Ubiquitin & Wnt: The diverse roles of ubiquitin in canonical Wnt signalling

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Contents

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The Wnt signalling pathway plays a major role in both the embryonic development and adult homeostasis of metazoans. Canonical Wnt signalling, which converges on the regulation of β-catenin stability, is involved in processes such as cell proliferation and fate determination. In accordance, imbalances in this pathway have been implicated in a myriad of human diseases. The post-translational modification of proteins with ubiquitin influences both the stability and function of many components of the Wnt signalling pathway. This review will focus on the mechanisms of ubiquitylation, its role in canonical Wnt signalling and finally its involvement in human pathogenesis.

1. Introduction

Since its discovery in 1982, signalling induced by the Wnt family of secreted proteins has become a major subject for scientific research. With its implication in a wide variety of biological processes such as stem cell maintenance, the establishment and upkeep of polarity, both embryonic and adult development, cell death, communication and migration, it comes as no surprise that Wnt signalling is also a key player in human pathogenesis. Most notably, defective Wnt signalling has been associated with the development of many types of cancer, and also with neurodegenerative diseases (MacDonald *et al.*, 2009; Clevers and Nusse, 2012).

The initial discovery of a Wnt gene was accomplished during a search for preferable genomic sites of integration for the Mouse Mammary Tumour Virus (MMTV), which causes mammary carcinogenesis. The *int1* site was found to be strongly favoured for MMTV integration, and the associated proto-oncogene turned out to be the secreted protein *Wnt1* (Nusse and Varmus, 1982). Convergingly, the *Drosophila* gene *wg*, which is involved in embryonic development, was identified as a homolog of this gene (Chopra, 1976; Niisslein-volhard and Wieschaus, 1980; Rijsewijk *et al.*, 1987). Together, *int-1* and *wg* were combined and they are now commonly referred to as Wnt. Many of the genes involved in Wnt signalling were found in forward genetic screens that were set up to identify factors that influenced patterning in the early embryo, and in 1995 a Nobel price was awarded for this work (Nüsslein-volhard and Wieschaus, 1980). With the subsequent identification of genes that led to defects in tissue patterning and axis formation when mutated in *Drosophila* and *Xenopus*, such as *armadillo* (Riggleman *et al.*, 1989), *dishevelled* (Klingensmith *et al.*, 1994), *shaggy/zeste-white3* (Siegfried *et al.*, 1992), *frizzled* (Bhanot *et al.*, 1996) and *arrow* (Wehrli *et al.*, 2000), the mechanisms of Wnt signalling were gradually established.

This initial mode of Wnt signalling, in which transcription is influenced by the activation of nuclear transcription factors, is also referred to as the canonical or Wnt/β-catenin pathway. In more recent years, Wnt signalling has also been associated with the activation of so-called non-canonical pathways, which do not necessarily rely on altering transcript levels. Rather, they regulate the cytoskeleton and intracellular calcium levels in a more direct manner. This review will focus on canonical Wnt signalling, which has been the subject of most Wnt-related studies. For discussion of the non-canonical pathways the reader is referred to the following reviews: Simons and Mlodzik (2008); Lai *et al.* (2009); De (2011) & Clark *et al.* (2012).

The canonical Wnt signalling pathway converges on the regulation of the transcription factor β-catenin. In the absence of Wnt signalling, β-catenin is continuously synthesized and degraded by the cytoplasmic β-catenin destruction complex. This protein complex consists of the adenomatous polyposis coli protein (APC), which was identified in patients with familial adenomatous polyposis (FAP) a hereditary type of cancer (Kinzler *et al.*, 1991; Nishisho *et al.*, 2013), Axin, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). Although the destruction complex has been the subject of many divergent studies, its exact functional dynamics have not been completely elucidated. In the current canonical Wnt signalling model (see **Figure 1**), CK1 and GSK3 phosphorylate β-catenin, which leads to its recognition by the F-box & WD repeat containing protein β-TrCP. This protein is a component of the Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex. $SCF^{\beta\text{-}TrCP}$ subsequently binds to β-catenin and ubiquitylates the protein at multiple residues. These modifications function as a signal that leads to the proteasome-mediated degradation of β-catenin, *inter alia*. With the activation of Wnt signalling the degradation of β-catenin is prohibited by inhibition of the destruction complex, for which multiple models have been proposed (Saito-Diaz *et al.*, 2013). This process is initiated by binding of the Wnt ligand to its membrane-bound receptor Frizzled and its co-receptors LRP-5/6. Activation

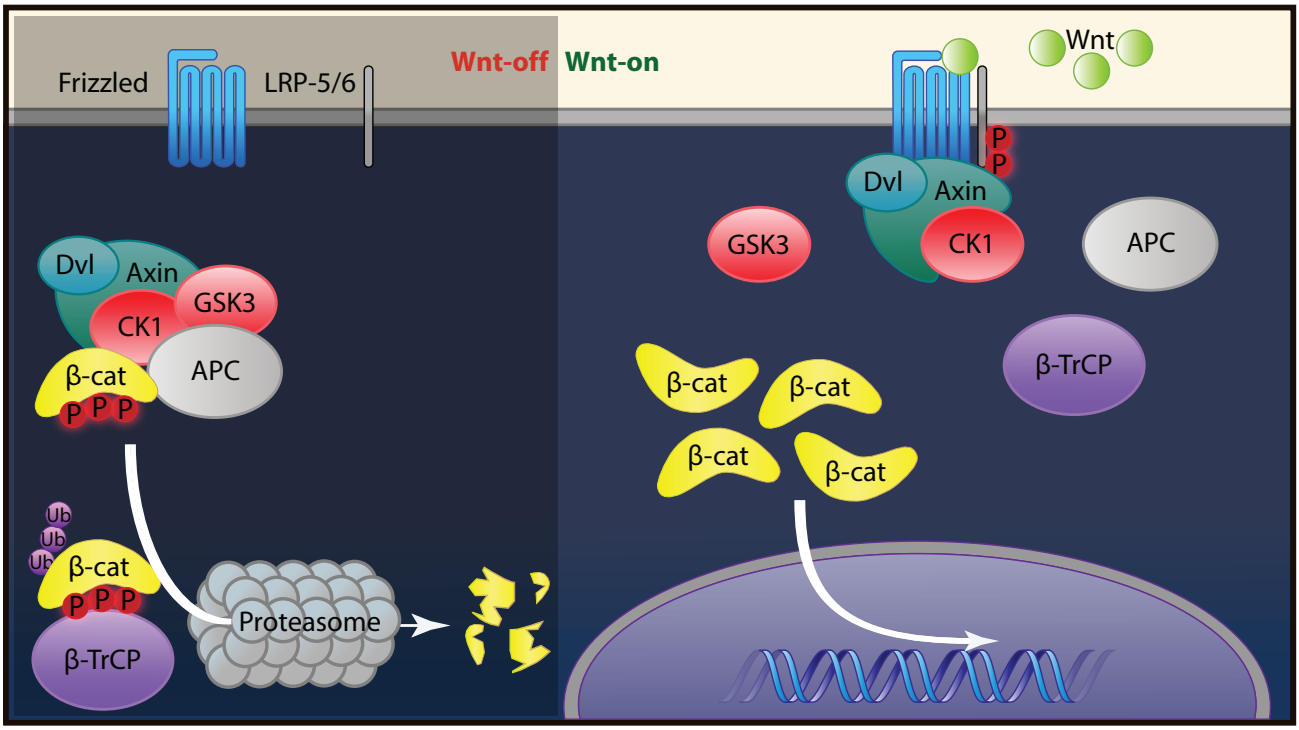

Figure 1. Schematic representation of the current Wnt signalling model.
During the Wnt-off phase, the destruction complex mediates the degradation of β-catenin in cooperation with β-TrCP and the proteasome. In the presence of Wnt ligand, the destruction complex is inhibited via its interaction with the Wnt receptors. This leads to the accumulation of β-catenin, which translocates to the nucleus where it regulates the expression of Wnt target genes. Figure reconstructed and adapted from Clevers and Nusse (2012).

of Frizzled leads to the recruitment of cytoplasmic dishevelled (Dsh/Dvl) and the subsequent inhibition of the destruction complex (Zeng *et al.*, 2008; Li *et al.*, 2012). The inhibition of β-catenin ubiquitylation allows the protein to accumulate and enter the nucleus. In this cell compartment β-catenin binds to DNA-binding transcription factors of T cell factor (TCF) and lymphoid enhancer factor (LEF) families (Beelman *et al.*, 1996; Molenaar *et al.*, 1996), allowing it to regulate the transcription of Wnt target genes. TCF associates with Wnt responsive elements (WREs), which are found in a range of positions throughout the genome and thus allow for the regulation of many genes (Hatzis *et al.*, 2008). A large portion of these genes is involved in cell proliferation and differentiation (Hatzis *et al.*, 2008; Niehrs, 2010; Wray and Hartmann, 2012). In addition, components of the Wnt signalling pathway are regulated by β-catenin-dependent gene transcription, thus resulting in the formation of both positive and negative feedback loops. In a study using colorectal cancer cell lines more than 6000 WREs were identified, which were predicted to regulate the expression of as many as 400 genes (Hatzis *et al.*, 2008). A discussion of the specific genetic targets regulated by Wnt signalling is not in the scope of this review, and for further information on the topic the reader is referred to the following review: Cadigan (2012).

An essential factor in the regulation of Wnt signalling, which is of influence on many components of its canonical signalling pathway, is the post-translational ubiquitylation of proteins. Ubiquitin is an 8.5 kD protein that was identified in 1975 by Goldstein *et al.*, and which is ubiquitously – hence its denomination – expressed in animal tissues. The protein is evolutionarily conserved from yeast to plants and humans (Goldstein *et al.*, 1975), and its covalent attachment to substrates has a diverse range of functions in the regulation of cellular processes. These include its classical role as a flag that labels proteins for degradation by the proteasome (either in the case of excess or misfolding proteins), but also non-proteolytic roles, *e.g.* in cellular signalling, membrane traffic and protein localization, activity and complex formation (Haglund and Dikic, 2005; Varshavsky, 2006; Chen and Sun, 2009; Williamson *et al.*, 2013). The deregulation of ubiquitylation, similar to Wnt signalling, is involved in human pathogenesis, which is illustrated by, for example, its role in the development of breast cancer (Lipkowitz and Weissman, 2011). The indispensable function of ubiquitylation is further illustrated by its central role in the regulation of the cell cycle in eukaryotic cells (Petroski and Deshaies, 2005; Peters, 2006). Wnt signalling is no exception, for the regulation of proteins by ubiquitylation plays a major role in its dynamics, as has been reviewed in Tauriello and Maurice (2010).

