytic activity of Tankyrase in the context of IRAP4 [153].

Tankyrase has further cellular functions. It has been shown that Tankyrase is involved in glucose transport. In this process, Tankyrase associates with GLUT4 (glucose transporter type 4) vesicles through binding to the insulin responsive aminopeptidase (IRAP) [153]. The IRAP is required for the targeting of vesicles carrying the glucose transporter GLUT4 [154]. GLUT4 mediates the insulin-stimulated glucose uptake in adipocytes and muscle cells. In this context, Tankyrase acts as a positive regulator of insulin-mediated GLUT4 translocation from cytosolic vesicles to the cell surface to mediate glucose uptake [141].

Another role of Tankyrase is its influence on the cell cycle through its interaction with the nuclear mitotic apparatus protein (NuMA), associated to spindle poles in mitosis from prophase to

anaphase [155, 156]. NuMa is thought to be an important structural protein both for the nucleus and spindle poles [156]. A Tankyrase knockdown leads to defects in mitotic spindle functions and to defects in the microtubules [141]. GSK3 is involved in mitotic phosphorylation of Tankyrase [157] on Ser978, Thr982, Ser987 and Ser991 in the conserved [S/T]-X-X-X-[S/T] motif. Whether Tankyrase phosphorylation by GSK3 impacts Tankyrase function at the mitotic spindle through NuMa poly(ADP-ribosylation) remains to be studied.

Tankyrases are involved in telomere maintenance by poly(ADP)ribosylating TRF1 (which prevents telomerase activity on telomeres) and releasing TRF1 from telomeres [140, 151]. In this context it is noteworthy that there are further links between telomeres and Wnt/ $\beta$ -catenin signaling. One of them is TERT (telomerase reverse transcriptase), a catalytic subunit of telomerase, that was shown to directly regulate Wnt/ $\beta$ -catenin signal by participating as a co-factor in the  $\beta$ -catenin/Tcf transcriptional complex [158]. It has been shown that an overexpression of either TERT or  $\beta$ -catenin in mouse hair follicles results in a similar phenotype [159, 160, 161]. Hence, under certain conditions gene regulation by TERT and  $\beta$ -catenin might intersect [162].

As many other components of the  $\beta$ -catenin degradation complex, Tankyrase can be observed in the vicinity of the plasma membrane. Such localization is triggered by E-cadherin-mediated cell-cell adhesion, as shown on polarized epithelial MDCK cells [157]. Tankyrase recruitment to the lateral membrane follows a calcium initiated cell-cell adhesion and is reversed by calcium depletion. Inhibition of the poly(ADP)ribosylation of Tankyrase leads to its stabilization and accumulation near the lateral membrane [157]. An inhibition of Tankyrase also leads to an inhibition of EMT *ex vivo* [163], which indicates that Tankyrase may influence intercellular adhesion. Accordingly, the disruption of intercellular adhesion by calcium depletion leads to a Tankyrase release into cytoplasm.

Tankyrases have been identified as a promising target for inhibiting Wnt/ $\beta$ -catenin signaling. Several research groups have identified small molecules that inhibit Tankyrases and correspondingly Wnt/ $\beta$ -catenin signaling (Table 1) by stabilizing the destruction complex [37-40], [165, 166].

Tankyrase inhibitors can be classified into two groups that bind differentially to the PARP catalytic center: one group binds to the nicotinamide pocket whereas the other occupies predominantly the adjacent ADP pocket. The first group includes the Tankyrase selective XAV939, and many generic PARP inhibitors (PDB structures in Protein Data Bank, [www.rcsb.org](http://www.rcsb.org): 3KR8, 3MHJ, 3P0P, 3P0Q, 3MHK and 3U9H) [37, 165, 166]. These compounds usually have stacking interactions with the side chain of Tyr1071 and form two hydrogen bonds with Gly1032 (numbering for human Tankyrase 2). Tankyrase inhibitors that bind to the ADP pocket include IWR1, JW55, and JW74 (Table 1) [38-40]. These molecules participate in stacking interactions with the side chain of histidine (aa 1201 in Tankyrase 1, aa 1048 in Tankyrase 2) and in hydrogen bonding with the backbone amides of Tyr1213 (Tyr1060 in Tankyrase 2) and Asp1198 (Asp1045 in Tankyrase 2) in the adenine dinucleotide pocket (PDB structures in Protein Data Bank, [www.rcsb.org](http://www.rcsb.org): 1UDD, 1UA9 and 4DVI) [167-169]. An interesting binding mechanism is exerted by the compound PJ34 (PDB code, [www.rcsb.org](http://www.rcsb.org): 3UH2) in that two molecules of PJ34 (Table 1) can simultaneously bind to the Tankyrase PARP domain; one in the nicotinamide pocket, the other in the ADP pocket [170]. A profound review on ADP-(ribosylation) as old and new targets for cancer therapy is given in [171].

### The Wnt receptor complex

The Wnt signalosome is the the best studied system that counteracts  $\beta$ -catenin degradation and enhances  $\beta$ -catenin-mediated signaling. The Wnt signalosome does so by recruiting components of the destruction complex to the membrane, a process that is trig-

gered by binding of one of several Wnt morphogens to the transmembrane proteins Frizzled and LRP5/6. In the process, the Wnt signalosome itself is cleared from the plasma membrane by endocytosis.

Before Wnt morphogens can induce the Wnt signalosome, they mature by undergoing a number of post-translational modifications prior to being secreted. During post-translational maturation, Wnt morphogens undergo N-glycosylation in the endoplasmic reticulum (ER) [172-174], S-palmitoylation of the N-terminal residue Cys77 (mouse Wnt3a) [175] and acetylation with palmitoleic acid at Ser209, which is required for secretion [176, 177]. The functional implications of these post-translational modifications are not entirely understood. For example, some studies suggest that glycosylation is important for secretion, while other studies do not confirm such a link [172], [178]. Although palmitoleic modification may not be strictly required for secretion, it participates in Wnt binding to Frizzled receptors and in Wnt signal transduction [179]. Wnt proteins with a mutation in the cysteine that is the target for palmitoylation are not able to transduce Wnt signaling. It has been proposed that the hydrophobicity of palmitate and palmitoleic acid is required for Wnt to interact with cellular membranes, which is necessary for the interaction with Frizzled/LRP5/6 receptors [180]. The lipid modifications as well as the acceptor amino acids are highly conserved among different Wnt proteins in diverse organisms.

After posttranslational modifications in the ER, Wnt proteins are transported to the Golgi apparatus. From the Golgi apparatus, Wnt proteins are translocated to the cellular membrane with the assistance of the seven-pass transmembrane orphan G-protein coupled receptor Evenness interrupted (Evi)/Wntless (Wls) (GPR177 in mammals) that co-localizes to the Golgi apparatus, cellular membrane and endocytic vesicles [176, 181-183]. Evi/Wntless exports all Wnt proteins [184]. In *Drosophila* it has been shown that acylation of Wnts is required for their binding to Evi/Wls, while glycosylation and S-palmitoylation do not appear to be required [172, 181, 185]. In mammalian cells N-linked glycosylation is required for GPR177 localization to the Golgi apparatus and targeting to the plasma membrane [186, 187]. In mouse, deletion of GPR177 leads to axis formation defects and early fetal lethality [188]. Finally, Evi/Wls is cleared from the plasma membrane by endocytosis in a process that involves the GTPase Rab5 [164]. Clathrin-mediated endocytosis of Evi/Wls and endosomal sorting through the trans-Golgi network (TGN) appear to be required for the proper secretion of Wnt morphogens. Thus, disruption of these processes leads to an Evi/Wls accumulation on the plasma membrane and a downregulation of Wnt secretion [176, 189]. Lipidation and acidification of secretory vesicles was suggested to be important for Wnt secretion [176, 190] and a blockage of v-ATPase-mediated acidification of secretion vesicles leads to an accumulation of the Evi/Wls complex in vicinity of the cellular membrane and downregulates Wnt secretion [185]. Interestingly, the transcription of the mammalian GPR177 gene is enhanced by Wnt/ $\beta$ -catenin signaling [186].

Also central to the secretion of mature Wnt morphogens is the multipass membrane protein Porcupine (Porc) that interacts with the N-terminal domain of Wnt [191]. Loss of Porc leads to an accumulation of Wnts in the ER [192]. Porc function is antagonized by the protein Oto, which is a homolog of the *Drosophila* glycosylphosphatidylinositol (GPI)-inositol-deacylase PGAP1. Oto deacetylates Wnt proteins in the secretory pathway, leading to its retention in the endoplasmic reticulum [193]. A class of potent small molecule inhibitors called IWP (Table 1) that target Porc and thereby inhibit Wnt secretion was identified using high-throughput screening [38]. Diverse IWP analogs were reported recently [194].

Signaling of Wnts is limited to approximately 20 cellular layers from the source of secretion [195]. It has been shown that heparane sulfate proteoglycans (HSPG) are involved in Wnt signaling and stabilizing the activity of purified Wnt proteins through preventing their aggregation [196]. Since Wnt proteins/morphogens are insolu-

ble and highly lipophilic, they require a specialized transport system. One way to transport Wnt proteins are lipoprotein particles, which associate with lipid modified Wnts [197, 198]. Another interesting way to transport Wnts is a direct translocation from cell to cell through a series of exocytosis-endocytosis cycles [199]. Hence, Wnts can be transported between the cells via exosomal vesicles [198, 200].