Studies focussing on the mechanisms of ubiquitylation and Wnt signalling have experienced an increasing level of attention in recent years, mostly due to their relevance to human disease. In accordance, Wnt signalling has been shown to be hyperactivated in many types of cancer (Polakis, 1999, 2012; Clevers, 2006; Clevers and Nusse, 2012), which is often detected by the assessment of β-catenin levels in cancer cells. For example, over 90% of colorectal cancerous growths assessed

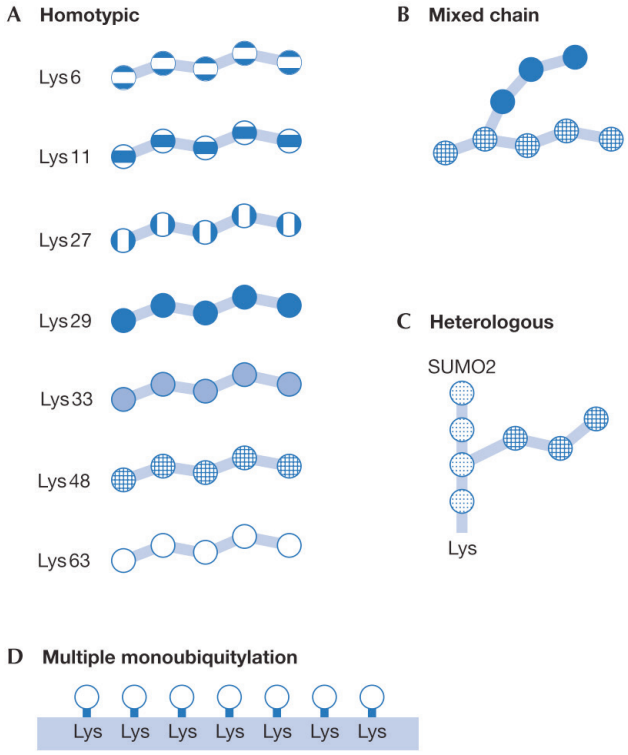

Figure 2. Schematic representation of the different varieties of ubiquitin modifications (Ikeda and Dikic, 2008).

(**A**) The seven types of homotypic chains, in which ubiquitin moieties are linked through the same lysine residue on each molecule.

(**B**) The mixed chain, consistent of multiple types of homotypic chains.

(**C**) A heterologous chain, in which ubiquitin-like proteins like SUMO are incorporated.

(**D**) Multiple monoubiquitylation, in which several substrate lysine residues are conjugated with single ubiquitin proteins.

show hyperactivation of β-catenin signalling (Niehrs, 2012; Polakis, 2012). Taking this information into account, this review will focus on the involvement of ubiquitylation events that directly or indirectly influence the cellular levels of β-catenin. In addition, this review will focus on the roles of these regulatory processes in human pathogenesis.

2. An overview of ubiquitylation

2.1 Ubiquitin: the basics

Four distinct genes in the human genome encode the ubiquitin protein. These are UBB, UBC, UBA52 and RPS27A. Ubiquitin can constitute up to 5% of the total protein level of a eukaryotic cell, which suggests that there is redundancy amongst the genes expressing this protein (Ryu *et al.*, 2007). The ubiquitin protein, which consists of 76 amino acids, is conjugated to lysine residues on its target molecules via its C-terminal glycine (glycine 76) residue, forming an iso-peptide bond (Kimura and Tanaka, 2010). These links are not formed with just non-ubiquitin proteins, as the protein itself also contains seven lysine residues (lysine 6, 11, 27, 29, 33, 48 & 63) that can be used to form different types of ubiquitin chains. Thus, proteins can be conjugated with several types of ubiquitin modifications, which will be discussed below.

2.1.1 Mono- and multiubiquitylation

The most basic type of post-translational protein modification based on ubiquitin moieties is monoubiquitylation, which is the conjugation of a protein with a single ubiquitin protein at one of its lysine residues. This modification is involved in the internalization of membrane-bound proteins such as receptor tyrosine kinases (RTKs), of which especially the epidermal growth factor receptor (EGFR) has been studied extensively due to its hyperactivation leading to cancer development (Madshus and Stang, 2009). Other functions comprise the intracellular sorting of vesicle-bound proteins, such as internalized RTKs that are recycled to the membrane via endosomal multivesicular bodies, or degraded by fusion of these vesicles with lysosomes (Haglund *et al.*, 2003a, 2003b; Hicke and Dunn, 2003). In these cases, ubiquitin modifications function as the sorting signals of the internalized proteins. In addition, monoubiquitylation is involved in the nuclear shuttling of proteins such as the tumour suppressor p53 (Brooks, 2004), inducing protein-protein interactions (Haglund *et al.*, 2003a), regulating protein activity in the case of factors involved in DNA repair mechanisms (Hoege *et al.*, 2002), and they can function in chromatin dynamics by histone modification (Hicke, 2001). The covalent modification of proteins with multiple single ubiquitin proteins on separate lysine residues is referred to as multi-monoubiquitylation, or multiubiquitylation (see **Figure 2D**). This type of modification has, for example, been associated with the regulation of myosin assembly in *C. elegans* (Gönczy, 2004; Hoppe *et al.*, 2004).

2.1.2 Polyubiquitylation

A third type of modification is the attachment of a ubiquitin chain to a single lysine residue on target proteins, which is called polyubiquitylation (Welchman *et al.*, 2005; Ikeda and Dikic, 2008). The seven aforementioned ubiquitin lysine residues are all used to conjugate ubiquitin moieties to one another. The specific combination of lysine residues that is used to create these chains determines the superstructure of the chain (see **Figure 2A**), and this structure in turn influences the function of the modification. Chains linked with solely lysine 11, 29 and 48 residues are generally associated with degradation of the substrate by the proteasome (Kim *et al.*, 2004; Verma *et al.*, 2004; Ikeda and Dikic, 2008). Linear lysine 63-linked chains, on the other hand, regulate the activation of signalling proteins involved in DNA repair and intracellular signalling (Spence *et al.*, 1995; Kerscher *et al.*, 2006), but are also involved in the lysosome-targeting of substrates (Mukhopadhyay and Riezman, 2007). In addition, chains mixed with different lysine residue linkage types have been identified, as well as heterologous chains, which are mixed with non-ubiquitin proteins (see **Figures 2B & C**) (Ikeda and Dikic, 2008). Interestingly, linear 'head-to-tail' ubiquitin chains can be formed as well, which are ubiquitin lysine residue-independent and instead consist of bonds between the C- and N-termini of ubiquitin moieties (Kirisako *et al.*, 2006). This type of modification is involved in, for example, non-proteolytic regulation of the transcription factor NF-κB (Iwai and Tokunaga, 2009).

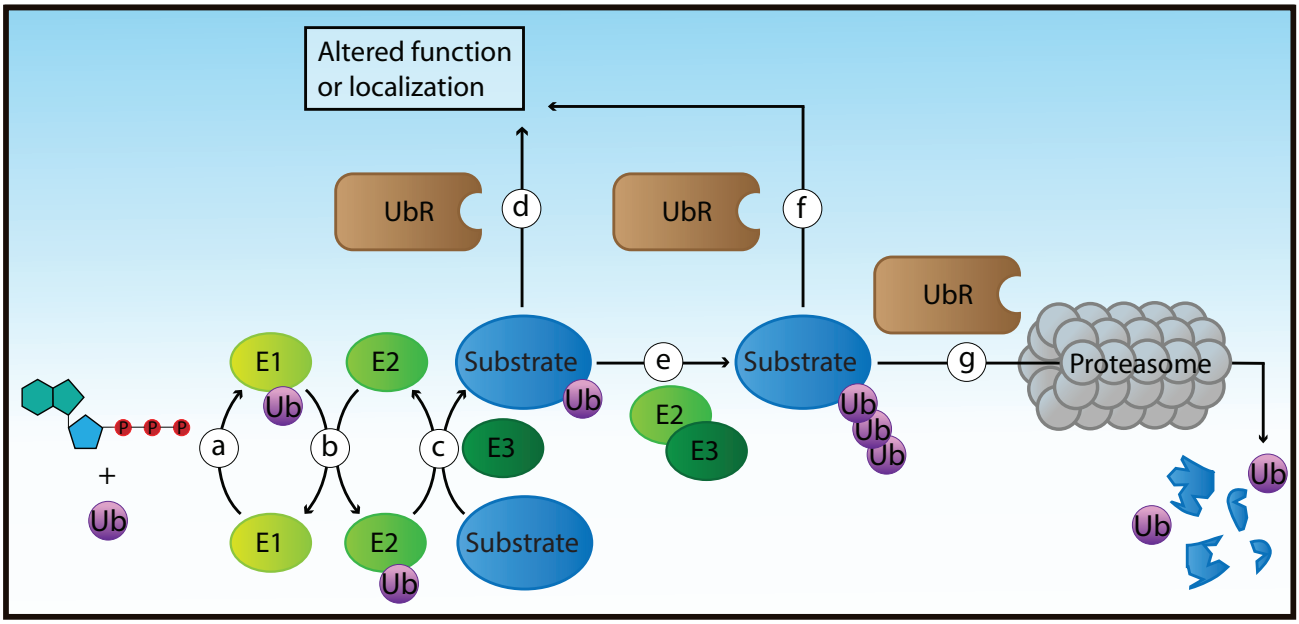

Figure 3. Schematic overview of ubiquitylation.

Initially, ubiquitin is activated with ATP and transferred to the E1 enzyme (**a**). Then, ubiquitin is transferred to the E2 ubiquitin-conjugating enzyme (**b**), and finally the E2 and E3 enzymes cooperate in the conjugation of ubiquitin to a target protein. Subsequently, the target protein either dissociates from the E3 enzyme (**d**), or the E2 and E3 enzymes mediate the further modification of the target protein (**e**), which leads to modifications such as those depicted in **figure 2A-C**. Depending on the type of ubiquitin modification, the substrate will either acquire an alternate function or localization (**f**), or be degraded by the proteasome (**g**). These alternate fates involve proteins that recognize specific ubiquitin modifications with their UBD (in this picture denominated as ubiquitin receptors, or UbRs). Figure reconstructed and adapted from Deshaies and Joazeiro (2009).

2.2 The ubiquitin ligases and deubiquitylating enzymes

The conjugation of ubiquitin groups to its target proteins is achieved with three separate reactions that are mediated by different enzymes (see **Figure 3**). The first step comprises the ATP-dependent activation of ubiquitin by E1 enzymes. The second step involves the conjugation of the ubiquitin group to an E2 ubiquitin-conjugating enzyme, and in the final step ubiquitin is ligated to a lysine residue on its target protein by an E3 enzyme. The E1, E2 and E3 enzymes are encoded by two, less than 40, and more than 600 genes in the human genome, respectively (Pickart and Eddins, 2004; Li *et al.*, 2008). The E3 enzymes are the main factors that determine substrate specificity of the ligation process, which explains their abundance and diversity. These enzymes are subdivided into two groups, namely those containing a HECT (Homologous to the E6-AP Carboxyl Terminus) and those containing a RING (Really Interesting New Gene) domain.

The two types of E3 ubiquitin ligases differ in their method of ubiquitin ligation. The HECT type first transfers the ubiquitin from the E2 enzyme to its acceptor site, after which it is transferred onto the substrate that is also bound to the E3 enzyme. The large group of RING E3s, on the other hand, do not directly bind ubiquitin. They mediate the transfer of ubiquitin directly onto the substrate, and they often consist of large multimeric protein complexes that contain adaptors and additional regulatory and specificity mediating proteins such as an Skp1, RBX1, Cullin and F-box protein in case of the SCF complex (Deshaies and Joazeiro, 2009; Rotin and Kumar, 2009). In addition to the E3 enzymes, a fourth group of ubiquitin ligases has been identified, which were named E4 enzymes (Koegl *et al.*, 1999). These proteins are involved in the elongation of ubiquitin chains on substrate proteins such as p53, in cooperation with E1, E2 and E3 enzymes (Grossman *et al.*, 2003; Hoppe, 2005; Shi *et al.*, 2009).

In addition to the conjugation of ubiquitin groups to substrate proteins, these modifications can also be reversed. A diverse group of ~100 deubiquitylating (DUB) enzyme-encoding genes has been identified in the human genome (Nijman *et al.*, 2005). These enzymes, which are classified as proteases, are involved in the removal of ubiquitin from substrate proteins and the remodelling of polyubiquitin chains. They can be functionally sub-divided into five groups, based on their functional domains: the cysteine proteases ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs) and Josephins, and the family of metallo-proteases that harbour a JAMM domain. In addition to removing ubiquitin groups from substrates, these proteins also process polyubiquitin chains and ubiquitin groups that are conjugated to ribosome subunits, which are the inactive forms of post-translation ubiquitin protein precursors. When processed by DUBs, these proteins yield mature monomeric ubiquitin proteins (Reyes-Turcu *et al.*, 2009).

2.3 The recognition of ubiquitin-based modifications

In order to exert an effect on cellular processes, modifications with ubiquitin proteins need to be recognized by their interacting partners. The proteins that are capable of interacting with ubiquitin are the ones that contain ubiquitin-binding domains (UBDs), of which at least 20 different varieties have

been described in over 150 proteins (Chen and Sun, 2009; Dikic *et al.*, 2009). Ubiquitin-interacting proteins use different structural UBDs to recognize the diverse group of ubiquitin modifications discussed above. In addition, the different UBDs associate with different moieties on the ubiquitin protein, and they form different binding interfaces with these moieties. This complicates the theoretical prediction of UBDs in proteins, and even more so when considering that certain UBDs preferentially interact with polyubiquitin chains that are linked through specific lysine residues. DUBs are an example of proteins that need to be able to discriminate between linkage types in order to fulfil their cellular function. For example, the DUB USP2 associates with and cleaves specifically lysine 48- and 63-linked ubiquitin chains. In addition, the DUB cylindromatosis (CYLD), which will be discussed later in this review, differentiates between linear, lysine 63- and 48-linked chains, cleaving only the first two types of polyubiquitin conformations (Komander *et al.*, 2009). Finally, ubiquitin-interacting proteins can harbour multiple UBDs, and the linker regions between them can determine their spatial confirmation and consequently their specificity for substrate binding (Chai *et al.*, 2004; Sims and Cohen, 2009).

The effects of ubiquitylation on cell signalling have been studied in a variety of signalling pathways. In addition to their role in stimulating proteolysis, their effects on the signalling effects of proteins have been studied in multiple cellular cascades, such as the T cell, TNF, NOD-like and RIG-I-like receptor pathways *inter alia* (Chen and Sun, 2009). The Wnt signalling pathway is no exception, as many of the components in this pathway have been associated with ubiquitin-mediated processes such as proteolysis, protein-protein interaction, cellular localization and endocytosis. The following sections of this review will be dedicated to discussing the roles of ubiquitylation in the direct and indirect regulation of β-catenin stability, Frizzled dynamics and finally its relevance to human pathogenesis.

3. Ubiquitin and the regulation of β-catenin stability

3.1 The kinases: CK1 & GSK3

As discussed above, the canonical Wnt signalling pathway converges on the regulation of cellular β-catenin levels, and aberrant β-catenin levels are associated with human disease. In the non-activated state of Wnt signalling, the cytoplasmic destruction complex binds and ubiquitylates β-catenin, leading to its proteasome-mediated proteolysis. This process requires the two interdependent kinases CK1 and GSK3β, which phosphorylate β-catenin. Phosphorylation is initiated by CK1, which targets β-catenin on its serine 45 residue. This modification acts as a priming step for the GSK3β-mediated phosphorylation of β-catenin on its serine 33, serine 37 and threonine 41 residues (Yost *et al.*, 1996; Amit *et al.*, 2002; Liu *et al.*, 2002; Yanagawa *et al.*, 2002).

The CK1 family presents a diverse group of kinases, which always phosphorylate substrates on serine and threonine residues. Seven members of this family have been identified in mammals, which include CK1α, β, γ1, γ2, γ3, δ & ε (Knippschild *et al.*, 2005; Price, 2006). Interestingly, the CK1 kinases have been implicated in both the positive and negative regulation of the Wnt signalling pathway (Saito-Diaz *et al.*, 2013). Positive regulation includes the phosphorylation of Dsh/Dvl (Yanagawa *et al.*, 1995; Peters *et al.*, 1999; Lee *et al.*, 2001; Gao *et al.*, 2002; Cong *et al.*, 2011), LRP6 (Zhang *et al.*, 2006; Zeng *et al.*, 2008), TCF/LEF (Kishida *et al.*, 2001; Lee *et al.*, 2001) and Axin (Kishida *et al.*, 2001; Gao *et al.*, 2002), while negative regulation includes the phosphorylation of β-catenin (Liu *et al.*, 2002; Hämmerlein *et al.*, 2005), APC (Gao *et al.*, 2002), Axin, and TCF/LEF. In addition, CK1 activity as well as its phosphorylation state are also regulated by Wnt signalling (Swiatek *et al.*, 2004).

The phosphorylation of β-catenin by CK1 and GSK3β leads to its ubiquitylation by the E3 ubiquitin ligase β-TrCP, which is a component of the SCF RING E3 ligase complex (Fuchs *et al.*, 2004). β-TrCP was found to recognize a specific dual phosphorylation pattern on β-catenin, also known as the destruction motif, which has the structure DpSG ϕ XpS that is also found on other ubiquitylated proteins (Winston *et al.*, 1999). After binding to β-catenin, β-TrCP induces its ubiquitylation at the positions lysine 19 and lysine 49 (Wu *et al.*, 2003). These modifications subsequently lead to the recognition of β-catenin by the proteasome, thus destroying the protein and keeping its cellular levels at a low steady state. Mutations in the destruction motif of β-catenin cause the protein to accumulate in the cell (Munemitsu *et al.*, 1996; Barth *et al.*, 1997; Pai *et al.*, 1997; Rubinfeld, 1997; Sparks *et al.*, 1998). In addition, such mutations have been identified in a multitude of cancer cell types, illustrating the importance of these residues in the regulation of β-catenin levels (Takahashi *et al.*; Dashwood *et al.*, 1998; de La Coste *et al.*, 1998; Iwao *et al.*, 1998; Sparks *et al.*, 1998).

3.2 The scaffolds: APC & Axin

In addition to CK1 and GSK3β, Axin and APC are also components of the β-catenin destruction complex. These proteins act as scaffolds to the complex as shown by their crystal structures when bound to β-catenin and GSK3β (Spink *et al.*, 2000; Dajani *et al.*, 2003; Xing *et al.*, 2003), thus identifying them as negative regulators of Wnt signalling. Their cellular function and the regulation of both proteins will be the next topic of discussion.

3.2.1: Axin

Axin is the least abundant protein and a rate-limiting factor of the destruction complex, as has been described in *Xenopus* (Lee *et al.*, 2003) and mammals (Li *et al.*, 2012). This scaffold interacts with multiple units of the complex, including β-catenin, APC, CK1 and GSK3β (Ikeda *et al.*, 1998; Sakanaka *et al.*, 1999; Liu *et al.*, 2002). The low abundance of Axin is probably related to its ubiquitin-mediated degradation (Yamamoto, 1999; Mao *et al.*, 2001; Tolwinski *et al.*, 2003), although its related E3 ligase is yet to be identified. The Frizzled co-receptors LRP5/6 are thought to be responsible for the downregulation of Axin, although it is unknown whether or not the degradation of Axin is needed for the initiation of Wnt signalling, and the exact mechanism by which this degradation occurs remains elusive. Conversely, in case of LRP6-mediated stabilization of β-catenin, GSK3 was shown to be the target rather than Axin (Cselenyi *et al.*, 2008). In this study, LRP6 is proposed to inhibit the phosphorylation

of β-catenin by GSK3β, which prohibits its degradation by the proteasome, independent of Axin degradation. This contradicts the findings of another study, in which LRP6 was presented as the negative regulator of the Axin protein (Kofron *et al.*, 2007). Interestingly, an earlier study showed that the degradation of Axin is also regulated by its GSK3β-induced phosphorylation, which increases the stability of Axin (Yamamoto, 1999). Possibly, both mechanisms apply and the regulation of Axin by LRP6 and GSK3β occurs through different mechanisms (Cselenyi *et al.*, 2008). In addition, APC has also been shown to be required for the turnover of Axin. This interaction was proposed to increase Wnt pathway robustness, or its ability to withstand fluctuations in the environment of the cell and its susceptibility to mutations. Following a theoretical analysis of experiments with *Xenopus* egg extracts by Lee *et al*., the low levels of Axin were proposed to stabilize the Wnt pathway when fluctuations in the levels of APC occur (Lee *et al.*, 2003). In addition to this, the low levels of Axin could isolate the Wnt signalling pathway from other pathways. Namely, components of the Wnt signalling pathway, such as GSK3β, Dvl and APC are involved in other cellular signalling pathways as well. The kinase GSK3β, for example, functions in glycogen synthesis, Hedgehog signalling, microtubule dynamics and apoptosis in addition to Wnt signalling (Forde and Dale, 2007). The low levels of Axin are thought to sequester the Wnt pathway from these other cellular functions, by not affecting the protein levels of its interactors to a degree that would influence their non-Wnt-related functions.