There are several models that describe the binding of Wnt morphogens to the Fz-LRP5/6 receptors to initiate Wnt/ $\beta$ -catenin signaling [7]. Historically, Wnt morphogens and Frizzled receptors were classified as canonical ( $\beta$ -catenin dependent) and non-canonical ( $\beta$ -catenin independent) proteins. However, closer scrutiny revealed that at least some of the Frizzled receptors and Wnt proteins can participate in both  $\beta$ -catenin dependent and independent signaling in a context-dependent manner [201-204]. LRP5 and LRP6 are thought to play redundant roles in the Wnt signalosome and are usually referred to as LRP5/6 [205, 206]. In humans, 19 Wnt morphogens and 10 Frizzled receptors are known to date. Mutations in Frizzled receptors were first identified in mutant *Drosophila* [207]. Later, it was found that Frizzled proteins belong to the family of seven-pass transmembrane receptors and bind Wnts [208, 209]. The most popular model proposes that Wnt binds to the transmembrane protein Frizzled and provides a link to the transmembrane protein LRP5/6. This binding forms the core of the Wnt signalosome and triggers a receptor oligomerization. Heterooligomerization of Fz-LRP5/6 is sufficient for activating Wnt/ $\beta$ -catenin signaling as demonstrated elegantly by studies involving chimeric Fz-LRP5/6 and Fz-Dkk proteins [210-212]. Evidence suggests that LRP6, in addition to participating positively in Wnt/ $\beta$ -catenin signaling, may also be engaged in an inhibitory role in  $\beta$ -catenin independent Wnt signaling [213, 214].

The oligomerization of the Wnt signalosome is enhanced on the intracellular side of the complex by Dishevelled, which oligomerizes through its DIX domain [215-217]. The Ser/Thr-rich motifs on LRP6 together with Dishevelled (Dvl) are then responsible for recruiting Axin and GSK3 to the Wnt signalosome [218-222], inducing Axin polymerization at the cytoplasmic side of the receptor complex [46]. In this process, the lipid kinases PI4KII and PIP5KI have been implicated in the formation of the Wnt signalosome and the translocation of Axin/GSK3 from the destruction complex to the plasma membrane [223]. It was shown in *Drosophila* that the recruitment of Dishevelled and Axin to the membrane is facilitated through G-proteins with trimeric Go-proteins acting as immediate transducer [224-227]. One of the Go subunits, Gao, uses an RGS domain to interact directly with Axin, recruiting it to the membrane [54, 228]. Gao also interacts with Rab5, an interaction that presumably promotes the internalization of the Wnt/Frizzled/LRP complexes [54, 229]. The Go subunit, G $\beta\gamma$ , recruits Dishevelled to the plasma membrane upon Wnt binding to the Frizzled receptors [54].

Together with Axin, two kinases - GSK3 and the primer kinases CK1( $\alpha$ ,  $\gamma$ ,  $\epsilon$ ) - are juxtaposed with LRP5/6 [230]. CK1-mediated phosphorylation acts as primer, which triggers GSK3-mediated phosphorylation. CK1 $\gamma$  and GSK3 phosphorylate PP(S/T)PX(S/T) repeats in the cytoplasmic C-domain of LRP5/6, a step that is crucial for rescuing  $\beta$ -catenin from degradation [53, 217, 219, 231]. Upon binding to LRP5/6, the kinases GSK3 $\beta$  and CK1 $\gamma$  switch from phosphorylating  $\beta$ -catenin to phosphorylating LRP5/6 [217, 232]. One model suggested that phosphorylation of Dishevelled-2 by CK1 $\epsilon$  increases its affinity to Frizzled receptors [219, 233]. CK1 $\epsilon$  was also shown to be required for Dvl-2 phosphorylation and its binding to LRP5/6 [230]. In turn, CK1 $\epsilon$  was shown to be directly activated by Wnt signaling through C-terminal dephosphorylation [234].

Recently, the seven-pass transmembrane protein TMEM198 was identified in *Xenopus tropicalis* and shown to associate with LRP6, recruiting CK1 to the receptor complex and promoting LRP6 phosphorylation [235]. Proline-directed kinases have also been

shown to be involved in LRP5/6 C-terminal phosphorylation including PKA, Pftk (Cdk14), MAPK (such as p38, ERK1/2, and JNK1) and G-protein-coupled receptor kinases (Grk5/6) [236, 237]. Recently it was also found that Wnt/ $\beta$ -catenin signaling cooperates with tyrosine signaling through FGFR2 (FGF receptor 2), FGFR3 (FGF receptor 3), EGFR (epidermal growth factor receptor) and TRKA kinases (Tyrosine kinase receptor type 1) [238]. Intriguingly, phosphorylated PP(S/T)PX(S/T) peptides alone, derived from the C-terminus of LRP5/6, are able to activate Wnt signaling through a direct inhibition of GSK3 [222, 239].

The release of  $\beta$ -catenin from phosphorylation by CK1 $\alpha$  and GSK3 may not be the only mechanism for the Wnt signalosome to regulate  $\beta$ -catenin levels. It was found that LRP6 can stabilize  $\beta$ -catenin indirectly through Axin degradation and GSK3 inhibition [231, 240]. Without the structural protein Axin, CK1 $\alpha$  and GSK3 cannot form a complex that phosphorylates  $\beta$ -catenin at the N-terminal end.

A further mechanism, by which the Wnt signalosome reduces  $\beta$ -catenin degradation, is an induced GSK3 internalization by multivesicular endosomes. This physically reduces the cytoplasmic presence of the kinase [241, 242, 243].

The LGR4, -5 and -6 G-protein coupled receptors were shown to associate with the Frizzled-LRP5/6 signalosome and mediate Wnt/ $\beta$ -catenin signaling in intestinal crypt cells. LGR receptors were previously considered to be orphan, but recently R-spondin was identified as their ligand [244, 245, 246].

Furthermore, the parathyroid hormone receptor was found to directly regulate  $\beta$ -catenin signaling through interactions with Dishevelled, but without an involvement of Frizzled receptors [247].

Most of the described receptors are expressed specifically in certain organs, or tissues. Thus parathyroid hormone receptors exert their function in kidneys and bones [248], while LGR4-6 are found in the stomach, in the stem cell compartment of the small intestine and in hair follicles [249]. The complex interface between various receptors and components of  $\beta$ -catenin signaling appear to allow an intricate adaptive regulation.

The Wnt signalosome has been a target for developing antibodies and small drug therapeutics. A monoclonal antibody against Wnt-1 has shown to induce apoptosis in cancer cell lines expressing the Wnt-1 protein [250]. Antibodies against Frizzled-5, developed by OncoMed, have shown anti-tumor properties [251]. The OMP-18R5 antibody, developed in collaboration between Bayer and OncoMed, has entered Phase I clinical trials. Furthermore, antibodies against Frizzled 10 (FZD10) may reduce osteosarcoma growth and metastasis [252].

A small molecule, which triggers the internalization of Wnt receptors, has been identified as the FDA approved antihelminthic drug Niclosamide (Table 1). Amongst other functions, Niclosamide was found to inhibit Wnt/Frizzled-1 signaling with an IC<sub>50</sub> of 0.5  $\pm$  0.05  $\mu$ M [253-255]. It also downregulates Dishevelled-2 (Dvl-2) [256] and induces LRP6 degradation in prostate and breast cancer cells [254]. Interestingly, Niclosamide has no reported toxicity against non-cancer cells [256].

### Dishevelled

Dishevelled participates in both  $\beta$ -catenin dependent and independent Wnt signaling. Three different Dishevelled proteins are known in humans, which have a similar size and domain organization. All Dishevelled proteins share three functional domains: an N-terminal DIX domain (named after Dishevelled and Axin), a central PDZ domain (Postsynaptic density 95, Discs Large, Zonula occludens-1) and a C-terminal DEP domain (Dvl, Egl-10, Pleckstrin).

The DIX domain is responsible for the polymerization of Dishevelled in the Wnt signalosome [257]. The resulting tetramerization of the Frizzled-LRP5/6 signal complex has been shown to be

required for the phosphorylation of the cytoplasmic tail of LRP5/6 [258]. The protein Ccd1, which also has a DIX domain, serves as a positive regulator of Wnt signaling by forming heterodimers with the Dishevelled DIX domain [259]. Recently, the ability of Axin to polymerize through its DIX domain was shown to be crucial for its function in the destruction complex, while a binding between the Axin DIX domain and its Dishevelled counterpart abrogates Axin polymerization. Hence, in addition of being important for the Wnt signalosome, heteromer formation through the DIX domain might be important for inhibiting the formation of the destruction complex by Dishevelled [46, 48], [260, 261].

The DEP domain of Dishevelled was suggested to mediate the interaction with membrane lipids [262] and to facilitate the interaction with Frizzleds through direct binding [263].

The PDZ domain of Dishevelled interacts with the cytosolic C-terminal tail of Frizzled [264]. This interaction can be counteracted by the Dapper (Dapper1 and Dapper3) proteins, which bind to the Dishevelled PDZ domain to prevent its interactions with Frizzled [216, 265, 266]. Proteins of Dapper family have been shown to promote Dishevelled degradation mediated by lysosomes instead of proteasomes [265, 267, 268]. Interestingly, Dishevelled was shown to promote Wnt5a-induced endocytosis of Frizzled by using the PDZ-domain to interact with the N-terminal region of  $\beta$ -arrestin 2 [269, 270]. Furthermore, Dishevelled 2 was shown to interact with a subunit of the clathrin adaptor protein AP2, micro2-adaptin. The interaction appears to be required for Frizzled 4 internalization [271].