Even though the direct mechanism of Axin downregulation by APC and LRP5/6 remains elusive, multiple factors that influence the ubiquitylation status of Axin have been identified in recent years. One mechanism by which Axin ubiquitylation is regulated is SUMOylation. SUMO stands for Small Ubiquitin-Like Modifier, a ubiquitin-related post-translational polypeptide modification that has been implicated in protein subcellular localization, turnover and activity (reviewed in (Hay, 2005)). The six C-terminal residues (KVEKVD) of the Axin protein have been related to this modification and its stability. The two lysine residues in this motif are targets of modification by three SUMO-1 conjugating E3 enzymes, PIAS1, PIASxβ and PIASy. The absence of SUMOylation on these residues leads to the increased susceptibility of Axin to be ubiquitylated on other regions of the protein in HEK293T cells, as deleting these residues increased the total amount of Axin ubiquitylation. In addition, absence of SUMOylation on these residues coincided with a several fold decrease of Axin half-life compared to the wild type protein (Kim *et al.*, 2008).

In search for the E3 ubiquitin ligases responsible for Axin ubiquitylation, authors Kim & Jho identified the smad ubiquitin regulatory factor 2 (Smurf2). Knockdown of Smurf2 increases cellular Axin levels and decreases its ubiquitylation, while transient expression of Smurf2 decreased Axin levels and increased its ubiquitylation. Axin was found to be ubiquitylated by Smurf2 on its lysine 505 residue, and recombinant Axin proteins which are mutated at this residue circumvent proteasomal degradation (Kim and Jho, 2010). In addition, Smurf2 was found to be responsible for the ubiquitylation of GSK3β, which leads to its proteasomal degradation and thus to the stabilization of β-catenin (Wu *et al.*, 2009). Thus, Smurf2 stimulates canonical Wnt signalling by targeting two components of the β-catenin destruction complex for degradation.

In another study focusing on Axin stability, the small molecule XAV939 was identified as an inhibitor of β-catenin-induced gene transcription. This inhibition occurs via the increased degradation of β-catenin by the stabilization of Axin. XAV939 was found to mediate this stabilization by inhibiting the proteins tankyrase 1 and tankyrase 2. These proteins are enzymes that mediate the poly-ADP-ribosylation (or PARsylation), and possibly the ubiquitylation of Axin, which leads to its proteasome-mediated destruction (Huang *et al.*, 2009a). In addition to this, a search for small-molecule inhibitors of Wnt signalling resulted in the identification of multiple inhibitors of Wnt response (IWR) compounds, of which one, IWR-1, is able to upregulate steady-state Axin levels through the inhibition of tankyrase proteins (Chen *et al.*, 2009; Saito-Diaz *et al.*, 2013). The E3 ubiquitin ligase that is responsible for the ubiquitylation of Axin following its PARsylation is the ring finger 146 (RNF146) protein, as identified by RNAi screening (Callow *et al.*, 2011). RNF146 forms a complex with Axin and tankyrase, recognizing PARsylation through its WWE domain (associated with ubiquitylation and PARsylation) (Zhang *et al.*, 2011c). Interestingly, the activity of tankyrase leads to the degradation of RNF146, and *vice versa*. RNF146 is thought to cause the (auto-)ubiquitylation of Axin, tankyrase and itself via tankyrase-mediated PARsylation (Callow *et al.*, 2011). Thus, tankyrase and RNF146 cooperate in the downregulation of Axin.

In addition to these negative regulators, a DUB for Axin has also been identified. Ubiquitin-specific protease 34 (USP34) was shown to be a positive regulator of Axin stability which functions downstream of the β-catenin destruction complex (Lui *et al.*, 2011). USP34 opposes the ubiquitylation of Axin via tankyrase, and thus this regulator could very well be the factor that opposes the RNF146-mediated ubiquitylation and degradation of Axin.

3.2.2 APC

The APC protein is not only involved in Wnt signalling, but also in other cellular functions such as cell migration, division and apoptosis. However, these different functions most likely depend on different subcellular populations of APC (Faux *et al.*, 2008). This idea is supported by studies showing that there is a notable difference between the behaviour of tumours with APC mutations affecting its role in β-catenin degradation, and those with wild type APC and hyperactivated β-catenin (Samowitz *et al.*, 1999; Harada *et al.*, 2002; Haigis *et al.*, 2004; Gounari *et al.*, 2005). Interestingly though, the role of APC in Wnt signalling remains elusive. Although many studies have linked APC to the degradation of β-catenin, the exact mechanism by which APC functions in the destruction complex is currently unknown. At least six non-mutually exclusive mechanisms have been proposed, which generally include the regulation of the subcellular localization of β-catenin and the destruction complex, as well as indirectly regulating the phosphorylation status of β-catenin. It is likely that, in a fashion similar to the function of the kinases CK1 and GSK3, APC will be involved in multiple steps of the Wnt signalling pathway (Saito-Diaz *et al.*,

2013). Notably, the oligomerization of Axin has been linked to the regulation of cellular APC levels (Choi *et al.*, 2004). These results, combined with the earlier discussed regulation of Axin by APC, lead to the conclusion that APC and Axin cross-regulate each other's turnover. Interestingly, the inactivation of APC and the subsequent activation of Wnt signalling can be compensated by the overexpression of Axin (Lee *et al.*, 2003). In accordance, the overexpression of APC in which the Axin-binding domain was deleted, a mutation that often occurs during carcinogenesis, was found to be more effective in promoting β-catenin degradation than wild type Axin (Hart *et al.*, 1998). An explanation for these results could be the model in which APC and Axin balance the steady state levels of β-catenin, even when either of these proteins is abnormally expressed (Lee *et al.*, 2003).

Contrary to the role of APC in β-catenin degradation, some mechanistic information is available on the factors that regulate the post-translational modification of APC. Notably, APC is ubiquitylated and subsequently degraded by the proteasome, and this process is inhibited by Wnt signalling. Axin overexpression was shown to further increase the degradation rate of APC (Choi *et al.*, 2004). Recently, HectD1 was identified as an E3 ubiquitin ligase that is responsible for the ubiquitylation of APC (Tran and Polakis, 2012; Tran *et al.*, 2013). HectD1 couples lysine 63–linked polyubiquitin chains to APC, which negatively regulate Wnt signalling by promoting complex formation by APC and Axin. Currently, the manner in which this interaction is promoted by lysine 63-linked polyubiquitylation, as well the factors regulating HectD1 within the Wnt pathway, are unknown. However, an APC-targeting DUB that counteracts the HectD1-mediated ubiquitylation of APC has been identified. TRAF-binding domain-containing protein, or Trabid, is a factor that was identified as a positive regulator of Wnt signalling in *Drosophila* and mammals. Trabid was initially shown to preferably bind and cleave lysine 63–linked polyubiquitin chains, and its activity is required for proper Wnt-induced, TCF-mediated gene transcription. In addition, Trabid binds to and deubiquitylates APC, but it acts downstream of the destruction complex (Tran *et al.*, 2008). While Trabid specificity for lysine 29 –and lysine 33–linked polyubiquitin chains was shown *in vitro* (Licchesi *et al.*, 2012), *in vivo* experiments indicate that Trabid cleaves lysine 63–linked chains as well. It is likely that the specificity of Trabid polyubiquitin chain–binding as well as its DUB activity is regulated by other, currently unknown factors (Tran *et al.*, 2013).

In addition to regulating complex formation by APC and Axin, ubiquitylation also regulates the turnover of APC. The E3 ligase that is responsible for this ubiquitylation has, however, not yet been identified. In contrast, a DUB that counteracts the degradation of APC has been found. The COP9 signalosome (CSN) regulates Cullin-RING E3 ubiquitin ligases (CRLs), and cooperates with the destruction complex in the degradation of β-catenin. Knockdown of the CSN was found to lead to the degradation of APC in colorectal cancer cell lines (Huang *et al.*, 2009b). In a wild type situation, APC is stabilised by the DUB ubiquitin-specific protease 15 (USP15), which is associated with the CSN. In addition, the CSN mediates the deneddylation of cullin proteins, which are scaffolding components of the CRLs. The modification of cullins with Nedd8, one of the ubiquitin-like proteins, inhibits the activity of CRL complexes. Thus, the CSN regulates both APC stability via the DUB USP15 and CRL complex activity via cullin deneddylation (Huang *et al.*, 2009b).

In addition, the HECT E3 ubiquitin ligase and tumour suppressor E3 isolated by differential display, or EDD, interacts with APC and stabilizes its protein levels, leading to the breakdown of β-catenin. Interestingly, overexpression of EDD not only increased APC protein levels, but also those of Axin (Ohshima *et al.*, 2007). In contrast, EDD also modifies β-catenin with lysine 11 and lysine 29–linked polyubiquitin chains, which increases its stability (Hay-Koren *et al.*, 2011). Lysine 11–linked polyubiquitin chains are abundant in *S. cerevisiae* (Peng *et al.*, 2003), and this modification occurs as often as lysine 48–linked chains, making up as much as 30% of the total cellular ubiquitin content (Xu *et al.*, 2009). Also, lysine 11–linked polyubiquitin chains are involved in cell signalling as well as promoting the proteasomal breakdown of cell cycle regulators, *inter alia* (Bremm and Komander, 2011). However, whether EDD promotes or inhibits Wnt signalling through the lysine 11 and lysine 29–linked polyubiquitylation of β-catenin and APC, or possibly both by different protein interactions and modifications, remains unclear.

3.3 Additional factors involved in the regulation of β-catenin turnover

In addition to the previously discussed factors that regulate β-catenin turnover, recent studies have implicated novel interactors in this process. For example, the protein casitas B-lineage lymphoma (c-Cbl) was identified as a novel RING-type E3 ubiquitin ligase that targets β-catenin. Contrary to β-TrCP, c-Cbl targets β-catenin during both the Wnt-on and Wnt-off phases. The dimerization of c-Cbl is required for its interaction with β-catenin, and Wnt signalling was found to promote c-Cbl dimerization and translocation to the nucleus in endothelial cells. Both in the cytoplasm and in the nucleus, dimerized c-Cbl binds the armadillo repeat motif of active β-catenin and mediates its ubiquitylation, which subsequently leads to its proteasome-mediated degradation. Interestingly, the ubiquitylation of β-catenin by c-Cbl is GSK3β-independent, in contrast to β-TrCP-mediated β-catenin ubiquitylation. The activity of c-Cbl attenuates Wnt signalling in endothelial cells, and it was shown to inhibit the expression of Wnt-target genes that are involved in angiogenesis (Chitalia *et al.*, 2013). Thus, c-Cbl was proposed to fulfil a dual role in Wnt signalling (see **Figure 4**): during the Wnt-off phase, this E3 ubiquitin ligase cooperates with the destruction complex to effectively mediate the degradation of β-catenin. During the Wnt-on phase, c-Cbl might function as an attenuator of β-catenin-induced gene expression, and it could also be involved in the controlled inhibition of β-catenin activity to prevent its hyperactivation. Further research should elucidate whether these are indeed the roles of c-Cbl in Wnt signalling, and whether the same functions are applicable in cell types other than those of the endothelium.

In addition to c-Cbl, focal adhesion kinase-family-interacting protein of 200 kDa (FIP200) was recently shown to interact with

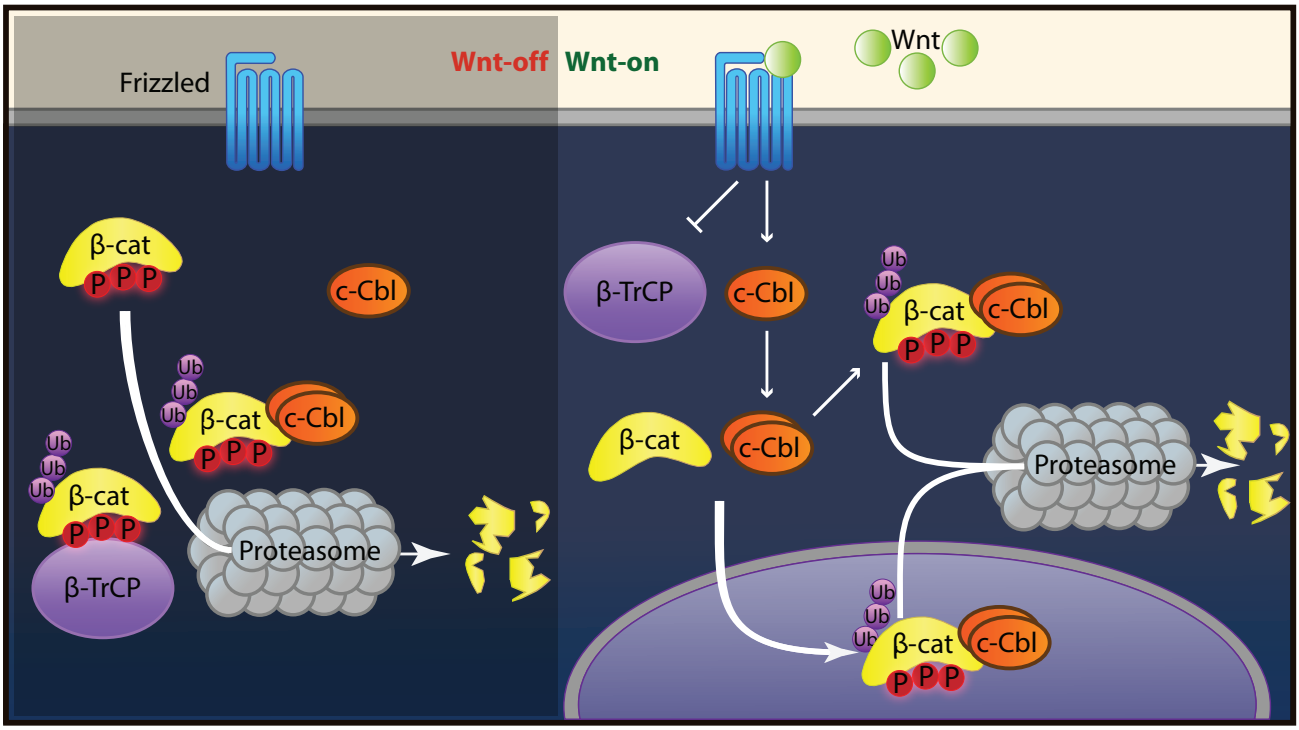

Figure 4. Schematic representation of c-Cbl function in Wnt signalling.

During the Wnt-off phase, dimerized c-Cbl and β-TrCP both regulate the ubiquitylation and subsequent proteasome-mediated degradation of cytoplasmic β-catenin. Upon the activation of Wnt signalling the degradation of β-catenin via β-TrCP is inhibited. In addition, c-Cbl dimerization is stimulated and the protein is translocated to the nucleus. In this compartment, c-Cbl also mediates the ubiquitylation and subsequent degradation of active β-catenin. Figure based on the model described in Chitalia *et al.* (2013).

β-catenin (Choi *et al.*, 2013). Interestingly, FIP200 also induces the ubiquitylation-dependent degradation of β-catenin in an APC-independent manner. Surprisingly, though, a mutant isoform of β-catenin that cannot be phosphorylated on its serine 33 residue is only scarcely targeted by FIP200. Thus, FIP200-mediated degradation of β-catenin is most likely GSK3β-dependent. In addition, the activity of FIP200 depends on its interaction with β-TrCP, which comes as a surprise given the independence of its function on APC. Two ubiquitin-binding motifs (UBMs) in the C- and N-termini of FIP200 could suggest that FIP200 acts as an adaptor protein in the ubiquitylation and degradation of β-catenin. This idea is further supported by the increase in ubiquitylation observed to coincide with FIP200-dependent degradation of β-catenin, and the fact that FIP200 has been shown to function as an E3 ubiquitin ligase scaffold in the TGF-β signalling pathway (Koinuma *et al.*, 2011). Interestingly, FIP200 has also been implicated in the stabilization of p53, the degradation of cyclin D1 and the induction of autophagy. It will be interesting to assess how these multiple roles of FIP200 regulate cellular behaviour in conjunction with Wnt signalling, especially when considering that FIP200 has been implicated in tumour suppression (Chano *et al.*, 2002; Melkoumian *et al.*, 2005; Choi *et al.*, 2013).

Recently, the ubiquitin-modifying enzyme A20 has also been implicated in β-catenin degradation. A20 has previously been shown to contain both an OTU domain for DUB, and a zinc finger (ZF) domain for E3 ubiquitin ligase activity. This DUB activity includes the removal of lysine 63-linked polyubiquitin chains, and the ZF domain mediates conjugation of lysine 48-linked polyubiquitin chains to substrates. In addition, both of these activities were previously shown to regulate NFκB signalling (Wertz *et al.*, 2004). Interestingly, A20 is often expressed at reduced levels in adenomas of the colon, and the frequency of mutations in the gene encoding this protein occurring in B cells has suggested that it functions as a tumour suppressor (Malynn and Ma, 2009; Shao *et al.*, 2013). In cells of the intestinal epithelium, A20 interacts with the β-catenin destruction complex, which leads to the promotion of β-catenin ubiquitylation and degradation. In agreement with these findings, the inactivation of A20 leads to increased expression of the Wnt-target genes and cell cycle regulators cyclin D1 and c-Myc (Shao *et al.*, 2013), the latter of which will be discussed in more detail further on in this review. Thus, although the exact mechanism by which A20 influences destruction complex-mediated ubiquitylation and degradation of β-catenin, this protein could be an interesting subject for further research due to its dual ubiquitin-editing activities.

While not directly associated with the stability of β-catenin, the Fanconi anemia, complementation group L (FANCL) protein has been implicated in β-catenin activity (Dao *et al.*, 2012). The 15 different FANC proteins are primarily associated with the DNA damage response, and their aberrant expression and/or activity leads to Fanconi anemia, a genetic disease that leads to bone marrow failure and different types of cancer *inter alia*. The 15 FANC proteins function in a large complex, the function of which converges on the E3 ubiquitin ligase activity of FANCL

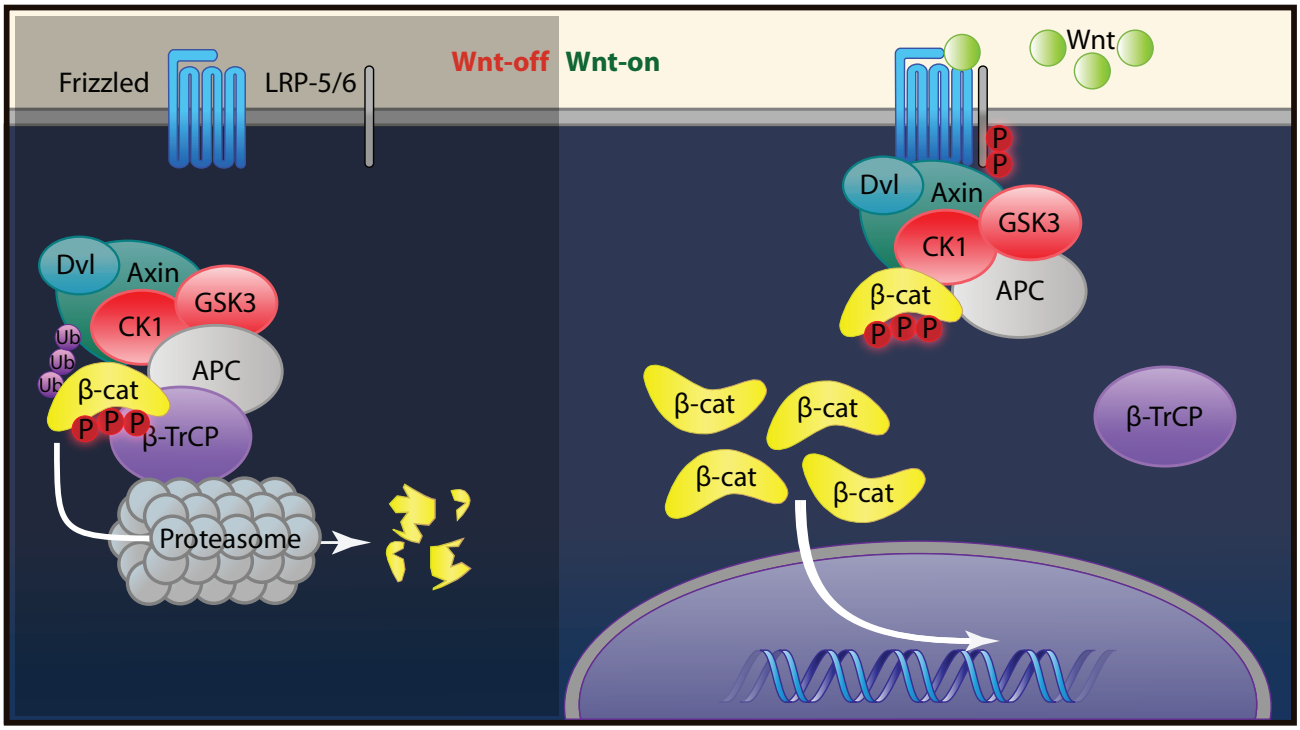

Figure 5. Schematic representation of the new Wnt signalling model.
In a new model proposed by Li *et al.*, the β-catenin destruction complex remains intact during the activation of Wnt signalling. Instead of dissociating, the complex associates with LRP and is saturated with phosphorylated β-catenin due to the inhibition of its ubiquitylation. Figure reconstructed and adapted from Clevers and Nusse (2012).