Phosphorylation modulates the activity of Dishevelled in the Wnt signalosome [272]. Three kinases, CK2, PAR1 and CK1 $\delta/\epsilon$  that respond to Wnt signaling, have been implicated in Dishevelled phosphorylation [273, 274]. For instance in mouse SN4741 neurons, both Wnt5a and Wnt3a have been shown to induce phosphorylation of Dishevelled-2 and Dishevelled-3 [273]. Based on loss-of-function and gain-of-function experiments a model of a stepwise phosphorylation of Dishevelled was suggested. First, Dishevelled is phosphorylated by the CK2/PAR1 kinases and then by CK1 [274]. It was proposed that CK1 $\epsilon$  can inactivate Dishevelled through phosphorylation [274].

The roles of Dishevelled in Wnt/ $\beta$ -catenin signaling go beyond stabilizing the Wnt signalosome and destabilizing the degradation complex. In *Xenopus* it has been demonstrated that mutations in the NLS of Dishevelled attenuate Wnt/ $\beta$ -catenin signaling [275]. Dishevelled translocates to the nucleus, where it interacts with the  $\beta$ -catenin/Tcf complex and participates in transcriptional regulation of  $\beta$ -catenin target genes [276, 277]. In the nucleus, Dishevelled can also form a complex with the histone deacetylase Sirtuin 1 (SIRT1), which supports the transcription of Wnt target genes. In accordance, the SIRT1 inhibitor cambinol negatively regulates Wnt signaling (Table 1) [278]. Sirtuin1 is a member of the sirtuins proteins family and possesses (NAD<sup>+</sup>)-dependent acetyl-lysine deacetylating activity.

Furthermore, Dishevelled proteins also participate in interactions that affect structural rearrangements of the cell [279]. Through its PDZ domain, Dvl-1 was shown to protect microtubules from depolymerization. It was furthermore demonstrated that the stabilization of microtubules by Dvl-1 is enhanced by GSK3 inhibition [280]. Studies in *C. elegans*, *Drosophila* and vertebrates have led to the conclusion that Wnt/ $\beta$ -catenin signaling may regulate the orientation of the mitotic spindle through Dishevelled [281-283].

Finally, autophagy has been proposed to inhibit Wnt signaling through Dishevelled degradation. It has been shown that an ubiquitination of Dishevelled by the Von Hippel-Lindau protein facilitates its binding to p62, which in turn assists an LC3-mediated recruitment of Dishevelled to autophagosomes [276]. In late stages of colon cancer, a negative correlation between Dishevelled expression and autophagy was observed [276].

The PDZ domain of Dishevelled has been used to develop small molecule inhibitors for Wnt/ $\beta$ -catenin signaling. A series of synthetic inhibitors were identified by virtual screening, QSAR and computer-based modeling on the basis of Scaffolds A and B (Table 1) [284-288]. These compounds interact with the groove of PDZ domain, which interacts with the Dapper proteins [216]. Compound J01-017a (Table 1) is currently the strongest Dishevelled binder, inhibiting Wnt signaling with a  $K_i$  of 1.5 $\pm$ 0.2  $\mu$ M [288]. Compound NSC668036 [289] imitates a Dapper protein and binds to the PDZ domain of Dishevelled. Compound 3289-8625 (Table 1) binds to the same pocket as NSC668036 in Dishevelled with a  $K_d$  of 10.6 $\pm$ 1.7  $\mu$ M [286].

### The Roles of Endocytosis in Wnt/ $\beta$ -Catenin Signaling

Endocytosis plays crucial role in most signaling pathways. In Wnt/ $\beta$ -catenin signaling, both clathrin- and caveolin-mediated endocytosis have been described [246, 290]. Clathrin-mediated endocytosis is mediated through vesicles that are coated by the clathrin protein, also referred to as clathrin-coated pits, while caveolin-mediated endocytosis is characterized by membrane proteins called caveolins which participate in the formation of membrane invaginations called caveolae.

It has been shown that the Wnt signalosome including Frizzled, GSK-3, Dishevelled and AXIN co-localizes with caveolae where the proteins involved in the signalosome are thought to be sequestered, preventing them from participating in the formation of a destruction complex [217, 243, 246, 290]. It remains unclear whether all Frizzled receptors can be processed through this endocytic pathway [290]. The involvement of caveolin in the endocytosis of LRP6/Frizzled was shown to be amenable to pharmacological inhibition by filipin (Table 1) [291].

Somewhat contradictory data are published on clathrin-mediated endocytosis [292]. For example, in the ventral cuticle of *Drosophila* larvae, clathrin-mediated endocytosis was reported to be required for the removal of the Wingless protein leading to a downregulation of the signal [293]. However, clathrin-mediated endocytosis was also claimed to be required for Wnt/ $\beta$ -catenin signaling as shown by using the endocytosis inhibitors hypertonic sucrose and chlorpromazine in L-cells [164, 242]. Interestingly, one of the important components of clathrin-mediated GPCR endocytosis is the clathrin-associated sorting protein (CLASP)  $\beta$ -arrestin, which was shown to be required for Wnt/ $\beta$ -catenin signaling [294].

For example, co-expression of  $\beta$ -arrestin and Dishevelled was shown to induce Wnt/ $\beta$ -catenin signaling [270, 295]. Furthermore, in *Xenopus* embryos it was shown that morpholinos against  $\beta$ -arrestin reduce endogenous  $\beta$ -catenin levels and interrupt induced axis duplication [270]. In this context it was demonstrated that  $\beta$ -arrestin can form a trimeric complex with Axin, and the N-terminus of Dishevelled [270]. It was also suggested that  $\beta$ -arrestin may couple Frizzled receptors to phosphorylated Dishevelled and thus participate in Wnt/ $\beta$ -catenin signal transduction. [290].

Blocking of endocytosis resulted in Dvl-2 degradation [269]. Thus, although endocytosis clears the Wnt signalosome from the cellular surface, there is an increasing evidence that clathrin-dependent endocytosis is in itself an important process in Wnt/ $\beta$ -catenin signal activation [164, 290, 292].

Endocytosis is not a process that is limited to the core Wnt signalosome. Recently, the endocytic adaptor disabled-2 (Dab-2) was shown to selectively recruit LRP6 to clathrin-dependent endocytosis whereby CK2-mediated phosphorylation of Ser1579 in LRP6 promotes its interactions with Dab-2 and the association with clathrin [296]. Clathrin-mediated internalization was also shown for LGR4-mediated  $\beta$ -catenin signaling, a process that could be disrupted by the small molecule clathrin inhibitor monodansylcadaverine (MDC)[244]. Curiously, the Wnt inhibitor Dkk1 also

triggers an internalization of LRP6 through clathrin-mediated endocytosis [164, 297].

Divergent consequences have been reported for the endocytosis of Wnt signalosomes on Wnt/ $\beta$ -catenin [298]. Endocytotic vesicles containing Wnt signalosomes may shuttle to early endosomes (EE) from which the receptor complex may be sequestered into intraluminal vesicles of multivesicular endosomes (MVEs). These can either be released as exosomes, whereby exocytosis itself can act as a signal transduction mechanism [199, 298], [299], or MVEs may fuse with lysosomes that lead to a degradation of the included proteins [298, 300, 301]. It is unclear to what stage during this process the Wnt signalosome will remain active, however, deactivation of Wingless was shown to occur after it accumulates in multivesicular endosomes which target it further for lysosomal degradation [302]. It was shown that in response to Wnt ligands or LRP6 overexpression, GSK3 in complex with LRP5/6 is delivered to the lumen of MVEs, separating GSK3 from its cytosolic substrates [241, 303]. Two proteins Hrs/Vps27 and Vps4 that are components of the endosomal sorting (ESCRT) machinery have shown to be required for MVE formation and it has been demonstrated that inhibition of Hrs or Vps4 leads to reduced Wnt/ $\beta$ -catenin signaling [241].

Finally, autophagy can negatively regulate Wnt pathway through the degradation of ubiquitinated Dishevelled which aggregates with LC3-mediated autophagosomes [276].

#### **Connections between the Wnt-Frizzled-LRP5/6 signalosome and the E-cadherin adhesion complex**

A close interaction between the Wnt signalosome and cell-cell junctions may exist both on a physical and functional level. N-cadherin [304] and E-cadherin [305] were shown to be able to directly associate with Lrp5/6. Binding of Wnt morphogens to the Frizzled-LRP5/6 induces a CK1 $\epsilon$ -dependent phosphorylation of both LRP5/6 and E-cadherin [306] inducing a dissociation of LRP5/6 from E-cadherin [230]. Furthermore, Wnt binding to Frizzled-LRP5/6 induces the phosphorylation of CK1 $\epsilon$  dependent phosphorylation of p120 (at Ser268 and Ser269) leading to the dissociation of p120 from the E-cadherin complex [230]. This process was shown to be sensitive to the specific CK1 $\delta/\epsilon$  inhibitor IC261 (Table 1) [307, 308]. IC261 was shown to bind tubulin and act as an inhibitor of microtubules polymerization [308]. Intriguingly, p120 that has a major cytoplasmic function at the cytoplasmic end of E-cadherin, also interacts with the Wnt signalosome making it an important linker protein [305]. A depletion of p120 prevents interactions between CK1 $\epsilon$  and LRP5/6, disrupting LRP5/6 phosphorylation and AXIN recruitment to the LRP5/6 signalosome, leading ultimately to increased  $\beta$ -catenin degradation [305]. An absence of p120 also has been reported to disrupt CK1 $\epsilon$ -mediated phosphorylation of Dvl2 [305]. In turn, signaling through the proteins Dishevelled and Frodo regulates the stability of p120 [309].