(Moldovan and D'Andrea, 2009). While the inactivation of this complex has been linked to complications in the cellular DNA damage response mechanism, the direct link of FANCL inactivation to the failure of hematopoietic stem cell regulation had not previously been elucidated. Dao *et al*. recently demonstrated that FANCL is responsible for the modification of β-catenin with lysine 11-linked polyubiquitin chains. This modification was found to increase the ability of β-catenin to promote transcription via its nuclear interactor LEF. In addition, the inactivation of FANCL results in the diminished ability of β-catenin to stimulate the expression of the Wnt targets cyclin D1 and c-Myc (Dao *et al.*, 2012). These are interesting findings, especially when taking into account that the effects of such atypical ubiquitin modifications have often not, or only marginally, been characterized in cellular processes, as will be address further on in this review as well.

A mechanism similar to that of the previously discussed protein c-Cbl is found in the activity of E3 ubiquitin ligase gene for apoptosis and differentiation in epithelia (jade-1). This protein is predominantly expressed in the kidneys and has been implicated in the development of renal cancer as a tumour suppressor. Like c-Cbl, jade-1 ubiquitylates β-catenin during both the on –and off-phase of canonical Wnt signalling. The jade-1-mediated ubiquitylation of β-catenin is dependent on phosphorylation of β-catenin by GSK3β (Chitalia *et al.*, 2008). Jade-1 is translocated to the nucleus by the nephrocystin-4 protein (NPHP4), which also promotes its stabilization (Borgal *et al.*, 2012). In addition, the Von Hippel-Lindau protein (pVHL), also an E3 ubiquitin ligase, is also required for the stabilization of jade-1, as well as its ubiquitylation of β-catenin. Mutations in pVHL are often identified as a major cause of renal cancer. Thus, the reported hyperactivation of canonical Wnt signalling in renal carcinogenesis might be due to the destabilization of jade-1 upon the mutational inactivation of pVHL (Zhou *et al.*, 2002; Chitalia *et al.*, 2008).

3.4 The inhibition of the β-catenin destruction complex

Following the initiation of Wnt signalling by the binding of Wnt to Frizzled, the cytoplasmic destruction complex is inactivated, allowing β-catenin to escape β-TrCP-mediated ubiquitylation and destruction by the proteasome. The manner in which the destruction complex, and especially the phosphorylation by GSK3β, is inhibited by activation of the Frizzled receptor is still under debate. Different models have been put forward to explain how β-catenin is protected from degradation by Wnt signalling, among which the most prominent are (Li *et al.*, 2012; Saito-Diaz *et al.*, 2013):

1) Disassembly of the destruction complex by recruitment of Axin and GSK3β by Dvl;
2) Phosphorylation of GSK3β at its serine 9 residue, leading to its inhibition;
3) Migration of the destruction complex to the cell cortex via LRP6 phosphorylation by CK1 and GSK3β and the Dvl-Frizzled interaction, creating a docking site for Axin and leading to the inhibition of GSK3β;
4) The degradation of Axin;
5) Inhibition of GSK3β by its transport to endosomes, specifically

multivesicular bodies (MVBs), sequestering it from proteins such as β-catenin;
6) The dephosphorylation of β-catenin by PP2A (Su *et al.*, 2008).

In contrast to these theories, which are based on the disassembly of the destruction complex or the interference with β-catenin phosphorylation, a recent article proposes a different mechanism of destruction complex inhibition (see **Figure 5**). Li *et al*. postulated that β-catenin is not only phosphorylated, but also ubiquitylated and degraded by the proteasome within an intact Axin complex (Li *et al.*, 2012). In this study, there was no observation of inhibited phosphorylation of β-catenin by CKI and GSK3β, or disassembly of the destruction complex upon the initiation of Wnt signalling. In the model that was put forward by Li *et al*., inhibition of β-catenin ubiquitylation results in the accumulation of phosphorylated β-catenin within the destruction complex, which ultimately leads to its saturation. Subsequently, β-catenin which is continually synthesized will not be incorporated in the destruction complex and be free to regulate gene transcription within the nucleus. Interestingly, the observations presented in this study were made using endogenous Axin levels. Considering the fact that Axin is expressed at rather low levels compared to the other proteins of the destruction complex, studying the dynamics of this complex is likely most reliable at endogenous Axin levels (Li *et al.*, 2012). In contrast to this study, it has also recently been observed that Wnt signalling inhibits the degradation of β-catenin upstream of, or coinciding with its phosphorylation steps (Hernández *et al.*, 2012). These conflicting results show that the inhibition of the destruction complex is most likely a complicated step in the Wnt regulation pathway, and it is feasible that multiple mechanisms of inhibition are involved.

3.5 The signalling hub: dishevelled

The cytoplasmic protein dishevelled is an adaptor that is involved in Wnt-induced signal transduction. When originally discovered in *Drosophila*, mutations in the *dsh* gene were found to convey the same developmental abnormalities as *wg* mutations (Klingensmith *et al.*, 1994). Three separate genes, namely Dvl1, Dvl2 & Dvl 3, encode the vertebrate homologs of Dvl. Their products play overlapping and interdependent roles in Wnt signalling (Sussman *et al.*, 1994; Yang *et al.*, 1996; Semënov and Snyder, 1997; Lee *et al.*, 2008). Upon the initiation of Wnt signalling by binding of Wnt to Frizzled and LRP5/6, Dvl is recruited to the cytoplasmic compartment of this receptor complex and phosphorylated by CK1 (Yanagawa *et al.*, 1995; Yang-Snyder *et al.*, 1996; Semënov and Snyder, 1997; Peters *et al*., 1999; Rothbächer *et al.*, 2000; Umbhauer *et al.*, 2000; Wong *et al.*, 2003). This phosphorylation, however, was shown to be independent of LRP5/6 function (González-sancho *et al.*, 2004). In addition, Dvl is thought to function upstream of LRP6 in the *Drosophila* Wnt pathway (Tolwinski *et al.*, 2003), and Dsh can activate Wnt signalling via β-catenin in an Arrow (the *Drosophila* LRP5/6 homolog)-independent manner (Wehrli *et al.*, 2000). As discussed previously, the exact role of Dvl in Wnt signalling has not yet been elucidated. However, multiple functional domains have been identified in the Dvl protein. The Dishevelled, Egl-10 and Pleckstrin (DEP) domain, which is located towards the Dvl C-terminus, is involved in the binding of Frizzled, an interaction that is strengthened by the association with phospholipids (Wong *et al.*, 2000; Simons *et al.*, 2009). In addition, the Dvl DEP domain is needed for the induction of PCP-signalling via Frizzled (Axelrod *et al.*, 1998; Simons *et al.*, 2009), illustrating that Dsh/Dvl can act as a switch between different pathways induced by Wnt signalling. The N-terminal Dishevelled and Axin (DIX) domain, which is present in both proteins, mediates their hetero –and homodimerization (Rothbächer *et al.*, 2000; Schwarz-Romond *et al.*, 2007; Metcalfe *et al.*, 2010; Tauriello *et al.*, 2012). Finally, the central Post-synaptic density-95, Discs-large and Zonula occludens-1 (PDZ) domain is also required for the binding of Dvl to Frizzled (Semënov and Snyder, 1997; Umbhauer *et al.*, 2000; Wong *et al.*, 2003), and in addition it mediates the interaction of Dvl with a large number of other proteins such as kinases, phosphatases and adaptor proteins (Lee *et al.*, 2008). Dvl has been shown to regulate different processes that are connected with the Frizzled/LRP-5/6 receptor complex. For example, Dvl is required for the phosphorylation of LRP6 upon Wnt signalling, leading to the recruitment of Axin, and the clustering of the receptor complex (Bilic *et al.*, 2007; Metcalfe *et al.*, 2010). Clustering of LRP-5/6 is facilitated by the generation of PIP2 by PI4KIIa and PIP5KI, which are both proteins that are bound and activated by Dvl (Pan *et al.*, 2008). Finally, Dvl is required for the internalization of Frizzled, which is mediated by the recruitment of the Clathrin adapter AP-2 (Chen *et al.*, 2003; Yu *et al.*, 2007).

3.5.1 Dvl ubiquitylation

In addition to the previously discussed Wnt pathway components, the function and stability of Dvl are also regulated by ubiquitylation. Compared to these other components, many ubiquitin E3 ligases have been implicated in the ubiquitylation of Dvl. The role of these currently known regulators of Dvl ubiquitylation will be discussed below.

First off, the HECT type E3 ubiquitin-protein ligase HECW1 (NEDL1) was identified in a search for proteins related to familial amyotrophic lateral sclerosis (FALS). NEDL1 was found to bind Dvl1 via its C-terminal compartment, including the DEP domain and three proline-rich clusters that are only found in mammalian Dvl1. The ubiquitylation and subsequent degradation of Dvl1 is likely mediated by NEDL1 (Miyazaki *et al.*, 2004). However, except for this study, which uses overexpression methods, no information is available on the interaction of NEDL1 and Dvl1. In addition, NEDL1 is mostly expressed in neuronal tissues, and thus the significance of NEDL1 as a Wnt regulator is yet to be established by follow-up studies.

Another ubiquitin E3 ligase of Dvl was identified by mass spectrometry in a co-immunoprecipitating protein complex associated with Dvl. The Kelch-like 12 (KLHL12) E3 ubiquitin ligase was found to mediate Dvl ubiquitylation, which targets the latter for proteasomal degradation (Angers *et al.*, 2006). KLHL12 is a Broad Complex/Tramtrack/Bric à Brac (BTB) domain-containing protein. There are many proteins that carry this domain, but to date only two substrates for BTB proteins have been identified in mammals (Cullinan *et al.*, 2004; Kobayashi *et al.*, 2004; Zhang *et al.*, 2004; Furukawa and Xiong, 2005;

Hernández-Muñoz *et al.*, 2005). The BTB domain in KLHL12 is required for its interaction with Cullin-3, which is a scaffold protein that functions in this Cullin-RING type ubiquitin ligase complex. The substrate specificity proteins for this type of ligase complex are members of the BTB-domain protein family (Pintard *et al.*, 2003; Furukawa *et al.*, 2003; Krek, 2003; Xu *et al.*, 2003). The Kelch domain of KLHL12 was shown to be required for the interaction with the C-terminal region of Dvl. The interaction of KLHL12 with Dvl was shown to be stimulated by Wnt signalling, which appoints KLHL12 as yet another negative feedback component of this pathway. However, the manner in which KLHL12 is regulated is yet to be elucidated.

Recently, another protein that interacts with Dvl was identified. The Nedd4-like itchy E3 ubiquitin protein ligase (ITCH) was shown to be an inhibitor of Wnt signalling. This inhibition is achieved by the lysine 48–linked polyubiquitylation of Dvl2, which leads to its proteasome-mediated degradation. Notably, a prerequisite for this ubiquitylation is that Dvl2 is phosphorylated. The Dvl DEP domain and PPXY motif are needed for the interaction between Dvl-2 and ITCH. The PPXY domain is present in the C-termini of all three human Dvl proteins (Wei *et al.*, 2012), and it is preferably targeted for binding by the WW domains of HECT ubiquitin E3 ligases (Bernassola *et al.*, 2008).

Inversin, which was initially found to be one of the genes that are often mutated in the autosomal recessive cystic kidney disease nephronophthisis type 2 (Otto *et al.*, 2003), is thought to be a molecular switch between different Wnt pathways. This has been suggested in relation to results from overexpression studies, which indicate its role in the degradation of cytoplasmic, but not cortical Dvl1. This idea is further supported by the fact that inversin can promote the initiation of non-canonical Wnt signalling. The latter was shown by demonstrating the role of inversin in convergent extension movements in *Xenopus laevis* embryos, in which the protein acts through a pathway similar to *Drosophila* PCP signalling (Simons *et al.*, 2005). In addition, inversin was found to interact with anaphase-promoting complex subunit 2 (ANAPC2). This protein is a component of the anaphase-promoting complex, also known as the cyclosome (APC/C), which is a large, 13-subunit E3 RING-Cullin ubiquitin ligase and a major regulator of the cell cycle (Morgan *et al.*, 2002; Barford, 2011). Subsequently, an interaction between Dvl and ANAPC2 was shown to occur via the DEP domain of Dvl. Also, the APC/C complex was shown to be required for the ubiquitylation and subsequent proteasome-mediated degradation of Dvl, which leads to the inhibition of canonical Wnt signalling (Ganner *et al.*, 2009).

More recently, the HECT E3 ubiquitin ligase neural precursor cell expressed, developmentally down-regulated 4-like (NEDD4L, alternatively NEDD4-2) has been implicated in the ubiquitylation and degradation of Dvl2 (Ding *et al.*, 2013). NEDD4L induces the lysine 6, lysine 27 and lysine 29–linked, but interestingly enough not the proteasomal degradation-associated lysine 48–linked polyubiquitylation of Dvl2. Similar to the E3 ubiquitin ligase ITCH which was discussed earlier, NEDD4L contains four WW domains which allow for its association with the PPXY motif, which is likely to mediate its binding to Dvl2. NEDD4L phosphorylation by c-Jun N-terminal kinase 1 (JNK1), a component of the mitogen-activated protein kinase (MAPK) signalling pathway, is required for NEDD4L to ubiquitylate Dvl2. In addition, signalling induced by Wnt5a stimulates this phosphorylation step. This connection possibly constitutes another negative feedback loop in the Wnt signalling pathway.

In addition, the NEDD4L-related HECT E3 ubiquitin ligase NEDD4 was shown to mediate the ubiquitylation and degradation of Dvl1 (and possibly Dvl2, according to unpublished data mentioned in Ding *et al*. (2013)) (Nethe *et al.*, 2012; Ding *et al.*, 2013). In this study by Nethe *et al*., the activity of Dvl1 was related to the inhibition of cell-cell contacts (Elbert *et al.*, 2006), but not specifically to Wnt signalling. NEDD4 binds, in a fashion similar to NEDD4L, to the DEP domain of Dvl1 through its WW domains. Ubiquitylation occurs at a region rich in lysine residues that is located between the Dvl1 DIX and PDZ domains. NEDD4 is thought to cooperate with Rac1, which indirectly stimulates actin polymerization and as such influences the establishment of cell-cell contacts (Watanabe *et al.*, 2009), in the ubiquitylation and downregulation of Dvl1. However, the influence of this process on canonical Wnt signalling is currently unknown.

In human hepatocellular carcinoma cells (HPCs), the protein prickle-1 was shown to interact with Dvl3. Prickle-1 downregulates Wnt signalling by mediating the ubiquitylation of Dvl3, which leads to its degradation (Chan *et al.*, 2006). This interaction occurs through the WW domain of prickle-1 and the destruction motif (arginine-x-x-leucine) of Dvl3, which is located in the DEP domain. In HPCs, insufficient expression of prickle-1 leads to the overexpression of Dvl3 and an increase in cell growth. Thus, prickle-1 is another interesting factor in the regulation of the Wnt signalling pathway by mediating the turnover of Dvl.

Activation of AMP-activated protein kinase (AMPK) has the ability to suppress cell growth through the inhibition of canonical Wnt signalling. This inhibition is mediated by the degradation of Dvl3 via the proteasome system (Kwan *et al.*, 2013). Which ligases are responsible for this degradation is currently unknown.

Down syndrome critical region protein 5 (Dscr-5) is a strongly conserved protein that is often mutated in Down syndrome patients. In *Danio rerio* and *Xenopus laevis* embryos the knockdown of *dscr5* leads to the degradation of Dishevelled by ubiquitylation and the proteasome system (Shao *et al.*, 2009). Whether this is also applicable in humans is yet to be established.

The *Drosophila* naked cuticle protein, and its vertebrate homologs NKD1 and NKD2, have all been implicated in the inhibition of Wnt signalling. NKD2-induced inhibition is dependent on its myristoylation, which is a post-translational modification that targets proteins to the plasma membrane. NKD2 was found to interact with ubiquitylated Dvl1, which leads to the enhanced polyubiquitylation and degradation of both proteins (Hu *et al.*, 2010). In *Danio rerio* the Nkd1 homolog is involved in the degradation of Dvl (Schneider *et al.*, 2010), and it binds to β-catenin to prevent its accumulation in the nucleus, a function that is conserved in mammals (Van Raay *et al.*, 2011). In addition, the inhibition of Wnt signalling by NKD1-mediated Dvl1 degradation is also a likely function in human cells (Zhang

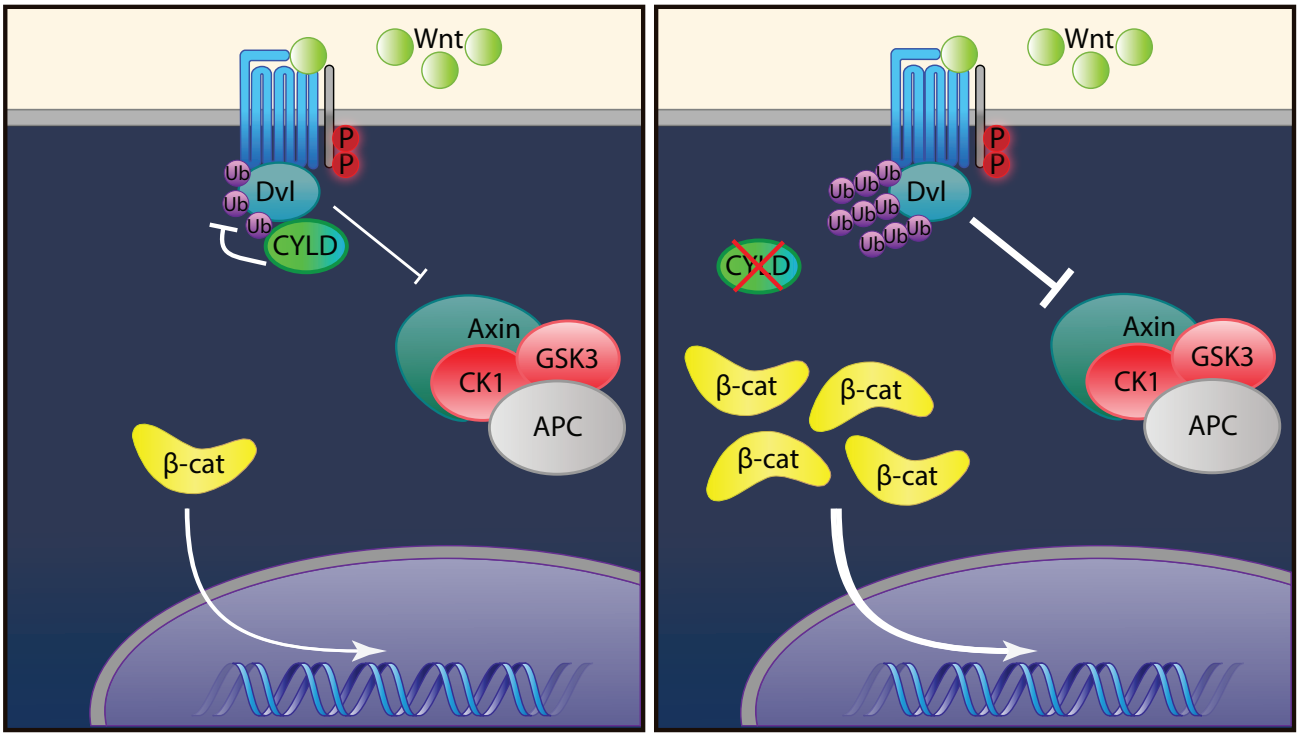

Figure 6. Schematic representation of CYLD function in Wnt signalling.