Evidence has also been presented that E-cadherin phosphorylation by CK1 $\epsilon$  in response to Wnt binding to Frizzled-LRP5/6 decreases the affinity of  $\beta$ -catenin to E-cadherin, leading to the release of  $\beta$ -catenin from its complex with E-cadherin. This process can provide an additional increase of the cellular threshold of free  $\beta$ -catenin. Hence, Wnt signaling can trigger synergistically a stabilization of  $\beta$ -catenin, a release of  $\beta$ -catenin from its complex with E-cadherin and a dissociation of p120 from E-cadherin.

An inhibitor of CK1 $\delta$  and CK1 $\epsilon$  - PF670462 (Table 1) was reported to be a potent inhibitor of Wnt/ $\beta$ -catenin signaling with an IC<sub>50</sub> of ~17 nM [308].

#### **Adherens complexes in the context of $\beta$ -catenin signaling**

Another major location for  $\beta$ -catenin are adhesion complexes that retain a significant cellular  $\beta$ -catenin pool. Indeed, E-cadherin containing adhesion complexes are supposed to be one of the key regulators of the cytoplasmic  $\beta$ -catenin pool [310]. A simple reduction of cellular E-cadherin was shown to be sufficient to increase

significantly free cellular  $\beta$ -catenin, and an abrogation of E-cadherin-mediated adhesion can correlate with an increase in the transcription of  $\beta$ -catenin target genes, a phenomenon that is often accompanied with cancer progression and poor prognosis [311-315]. Strikingly,  $\beta$ -catenin can be actively mobilized from adhesion complexes by Wnt-independent signaling pathways.

Adhesion complexes have multiple roles in the cell, including structural functions, protective functions and a role in signaling pathways, including prominently Wnt/ $\beta$ -catenin signaling [316-319]. The central component of adherens and tight junctions are cadherins, a family of single-pass transmembrane Ca<sup>2+</sup>-dependent proteins. Changes in mutual cadherin concentrations are referred to as cadherin switches. Hence, the increase of N-cadherins against E-cadherins is a hallmark of an epithelial to mesenchymal transition (EMT) both in normal development and in metastasis [320-323]. Cadherin switches are crucial for motility, invasiveness, migration and metastasis in cancer cells [324, 325]. In several works a reverse correlation between invasiveness of cancer cells and E-cadherin-mediated adhesion was shown [326, 327].

The extracellular N-terminal domain of E-cadherin mediates cell-cell adhesion and consists of five repeats that are stabilized by calcium ions [321, 328]. In the context of  $\beta$ -catenin-mediated signaling, the intracellular domain of E-cadherin is important. It connects, by its juxtamembrane domain (JMD), adhesion complexes to components of the cytoskeleton involving the armadillo proteins  $\beta$ -catenin and plakoglobin, as well as p120 and  $\alpha$ -catenin [329, 330]. The clustering of cadherins is regulated by p120 [331, 332]. Binding of p120 stabilizes cadherins and protects them from internalization and degradation [333, 334] and it has been proposed that the concentration of p120 is a direct limiting factor for cadherins pools [335]. Recruitment of p120 to one type of cadherins sequesters it from binding to another type of cadherins [335]. Hence, a knock-down of p120 reduces cadherin levels by facilitating their degradation, a process that can affect cellular  $\beta$ -catenin levels. p120 is also implied in multiple other ways that attenuate Wnt/ $\beta$ -catenin signaling as described below.

Centrally important for Wnt/ $\beta$ -catenin signaling is the competitive binding between  $\beta$ -catenin and plakoglobin to E-cadherin through the catenin-binding domain (CBD) [336-340]. All 12 armadillo repeats of  $\beta$ -catenin participate in this interaction. Another armadillo protein -  $\alpha$ -catenin - facilitates the interactions between E-cadherin and  $\beta$ -catenin, and anchors actin filaments to  $\beta$ -catenin/E-cadherin [15, 341]. Binding between  $\alpha$ -catenin and  $\beta$ -catenin occurs through the first two armadillo repeats of  $\beta$ -catenin [342]. Interactions between  $\beta$ -catenin and  $\alpha$ -catenin can be inhibited by Tyr142 and Tyr654 phosphorylation of  $\beta$ -catenin through the tyrosine kinases Fer and Fyn [343, 344].

C-terminal phosphorylation of  $\beta$ -catenin attenuates the affinity between  $\beta$ -catenin and E-cadherin and thus can contribute to regulate free cellular  $\beta$ -catenin levels [19, 311]. Hence, phosphorylation of Tyr654 regulates the orientation of the C-terminal tail of  $\beta$ -catenin, changing its position from closed to open. The induced conformational change enables a number of binding proteins to interact with  $\beta$ -catenin [345]. It has been demonstrated that a Tyr654Glu point mutation in  $\beta$ -catenin imitates the negative charge of a phosphorylation and reduces the affinity of  $\beta$ -catenin to cadherins. Tyr654 phosphorylation of  $\beta$ -catenin also enhances Ser675 phosphorylation by protein kinase A (PKA) [346]. Ser675 phosphorylation appears to promote the stability of  $\beta$ -catenin, and assists in its binding to the Creb Binding Protein (CBP) and as a consequence triggers an enhancement of  $\beta$ -catenin-mediated signaling [347], [348]. In addition, protein kinase B (AKT)-mediated Ser552 phosphorylation of  $\beta$ -catenin promotes its induction of transcription through Tcf/Lef [349-351].

The cellular kinase Src (c-Src) phosphorylates amino acids Tyr86 and Tyr654 in the C-terminus and in the last armadillo repeat

of  $\beta$ -catenin respectively [345]. This phosphorylation also structurally impairs  $\beta$ -catenin binding to E-cadherin and can lead to increased cellular  $\beta$ -catenin levels [19, 341, 345, 352]. An increase of Src levels leads to a disruption of intercellular adhesion and E-cadherin dysfunction, while an inhibition of Src by small molecules has the opposite effect [353-355]. Hence, the Src kinase inhibitor bosutinib (SKI-606) (Table 1) was shown to increase the membrane localization of  $\beta$ -catenin and intercellular adhesion [356-357] and bosutinib has shown promising results in Phase I clinical trials in advanced solid tumors [358].

A further mechanism by which  $\beta$ -catenin levels can be regulated at adhesion complexes is the Presenilin 1 (PS1)/ $\gamma$ -secretase system that can cleave the cytoplasmic domain of E-cadherin. The cleavage can be stimulated by calcium influx and has been reported to lead to a disruption of the E-cadherin- $\beta$ -catenin complex followed by an increase of cytoplasmic  $\alpha$ - and  $\beta$ -catenins [359, 360]. Furthermore, the cleaved cytoplasmic terminal fragment (CTF) of E-cadherin has been demonstrated to sequester free  $\beta$ -catenin from the cytoplasm by forming a physical complex with  $\beta$ -catenin. This complex may translocate to the nucleus and interfere directly with Tcf/Lef signaling [314, 361, 362]. Increased  $\beta$ -catenin levels in the cytoplasm and high levels of a sequestered cytoplasmic domain of E-cadherin were found to correlate with malignancy in esophageal squamous cell carcinoma [363]. Furthermore, it has been shown that tumor invasiveness can be correlated to an accumulation of E-cadherin in the nucleus [363, 364]. Accordingly, an overexpression of E-cadherin lacking a transmembrane and/or an extracellular domain was shown to stabilize cytoplasmic  $\beta$ -catenin levels [363]. Also increased levels of metalloproteinases can lead to a cleavage of the cytoplasmic domain of E-cadherin, as has been shown in metastatic prostate cancers [365], [366]. Alterations in E-cadherin, directly affect the anchoring of actin filaments and simultaneously influence signal transduction mediated through  $\beta$ -catenin and p120 [367, 368].

Intriguingly, X-ray structures of  $\beta$ -catenin with its binding partners ([www.rcsb.org](http://www.rcsb.org)) along with biochemical data show that the  $\beta$ -catenin binding proteins Tcf/ICAT/APC and APC/E-cadherin cannot bind  $\beta$ -catenin simultaneously [15, 42, 369]. APC and E-cadherin share the conserved sequence SxxxSLSSL that interacts with the armadillo repeats 3 and 4 of  $\beta$ -catenin, while APC, ICAT, Tcf and E-cadherin have a conserved Dx00x $\Phi$ x<sub>2,7</sub>E motif ( $\Phi$ -hydrophobic,  $\Phi$ -aromatic), which binds to the armadillo repeats 5-9 of  $\beta$ -catenin [15]. This is important since E-cadherin may compete with APC for binding to  $\beta$ -catenin or plakoglobin in a mutually exclusive manner [95, 370].

Interestingly, also EpCAM (Epithelial cell adhesion molecule), one of the first tumor-associated antigens identified, was shown to be a  $\beta$ -catenin dependent signal transducer, and  $\beta$ -catenin is involved in nuclear signaling by EpCAM itself [371]. It has been demonstrated that a proteolytic cleavage of EpCAM by Presenilin 2 releases EpICD, which forms a complex with  $\beta$ -catenin and Tcf/Lef leading to an induction of c-Myc and Cyclin A and E expression [371, 372].

Small molecules targeting cadherins have shown to affect cancer metastasis. The synthetic cyclic pentapeptide, ADH-1 (N-Ac-CHAVC-NH<sub>2</sub>) targets N-cadherins (it imitates the HAVD amino acid sequence of N-cadherin), increases cellular levels of E-cadherin, and has demonstrated efficacy in Phase I clinical trials against melanoma [373, 374]. Small molecules that influence C-terminal phosphorylation and thus mobilization of  $\beta$ -catenin are discussed below.

### Other Transmembrane Receptors Influencing Wnt/ $\beta$ -Catenin Signaling

The hepatocyte growth factor/scatter factor (HGF) is involved in regulating morphogenesis, embryonal development and regenerative processes [375, 376], and an activation of HGF signaling dur-

ing tumorigenesis can promote proliferation, angiogenesis and motility [377, 378]. c-Met, the tyrosine kinase receptor of HGF has been linked to  $\beta$ -catenin signaling [379, 380] and it has been shown that HGF/c-Met can activate  $\beta$ -catenin signaling independent from Wnt signaling [381] at the site of E-cadherin containing junctions. Binding of HGF to c-Met triggers an autophosphorylation at Tyr1234 and Tyr1235, which in turn mediates a tyrosine kinase-mediated Tyr654 and Tyr670 phosphorylation of  $\beta$ -catenin [382] inducing the dissociation of  $\beta$ -catenin from E-cadherin [352, 383]. Similarly, Tyr142 and Tyr654 phosphorylation by the FLT3/ITD kinase (Fms-like Tyrosine Kinase-3) leads to a dissociation of  $\beta$ -catenin from its complex with E-cadherin [344, 384]. Tyr654 phosphorylation is also required for  $\beta$ -catenin binding to Tcf4, adding to the synergistic effect of c-Met-mediated  $\beta$ -catenin phosphorylation [344]. Accordingly, Imatinib (Table 1), a tyrosine kinase inhibitor, was shown to reduce Wnt/ $\beta$ -catenin signaling [385]. The small molecule PHA665752 (Table 1), which inhibits c-Met-mediated phosphorylation, has shown to act inhibitory on HGF induced  $\beta$ -catenin signaling [379, 386].

Also the Endothelin A receptor (ET(A)R), through Src-dependent EGFR (epidermal growth factor receptor) transactivation, causes a Tyr654 phosphorylation of  $\beta$ -catenin leading to its mobilization from E-cadherin [387]. Moreover, the receptor tyrosine kinases FGFR2, FGFR3, EGFR and TRKA have recently been shown to increase cytoplasmic  $\beta$ -catenin concentrations via a Tyr142 phosphorylation that releases  $\beta$ -catenin from cadherin complexes [238].

In addition to mobilizing  $\beta$ -catenin, a C-terminal phosphorylation of  $\beta$ -catenin protects the protein from Ser/Thr phosphorylation in the degradation complex and thus can lead to increased cytoplasmic levels of  $\beta$ -catenin [380], [388]. It has also been shown that HGF could activate  $\beta$ -catenin signaling through inducing a degradation of E-cadherin which again would lead to a mobilization of  $\beta$ -catenin [366]. The matrix metalloproteinase-7 (MMP-7), a downstream target of Wnt/ $\beta$ -catenin signaling, participates in HGF-induced degradation of E-cadherins. [366]. Furthermore, HGF signaling may also alter  $\beta$ -catenin thresholds secondarily through regulating Snail leading to a repression of the transcription of E-cadherin which in turn leads to a reduced  $\beta$ -catenin pool at cellular junctions [389, 390]. HGF/c-Met-mediated stabilization of  $\beta$ -catenin has been associated with several types of tumors [391]. The small molecule PHA665752 (Table 1), which inhibits c-Met-mediated phosphorylation, has shown to act inhibitory on HGF induced  $\beta$ -catenin signaling [379, 386].

Phosphorylation of E-cadherin by CK1 $\delta$  at Ser846 also reduces its binding to  $\beta$ -catenin [306]. Interestingly, a phosphorylation of E-cadherin and  $\beta$ -catenin (Thr112 and Thr120 by PKD1) can also lead to the opposite effect: to stimulate  $\beta$ -catenin/E-cadherin complex formation [341, 392, 393]. Accordingly, it has been shown that downregulation of PKD1 is associated with advanced prostate cancers [393].

### The $\beta$ -Catenin Tcf/Lef Transcription Complex

Besides its implications in junctions, the main effector function of  $\beta$ -catenin is in the nucleus. Here it regulates transcription through interactions with a number of transcription factors, including predominantly Tcf/Lef, Hif-1 and possibly also Oct4.  $\beta$ -catenin may also have a more unspecific role on transcription regulation through interactions with chromatin. The shuttling of cytoplasmic  $\beta$ -catenin to the nucleus and back to the cytoplasm is not entirely understood. A picture emerges, where the nuclear uptake of  $\beta$ -catenin can be enhanced by the context-dependent C-terminal phosphorylation of  $\beta$ -catenin at S675 by PKA. Evidence suggests that the export of  $\beta$ -catenin from the nucleus to the cytoplasm can be GSK3-dependent [394]. Both kinases are well-explored drug targets. A further mode of  $\beta$ -catenin transport to the nucleus was proposed to be a binding between  $\beta$ -catenin and Tcf/Lef in the cy-



toplasm, followed by its transfer to the nucleus [12, 13, 395]. Other mechanisms that influence the shuttling of  $\beta$ -catenin to the nucleus have been discussed earlier.

In the nucleus, the interaction between  $\beta$ -catenin and the zinc finger transcriptional factors of the Tcf/Lef family has been described and is seen as the classical regulatory unit for Wnt/ $\beta$ -catenin target genes (<http://www.stanford.edu/~russel/wntwindow.html>). All members of the Tcf/Lef family (Tcf-1, Tcf-3, Tcf-4 and Lef1) contain an N-terminal binding domain for  $\beta$ -catenin, followed by a context-dependent regulatory domain (CRD) with binding sites for the co-repressor Groucho (Gro), a HMG-box DNA-binding domain, and a C-terminal domain with binding sites for the co-repressor C-terminal binding protein (CtBP) [396, 397]. Lef1 in general acts as a transcriptional activator in complex with  $\beta$ -catenin. Tcf3 is considered to be predominantly a transcriptional repressor. Tcf-1 and Tcf-4 have been claimed to execute context dependent dual activator or repressor roles [396]. In mice, Tcf-3 represses Wnt/ $\beta$ -catenin signaling either through a competitive physical interaction with  $\beta$ -catenin or via competition for Tcf/Lef binding sites on DNA [396, 398]. Further diversity of family members may be created by alternative splicing [399].

In the absence of  $\beta$ -catenin, members of the Tcf/Lef family form a complex with co-repressors such as Groucho, CtBP, and HDAC leading to a repression of the transcription complex [5, 400-402].  $\beta$ -catenin directly displaces Groucho/TLE from Tcf/Lef by binding to a N-terminal low-affinity binding site that overlaps with the Groucho/TLE-binding site rendering the Tcf/Lef into a transcription activator [403]. It has been reported that the interactions between  $\beta$ -catenin and Tcf/Lef are charge-dependent and occur through the formation of salt bridges between Lys amino acids of  $\beta$ -catenin and Glu amino acids of Tcf [404]. The histone acetyltransferase CREB binding protein (CBP) attenuates the complex and acts as a context-dependent transcriptional regulator [394].

Also, the armadillo protein plakoglobin is able to associate with the Tcf/Lef transcriptional complex, although with less affinity than  $\beta$ -catenin, and early reports claim that both proteins are able to activate Tcf/Lef reporters [405-407]. Plakoglobin was also shown to promote transcriptional activity independently from  $\beta$ -catenin [407], and although plakoglobin was shown to be less potent to activate Wnt/ $\beta$ -catenin downstream genes, c-Myc expression is significantly elevated by plakoglobin [408]. Interestingly, similar to  $\beta$ -catenin, ectopic over-expression of plakoglobin was shown to lead to axis duplication in *Xenopus* [409]. Further indications for a functional redundancy between the two structurally related proteins come from mouse studies showing that mice lacking plakoglobin do not show developmental apparent abnormalities. Furthermore, a decrease of plakoglobin in *Xenopus* does not affect embryonic axis formation [405].

p120 also affects Wnt/ $\beta$ -catenin-mediated transcription. In the absence of phosphorylated p120, the zinc finger transcription factor Kaiso binds to the HMG domain of Tcf, forming a co-repressor complex together with histone deacetylase (HDAC). Phosphorylation of p120 causes its dissociation from E-cadherin, its entrance to the nucleus and binding to Kaiso. In consequence, Kaiso loses its role as a co-repressor [230]. Kaiso binding sites are frequently located near Wnt responsive elements of several  $\beta$ -catenin target genes including Siamois, c-Myc and cyclin D1 [410-413]. Notably, this process was shown to be enhanced by Wnt-signaling indicating that several armadillo components that are present in adhesion junctions could be involved in mediating a convergent signaling program.

The Tcf/Lef transcriptional complex has a multitude of further binding partners [402]. Proteins like Pontin52 [414], the TATA-binding protein [415], Bcl-9/Legless, and Pygopus [416, 417] have all shown to promote the formation of a  $\beta$ -catenin/Tcf complex. Chibby (Cby), a small (126 aa) protein antagonizes Wnt/ $\beta$ -catenin

signaling by forming a ternary complex with protein 14-3-3 $\zeta$  and  $\beta$ -catenin [418, 419]. The protein TC-1, associated with thyroid cancer, in turn can bind to Cby and inhibit its interactions with  $\beta$ -catenin, leading to an upregulation of  $\beta$ -catenin target genes [420, 421]. Further tissue-specific proteins like Osterix (osteoblasts-specific transcription factor) are also able to repress the transcriptional complex through a disruption of Tcf binding to DNA [422]. As earlier described, Dishevelled in response to Wnt signaling may also localize to the nucleus [275, 423] where it forms a quaternary functional complex with Tcf/Lef and c-Jun, whereby c-Jun acts as scaffold [277]. Further proteins, associated with the Tcf/Lef complex and regulate its activity are reviewed in [396, 402].

Tcf/Lef-mediated transcription is target of numerous regulative covalent modifications like phosphorylation, SUMOylation, ubiquitination, and acetylation [201, 396]. It has been shown that CK1 $\delta$  and CK2 phosphorylate Lef-1 [424] leading to a disruption of interactions between  $\beta$ -catenin and Lef-1, but not between Lef-1 and the template DNA. Hence, CK1 $\delta$ -mediated phosphorylation results in a transcriptional repression of Lef-1/ $\beta$ -catenin target genes. In contrast, it has been demonstrated that Ser42 and Ser61 phosphorylation of Lef-1 by CK2 enhances Lef-1/ $\beta$ -catenin-mediated transcription [424, 425]. Initially it was supposed that CK2-mediated phosphorylation increases Lef-1 affinity to  $\beta$ -catenin. However, affinity studies have shown that Lef-1 phosphorylation does not affect its binding to  $\beta$ -catenin [426]. Instead, it was found that CK2-mediated phosphorylation leads to a decrease of Lef-1 interactions with the Gro/TLE1 co-repressor [427].

The Nemo-like kinase (NLK) phosphorylates amino acids Thr155 and Ser166 of Lef-1 and amino acids Thr178 and Thr189 of Tcf-4 which have been shown to lead to a reduced DNA-binding [20, 428, 429]. Thus, NLK has been proposed to be a negative regulator of  $\beta$ -catenin/Tcf controlled transcription [430]. Indeed, in human embryonic kidney 293 (HEK293) cells and the cervical epithelioid carcinoma cell line HeLa, NLK inhibits  $\beta$ -catenin-regulated target genes expression [428, 431]. Curiously, in zebrafish midbrain and mammalian neural progenitor cell (NPC)-like cell lines, NLK-mediated phosphorylation of Lef-1 upregulates Wnt signaling [432].

The E3 ligase PIASy SUMOylates Lef-1, which could lead - context dependent - to either an activation or to an inhibition of Lef-1 [72, 433].

Different small molecule inhibitors that act at the level of the Tcf/Lef transcription complex have been reported. ICG-001 selectively binds to CBP, but not to the closely-related protein p300. ICG-011 disrupts the interaction of CBP with  $\beta$ -catenin and down-regulates target genes expression [434]. Recently, ICG-001 has shown to be able to block EMT (epithelial to mesenchymal transition) induced by TGF $\beta$ 1 in a RLE-6TN rat lung epithelial-T-antigen negative cell line. [435]. ICG-001 has reached Phase I clinical trials [436]. During a high-throughput screening the approved FDA diuretic ethacrynic acid (Table 1) was found to down-regulate Wnt/ $\beta$ -catenin signaling by inhibiting the formation of the  $\beta$ -catenin/Lef-1 complex [437, 438] in chronic lymphocytic leukemia (CLL) cells, although at a low IC<sub>50</sub> (Table 1). A number of ethacrynic acid derivatives have since been synthesized leading to a significant potency improvement [439].

Several small molecules were found among natural products that disrupt the  $\beta$ -catenin/Tcf-4complex: CGP049090, PKF118-310, PKF115-584 and ZTM000990, all with an IC<sub>50</sub> slightly below 1  $\mu$ M (Table 1) [440-442]. Docking studies have shown that the assumed binding site for these compounds in  $\beta$ -catenin corresponds to a cavity located between amino acids Arg469, Lys435, Lys508, Glu571 and Arg515 which interacts with Tcf-4 [443]. A further set of compounds, PNU-74654 and BC21 (Table 1), also inhibits the interactions between  $\beta$ -catenin and Tcf. The binding of BC21 to  $\beta$ -

catenin was shown to depend on the polar amino acids Lys435, Arg469, Lys508, Arg515, and Glu571 of  $\beta$ -catenin [443, 444].

In addition to Tcf/Lef, the oxygen sensing zinc finger transcription factor Hif-1 $\alpha$  has been pointed out as a central regulatory element in  $\beta$ -catenin signaling. In an oxygen rich environment, Hif-1 $\alpha$  gets hydroxylated through the HIF prolyl hydroxylase, triggering a subsequent ubiquitination by the von Hippel-Lindau protein (pVHL) that targets Hif-1 $\alpha$  for rapid degradation in the proteasome [445]. Strikingly, the von Hippel Lindau protein has also been implicated in promoting the degradation of cytoplasmic  $\beta$ -catenin, while maintaining the expression of E-cadherin [446]. In contrast, it was shown that PI3K (Phosphoinositide 3-kinase) through MAPK induces Hif-1 $\alpha$  signaling [447]. A second positive regulatory pathway has been described for PI3K/mTOR that is involved in regulating Hif-1 $\alpha$  protein synthesis through AKT/PKB (protein kinase B), mTOR (mammalian target of rapamycin) and S6K (p70 S6 kinase) [448]. It has recently been demonstrated that the PI3K inhibitor GDC-0941 represses Hif1 $\alpha$  and Hif-2 $\alpha$  expression and activity [449].

In a low oxygen environment – as frequently found in stem cell niches – Hif-1 $\alpha$  forms a complex with ARNT (the constitutive active form of Hif-1 $\alpha$ ) and enters the nucleus where it competes with Tcf/Lef proteins for  $\beta$ -catenin binding [450]. Hence, it has been proposed that while under normoxic conditions  $\beta$ -catenin binds predominantly to Tcf/Lef and activates classical Wnt/ $\beta$ -catenin downstream genes, under hypoxic conditions, Hif-1 $\alpha$  may recruit  $\beta$ -catenin to alternative binding sites at promoters e.g. promoters that enhance tumor survival [446]. Strikingly, the promoters of genes of Tcf-1 and Lef-1 contain hypoxia response elements (HREs) [451] and Hif-1 $\alpha$  is directly involved in regulating Tcf/Lef protein abundance [451]. Hif-1 $\alpha$  also is involved in regulating the transcription of proteins implied the destruction complex, and has been shown to negatively regulate the transcription of APC via hypoxia-responsive elements, which could lead to increased cellular  $\beta$ -catenin levels. In a feedback loop APC mediates a repression of Hif-1 $\alpha$  while APC depletions result in increased Hif-1 $\alpha$  levels [452]. Furthermore, GSK3 has been reported to phosphorylate and destabilize Hif-1 $\alpha$  [132].

### Cross-talk with other Pathways: Interconnections Between Inflammation and Wnt/ $\beta$ -Catenin Signaling

Both COX activity and Wnt/ $\beta$ -catenin signaling are interconnected and have been associated with tumorigenesis. An inhibition of the cyclooxygenases COX-1 and COX-2 leads to reduction of prostaglandin synthesis including the pro-inflammatory prostaglandin E2 (PGE2) [453]. PGE2 activates a signaling cascade through the EP2 and EP4 receptors that leads to a PKA dependent Ser552 and Ser675 phosphorylation of  $\beta$ -catenin. The phosphorylation promotes  $\beta$ -catenin stabilization and nuclear uptake [52, 347, 348]. Strikingly, it was shown that Tyr654 phosphorylation of  $\beta$ -catenin, e.g. by receptor tyrosine kinases [311, 454], facilitates a Ser675 phosphorylation by PKA [346]. Hence, a model has been proposed whereby an activation of c-Met induces Tyr654 phosphorylation of  $\beta$ -catenin, which leads to a dissociation of  $\beta$ -catenin from adhesion complexes followed by a possible phosphorylation by PKA on Ser674, which enhances nuclear uptake of  $\beta$ -catenin and a recruitment of transcription factors [346]. In addition, it was shown that PGE2 interaction with EP1-4 receptors leads to activation of G $\alpha$ , which competes with APC for binding to the RGS domain of Axin [455]. Hence, PGE2 may also contribute to a Wnt-independent destabilization of the  $\beta$ -catenin destruction complex [455]. Finally, PKA-mediated phosphorylation of GSK3 may also lead to increased  $\beta$ -catenin levels [456].

Recently a connection between prostaglandin H2 and Wnt/ $\beta$ -catenin signaling was shown on the transcriptional level. Hence, the gene encoding 15-prostaglandin dehydrogenase, which dehydrogenates prostaglandin H2, was shown to be repressed by  $\beta$ -catenin

[457]. Furthermore, mutated APC can lead to an elevated COX2 gene expression [458].

A number of anti-inflammatory COX-2 inhibitors were reported to affect Wnt signaling [459]. In this context, it has been demonstrated that an inhibition of COX enzymes by non-steroid anti-inflammatory drugs or aspirin can reduce the risk of Wnt/ $\beta$ -catenin dependent colorectal cancers significantly [460]. Furthermore, it was shown that Rp-8-Br-cAMP (Table 1), a small molecule inhibitor of PKA, reduces the translocation of  $\beta$ -catenin to the nucleus and reduces the expression of Wnt/ $\beta$ -catenin target genes [461].

### TGF $\beta$ and $\beta$ -Catenin Signaling

The TGF $\beta$  signaling pathway belonging to the same protein superfamily as bone morphogenic proteins (BMP) and Nodal, is involved in multiple biological processes including proliferation, apoptosis and cancerogenesis [462-464]. TGF $\beta$  and Wnt/ $\beta$ -catenin signaling are interconnected at several levels through Smad proteins. In particular the TGF $\beta$  inhibitory protein Smad7 is regulated by components of the Wnt/ $\beta$ -catenin signaling pathway but it also affects Wnt/ $\beta$ -catenin signaling [464, 465]. For instance it has been shown that Axin assists in the degradation of Smad7 through serving as a scaffold for the E3 ligase Arkadia, which ubiquitinates Smad7 and targets it for degradation [464]. Also the ubiquitin ligase Smurf2, which belongs to the HECT class of ubiquitin ligases, binds Smad7 and targets the TGF $\beta$  receptors for degradation [466]. Interestingly, Smurf2 has also been suggested to act as an ubiquitin ligase for Axin, and a knockdown of Smurf2 leads to a reduction of  $\beta$ -catenin/Tcf reporter activity [467]. Furthermore, it was shown in mouse keratinocytes that Smad7 associates with  $\beta$ -catenin and enhances its degradation by recruiting the E3 ubiquitin ligase Smurf2 [468]. Hence, a knockdown of Smad7 leads to an increase of  $\beta$ -catenin-mediated signaling [468]. Smad7 was also shown to interact with the  $\beta$ -catenin-Tcf/Lef transcriptional complex and to regulate apoptosis in a TGF $\beta$  dependent manner [469]. It has been proposed that Smad7 selectively downregulates the mobile pool of  $\beta$ -catenin while it upregulates the pool of  $\beta$ -catenin that interacts with E-cadherin [468, 470, 471].

Smad7 is not the only representative of the Smad family that affects  $\beta$ -catenin. Smad4 in complex with its receptor R-Smad interacts with  $\beta$ -catenin in the nucleus. In chondrocytes it was shown that the C-terminal domain of Smad3 interacts with the N-terminal and central domains of  $\beta$ -catenin in a TGF- $\beta$ -dependent manner [81].

Finally, the TGF $\beta$  pathway was shown to induce phosphorylation of  $\beta$ -catenin at Tyr654 through an activation of Src kinase(s), influencing both the presence in junctional complexes, and the nuclear localization of  $\beta$ -catenin [163].

### Interconnections Between PDGF and $\beta$ -Catenin Signaling

Platelet-derived growth factor (PDGF) regulates cellular division and participates in angiogenesis. PDGF treatment leads to a phosphorylation of the p68 helicase, which facilitates the nuclear translocation of  $\beta$ -catenin and its interaction with the Tcf/Lef complex [472]. Interestingly, in a prostate cancer model, PDGF has shown to promote the formation of a nuclear transcription complex including  $\beta$ -catenin and Hif-1 $\alpha$ , establishing a link between PDGF signaling, hypoxia and  $\beta$ -catenin [473]. In an apparent feedback loop, the extracellular Wnt inhibitor sFRP1 was shown to increase the expression of platelet-derived growth factor-BB (PDGF-BB) in mesenchymal stem cells (MSC) [474].

### Interconnections Between Notch and $\beta$ -Catenin Signaling

Both Notch and Wnt/ $\beta$ -catenin signaling are interconnected. Recently it was shown that membrane-associated uncleaved Notch directly interacts with  $\beta$ -catenin, serving as a protein trap and down-regulating the cellular levels of  $\beta$ -catenin. This process has been demonstrated to require the endocytic adaptor protein Numb and

lysosomal activity [475]. In turn, the Notch ligand Jag1, which is a target of Wnt/ $\beta$ -catenin signaling, functions as a Wnt-dependent Notch activator [476].

### CONCLUDING REMARKS

Although Wnt/ $\beta$ -catenin is a major signaling pathway with very significant implications in a broad range of diseases, addressing the pathway through small drugs or therapeutic antibodies is still at its infancy. Despite the description of a multitude of interesting bio-targets in the pathway, together with the identification of reagents that interfere with these bio-targets, it is by no means clear which bio-targets in the pathway may give a lead in drug discovery.

Creating specific agents to any bio-target is a challenge. In addition, several of the known bio-targets in the Wnt/ $\beta$ -catenin pathway are also implied in a multitude of other pathways raising specificity issues. Since the Wnt/ $\beta$ -catenin signaling pathway is highly complex, a number of back-up mechanisms as well as feedback loops exist. In this context it is also necessary to bear in mind that  $\beta$ -catenin is a member of a larger protein family – the armadillo protein family – and other members of that family, including p120 and plakoglobin, may have functions that are supportive to, or overlapping with  $\beta$ -catenin. Although it is not settled where the best druggable bottlenecks in the pathway may be, or if such bottlenecks exist at all, three main interference points in the pathway are at current predominantly explored: (i) the Wnt signalosome, (ii) the destruction complex and (iii)  $\beta$ -catenin targets and interactions in the nucleus. In a broader sense, other manipulations including altering cellular junctions, influencing prostaglandine-mediated signaling, affecting HGF signaling and mechanisms that influence Hif-1 $\alpha$  levels are important in the context of addressing Wnt/ $\beta$ -catenin signaling.

Hence, it is not clear to what extent the pathway could be silenced by a single therapeutic agent and whether it may ultimately be necessary to regulate the pathway at two or multiple points simultaneously in an approach that may be termed “cloud inhibition”. Indeed, a combination of an EGFR and Tankyrase inhibition recently revealed a close functional correlation of both pathways and confirmed the synergistic effect of a dual antagonistic treatment in lung cancer cells [477]. Zibotentan, a ET(A)R antagonist when combined with the EGFR inhibitor gefitinib, reduces  $\beta$ -catenin activity [387]. Furthermore, it has been shown that  $\beta$ -catenin-mediated resistance to PI3K and AKT inhibitors can be reversed by XAV939 (Table 1), a PARP/Tankyrase blocker [478].

However, even if Wnt/ $\beta$ -catenin signaling can be reduced more stringently with combinations of inhibitors, it is important to note that a complete inhibition of Wnt/ $\beta$ -catenin signaling may not be desirable as it may be necessary to maintain a basic Wnt/ $\beta$ -catenin activity in an organism to ensure the viability of natural Wnt dependent cells.

Despite of all the described issues, significant excitement is sensed in the field that developing reagents altering Wnt/ $\beta$ -catenin signaling can provide valuable new therapeutic tools which will allow to address disease conditions that have hitherto escaped therapeutic success.

### CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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### ABBREVIATIONS

ADP-HPD	=	Adenosine 5'-diphosphate (hydroxymethyl) pyrrolidinediol
ADH-1	=	Peptide N-Ac-CHAVC-NH2
APC	=	Adenomatous polyposis coli
AP-1	=	Activator protein 1 (AP-1), transcription factor
AP-2alpha	=	Transcription factor activator protein
Micro2-adaptin	=	Subunit of clathrin AP2 adaptor protein
ADP	=	Adenosine diphosphate
Axam	=	Axin associating protein
Axin2	=	Axis inhibition protein 2
$\beta$ -TrCP	=	$\beta$ -transducin-repeat-containing protein
Bcl-9	=	B-cell CLL/lymphoma 9 protein
B56 $\alpha$	=	Protein Phosphatase 2A subunit
CBD	=	Catenin-binding domain
CBP	=	Protein cAMP response element-binding (CREB)-binding protein
Ccd1	=	Coiled-coil-DIX1
Cer	=	Cerberus
c-Jun	=	Transcription co-factor
Diversin	=	Planar cell polarity protein, containing ankyrin domain
DIX domain	=	Dishevelled/Axin homologous domain
CK2	=	Casein kinase 2
CDK1	=	Cyclin-dependent kinase 1
CLASP	=	Clathrin-associated sorting protein
CK1 $\alpha$	=	Casein kinase 1 $\alpha$
CK1 $\gamma$	=	Casein kinase 1 $\gamma$
CK1 $\delta$	=	Casein kinase 1 $\delta$
CK1 $\epsilon$	=	Casein kinase 1 $\epsilon$
c-Met	=	Hepatocyte growth factor receptor (HGFR)
c-Myc	=	Myelocytomatosis virus oncogene cellular homolog
CRD	=	Context-dependent regulatory domain in proteins of Tcf/Lef family
CREB	=	Cyclic-AMP response element-binding protein
CtBP	=	C-terminal binding protein
CTF	=	Cytoplasmic terminal fragment
Dab-2	=	Endocytic adaptor disabled-2
Dapper	=	Dishevelled-associated antagonist of $\beta$ -catenin
DEP domain	=	Dvl, Egl-10, Pleckstrin
DIX domain	=	Dishevelled/Axin homologous domain
DKK	=	Dickkopf
Dvl/Dsh	=	Dishevelled
EE	=	Early endosomes
EB1	=	Microtubule-associated protein RP/EB family member 1
EBNA-1	=	Epstein-Barr nuclear antigen 1
EGFR	=	Epidermal growth factor receptor
EMT	=	Epithelial-mesenchymal transition
EpCAM	=	Epithelial cell adhesion molecule

ESCRT	=	Endosomal sorting complex required for transport	JW74	=	Inhibitor of tankyrases, small molecule
ET(A)R	=	Endothelin A receptor	Kaiso	=	Bi-modal DNA-binding protein, transcription regulator
Ex vivo	=	Experiments conducted with tissues outside of the organism, but in conditions close to natural	Kd	=	Dissociation constant
Evi/Wls	=	Evenness/Wntless	Ki	=	Inhibition constant
FDA	=	Food and drug administration	LC3	=	Light chain of the microtubule-associated protein 1
Fer	=	Proto-oncogene protein tyrosine kinase	LEF	=	Lymphoid enhancer factor-1; transcription factor
FGF	=	Fibroblast growth factor	LGR4, 5	=	Leucine-rich repeat-containing G protein-coupled receptor 4, 5
FGFR2	=	Fibroblast growth factor receptor 2	LRP5/6	=	Low-density lipoprotein receptor-related proteins 5/6
FGFR3	=	Fibroblast growth factor receptor 3	MAPK	=	Mitogen-activated protein kinase
FLT3/ITD	=	Cluster of differentiation antigen 135 (CD135), also known as Fms-like tyrosine kinase 3	Mcl-1	=	Myeloid cell leukaemia 1
FRAT1	=	Frequently rearranged in advanced T-cell lymphomas 1 proto-oncogene	MDC	=	Monodansyl-cadaverine
FRAT2	=	Frequently rearranged in advanced T-cell lymphomas 2 proto-oncogene	MDCK cells	=	Madin-Darby canine kidney (MDCK) cells
FZ, FZD	=	Frizzled, receptor of Wnt proteins	MEKK1	=	MAP kinase kinase kinase 1
Frodo	=	Signaling adaptor protein	MEKK4	=	MAP kinase kinase kinase 4
FYN	=	Proto-oncogene protein tyrosine kinase	MM	=	Multiple myeloma
G $\alpha$ , G $\beta$ , G $\gamma$ , Go	=	Guanine nucleotide binding proteins	MMP-7	=	Matrix metalloproteinase-7
GPI	=	Glycophosphatidylinositol	MSC	=	Mesenchymal stem cells
GLUT4	=	Glucose transporter type 4	MVEs	=	Multivesicular endosomes
GPCR	=	G-protein coupled receptor	NLS	=	Nuclear localization signal sequences
G proteins	=	Guanine nucleotide-binding proteins	NAD <sup>+</sup>	=	Nicotinamide adenine dinucleotide
Grk5/6	=	G-protein-coupled receptor kinases	NLK	=	Nemo-like kinase
GRB14	=	Growth factor receptor-bound protein 14	NuMa	=	Nuclear mitotic apparatus protein 1
GPR177	=	G protein-coupled receptor 177 (GPR177)	Oct-4	=	Octamer-binding transcription factor 4
GSK3	=	Glycogen synthase kinase 3	OMP-18R5	=	OncoMed Pharmaceuticals 18R5
GTPase	=	GTPase-activating protein	Osterix	=	Osteoblast specific transcription factor
hDLG1	=	Human disks large homolog 1	PAR1	=	Prader-Willi/Angelman region-1
HECT	=	Domain homologous to the E6-AP carboxyl terminus	PARG	=	Poly (ADP-ribose) glycohydrolase
Hif-1 $\alpha$	=	Hypoxia-inducible factor 1 $\alpha$	PARP	=	Poly (ADP-ribose) polymerase
HGF	=	Hepatocyte growth factor	PCNA	=	Proliferating cell nuclear antigen
HGFR	=	Hepatocyte growth factor receptor	p62	=	Nucleoporin 62, a protein complex associated with the nuclear envelope
HPS domain	=	His/Pro/Ser domain of tankyrase	PDB	=	Protein Data Bank, www.rcsb.org
HRS/VPS27	=	Hepatocyte growth factor receptor tyrosine kinase substrate	PDGF	=	Platelet-derived growth factor
HSPG	=	Heparane sulfate proteoglycans	PDZ domain	=	Postsynaptic density 95, Discs Large, Zonula occludens-1
HUWE1	=	HECT-type ubiquitin E3 ligase 1	PFTK (CDK14)	=	Serine/threonine-protein kinase PFTAIRE-1
IC <sub>50</sub>	=	Half maximal (50%) inhibitory concentration (IC) of a substance	PGAP1	=	Glycosylphosphatidylinositol (GPI)-inositol-deacylase
ICAT	=	Inhibitor of $\beta$ -catenin and Tcf4	PGE2	=	Prostaglandine E2
I-MFA	=	Inhibitor of myogenic basic helix-loop-helix transcription factors	PIAS	=	Protein inhibitor of activated STAT-1, E3 SUMO-protein ligase
IRAP	=	Insulin-responsive amino peptidase	PI3K, PI4KII, PIP5KI	=	Phosphatidylinositol kinases
IWP	=	Inhibitor of Wnt production, small molecule	PKA	=	Protein kinase A
IWR	=	Inhibitor of Wnt response, small molecule	PKB (AKT)	=	Protein kinase B
Jag1	=	Jagged 1 protein	PKC	=	Protein kinase C
JMD	=	Juxtamembrane domain	PKD1	=	Protein kinase D1
JNK	=	c-Jun N-terminal kinase	PLK1	=	Serine/threonine-protein kinase, also known as polo-like kinase 1
			PORC	=	Porcupine

PP1	=	Protein phosphatase 1
PP2A	=	Protein phosphatase 2A
PP2C	=	Protein phosphatase 2C
PRMT1	=	Arginine methyltransferase
PS1	=	Presenelin 1
PTP-BL	=	Protein tyrosine phosphatase
p38 MAPK	=	p38 mitogen-activated protein kinase
p70S6K	=	p70 ribosomal S6 kinase
p90RSK/MAPKAP	=	p90 ribosomal S6 kinase/MAPK-activating protein
p120	=	Armadillo repeats containing protein, referred to also as catenin $\delta$
QSAR	=	Quantitative structure-affinity relationship
Rab5	=	Ras superfamily of monomeric G proteins, localized to clathrin vesicles and early endosomes
RGS domain	=	Regulators of G protein signaling
RNF146	=	RING finger protein 146 ubiquitin ligase
R-Smad	=	Receptor of Smad 4
R-spondin	=	Secreted activator protein
SAM domain	=	Sterile alpha motif
SAR	=	Structure-activity relationship
SFRP	=	Soluble Frizzled-related protein
SIRT1	=	Histone deacetylase Sirtuin 1
SN4741	=	Dopaminergic neurons derived from transgenic mouse embryos
Smad3	=	Mothers against devapentaplegic 3
Smad4	=	Mothers against devapentaplegic 4
Smad5	=	Mothers against devapentaplegic 5
SNAIL	=	Zinc-finger transcription factor
c-SRC	=	Cytoplasmic SRC tyrosine kinase (Src - abbreviation of sarcoma)
STF	=	Super TOPFlash reporter containing Tcf/Lef binding sites
SUMO	=	Small Ubiquitin-like Modifier protein
SUMOylation	=	Post-translational modification of proteins by adding SUMO
TAB182	=	Tankyrase 1 binding protein 182
TATA-binding protein	=	Transcription factor, which binds TATA DNA sequence
Tankyrase	=	TRF-1-interacting ankyrin related ADP-ribose polymerase
TC-1	=	Thyroid cancer protein 1
TCF	=	T- cell factor; transcription factor
TERT	=	Telomerase reverse transcriptase subunit
TGF $\beta$	=	Transforming growth factor $\beta$
TGN	=	Trans-Golgi network
TLE1	=	Transducin-like enhancer protein 1
TMEM198	=	Transmembrane protein 198
TRF1	=	Telomeric repeat binding factor 1
TRKA kinases	=	TRK1-transforming tyrosine kinase protein or Trk-A
RNF146	=	Poly(ADP-ribose)-directed E3 ligase
ROR	=	RAR-related orphan receptor
RYK	=	Related to receptor tyrosine kinase
V-ATPase	=	Vacuolar-type H <sup>+</sup> -ATPase

WIF-1	=	Wnt inhibitory factor-1
WNT	=	Wntless/Int1 signaling protein
WWE domain	=	Common interaction module in protein ubiquitination and ADP ribosylation, has 3 conserved amino acids - two tryptophans and glutamate
Xpo1(Exportin 1)	=	Protein, participating in the nuclear export

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