(**Left panel**) In a wild type situation, CYLD mediates the cleavage of lysine 63-linked polyubiquitin chains that are conjugated to the DIX domain of Dvl. In the absence of these chains, Dvl no longer recruits the destruction complex to the cell membrane. Thus, in this model CYLD is involved in the attenuation of Wnt signalling. (**Right panel**) When *cyld* expression is knocked down, lysine 63-linked polyubiquitin chains are conjugated to the DIX domain of Dvl, which allows for the recruitment and inhibition of the destruction complex. This subsequently leads to the accumulation of β-catenin and an enhanced Wnt signalling response. Figure based on the model described in Tauriello *et al*. (2010).

et al., 2011a). However, whether this degradation occurs via ubiquitylation and the proteasome system has not yet been established.

Malin is another negative regulator of Wnt signalling. The gene encoding this protein is mutated in ~40% of Lefora disease patients, which is an autosomal recessive disorder associated with progressive myoclonus epilepsy. Malin interacts with and downregulates Dvl2 protein levels. This downregulation is mediated by the lysine 48- and lysine 63-linked polyubiquitylation of Dvl2 *in vivo*, which probably leads to the autophagy and/or proteasomal degradation of the protein (Sharma *et al.*, 2012). Autophagy has been shown to regulate Dvl1, Dvl2 and Dvl3 degradation. The previously discussed E3 ubiquitin ligase pVHL binds to Dvl2 via its DEP domain and induces Dvl2 ubiquitylation, which leads to the autophagy of Dvl2. The significance of these results to canonical Wnt signalling remain to be established, especially considering that these experiments were conducted under conditions of starvation (Gao *et al.*, 2010).

3.5.2 Dvl-targeting DUBs

In addition to the vast array of Dvl interactors that mediate its ubiquitylation and degradation, DUBs that target Dvl have been identified as well. Encoded by the cylindromatosis gene, the CYLD protein was shown to be a negative regulator of canonical Wnt signalling (Tauriello *et al.*, 2010). CYLD was initially identified as a tumour suppressor (Bignell *et al.*, 2000), and its mutation leads to the hyperactivation of Wnt signalling both in cultured and primary tumour cells. CYLD was further shown to act as a lysine 63–linked polyubiquitin chain regulator of Dvl. This de-ubiquitylation occurs in the DIX domain of Dvl, thus possibly influencing its ability to polymerize and to interact with proteins that mediate processes such as Frizzled endocytosis and downstream signalling (see **figure 6** for the proposed model) (Schwarz-Romond *et al.*, 2007; Yu *et al.*, 2007; Tauriello *et al.*, 2010). Which E3 ubiquitin ligase mediates the ubiquitylation of Dvl with the lysine 63–linked polyubiquitin chains that are affected by CYLD is yet to be discovered. Thus far, the only candidate discussed above would be Malin, which was shown to enhance this type of ubiquitylation of Dvl2 (Sharma *et al.*, 2012).

Recently, mutations in the *gumby* (previously *Fam105b*) gene were shown to cause aberrant angiogenesis phenotypes during embryonic development in mice. The gumby protein was found to be an OTU-domain containing DUB that interacts with Dvl2 via its N-terminus. Notably, this DUB specifically targets linear polyubiquitin chains and associates with RNF31, which is involved in the construction of linear polyubiquitin chains via the linear ubiquitin assembly complex (LUBAC). Although gumby and LUBAC were shown to modulate canonical Wnt signalling by increasing and respectively decreasing the levels of Dvl2 protein and TCF/LEF-induced gene activation, their exact mechanisms of action in relation to this pathway remain to be determined (Rivkin *et al.*, 2013).

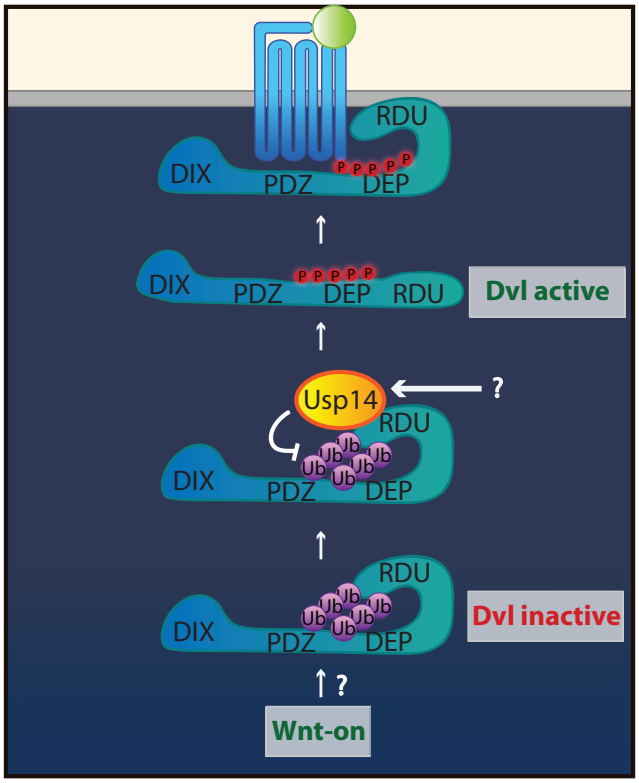

Figure 7. Schematic representation of USP14 function in Wnt signalling.

In the absence of Wnt ligand, Dvl expression is kept at a steady state level via its lysine 48-linked polyubiquitylation and degradation (not shown). During the Wnt-on phase, lysine 63-linked polyubiquitin chains are conjugated to Dvl by an unknown E3 ubiquitin ligase. These modifications induce a conformational change in Dvl by linking its DEP and RDU domains, which in turn allows for Usp14 to bind Dvl. Usp14 cleaves the lysine 63-linked polyubiquitin chains conjugated to Dvl, which reverts the conformational change and allows for Dvl phosphorylation to occur. Subsequently, this activated variant of Dvl binds to Frizzled and mediates the activation of Wnt signalling. Figure based on the model described in Jung *et al*. (2013).

Finally, USP14 was recently identified as a positive regulator of canonical Wnt signalling. In contrast to the deletion of CYLD, the inhibition of USP14 leads to impaired signalling downstream of Dvl (Tauriello *et al.*, 2010; Jung *et al.*, 2013). Interestingly, in both cases inactivation leads to the accumulation of polyubiquitin chains on Dvl. USP14 was identified as a DUB that is associated with a C-terminal Dvl regulatory domain of ubiquitylation (RDU), the deletion of which leads to the accumulation of lysine 63–linked polyubiquitin chains. In addition, the study shows that there is a correlation between the levels of USP14 and β-catenin in colon cancer cells. Possibly, the RDU is a domain that interacts with USP14 to mediate the regulation of lysine 63–linked polyubiquitin chains on Dvl. This de-ubiquitylation of Dvl was shown to be required for its subsequent phosphorylation and the activation of downstream signalling (see **Figure 7** for the proposed model) (Jung *et al.*, 2013).

In conclusion, many interactors of Dvl have been identified, including a variety of E3 ubiquitin ligases and DUBs. However, many of these proteins have been identified only recently, which explains why possible connections between these regulators are yet to be established. It will be interesting to see which of these modifiers of Dvl ubiquitylation are ubiquitously involved in canonical Wnt signalling, and which serve different, possibly more tissue-specific functions.

4. Ubiquitylation and the regulation of Wnt receptors

4.1 Frizzled and its co-receptors

In order for an intracellular signalling cascade to be initiated, Wnt ligands that are present in the surrounding milieu of the cell need to be recognized by membrane-bound receptors. The main Wnt receptors are those of the Frizzled family, which consists of G-protein-coupled receptor (GPCR)-like proteins that contain seven transmembrane domains (Bhanot *et al.*, 1996; Hsieh *et al.*, 1999). In accordance with their GPCR-like structure, some studies have suggested that G proteins, which mediate the intracellular signalling initiation for GPCRs, are involved in Wnt signalling (Katanaev *et al.*, 2005; Liu *et al.*, 2005; Jernigan *et al.*, 2011). However, the mechanisms by which they act upon receptor activation by Wnt remain elusive. In addition to Frizzled, the vertebrate co-receptors LRP-5/6 (or the single homolog Arrow in *Drosophila* (Wehrli *et al.*, 2000)) are involved in the initiation of Wnt signalling. LRP-5 and LRP6 are the partially redundant co-receptors that associate with Frizzled upon Wnt binding (Pinson *et al.*, 2000; Tamai *et al.*, 2000). Intriguingly, the *C. elegans* genome contains homologs for the frizzled receptor and dishevelled adaptor for example, but an LRP/Arrow-like co-receptor has not been identified. This might suggest that different mechanisms of signalling cascade initiation upon Wnt activation have emerged during evolution (Phillips and Kimble, 2009). Although the structures of LRP5 and LRP6 are very similar, their roles during animal development are possibly divergent (He *et al.*, 2004; Mi and Johnson, 2005). In addition, the affinity of the LRPs for the different Wnt molecules varies between them (Bourhis *et al.*, 2010; Chen *et al.*, 2011). Upon the activation of frizzled and its co-receptors phosphatidylinositol (4,5)-biphosphate (PIP2), a membrane phospholipid important in different signalling cascades, is produced. This production of PIP2 is required for the subsequent clustering of LRP-5/6, their phosphorylation and interaction with Axin, and thus the initiation of the Wnt signalling pathway (Baig-Lewis *et al.*, 2008; Pan *et al.*, 2008). In addition, it has been demonstrated that LRP6 is internalized via the caveolin pathway upon the initiation of Wnt signalling, and interestingly, this internalization is required for the accumulation of β-catenin (Yamamoto *et al.*, 2006).

The expression levels of the Wnt (co-)receptors at the plasma membrane are tightly regulated. The role of ubiquitin modifications in this context has long remained elusive, but it has received increasing attention over recent years. This section will cover the various mechanisms in which the Wnt receptors are regulated by ubiquitylation. Other Wnt receptor-related topics like the various Wnt agonists and antagonists, the post-translational modification of Wnt molecules to enable their signalling activity, and the mode of interaction between these

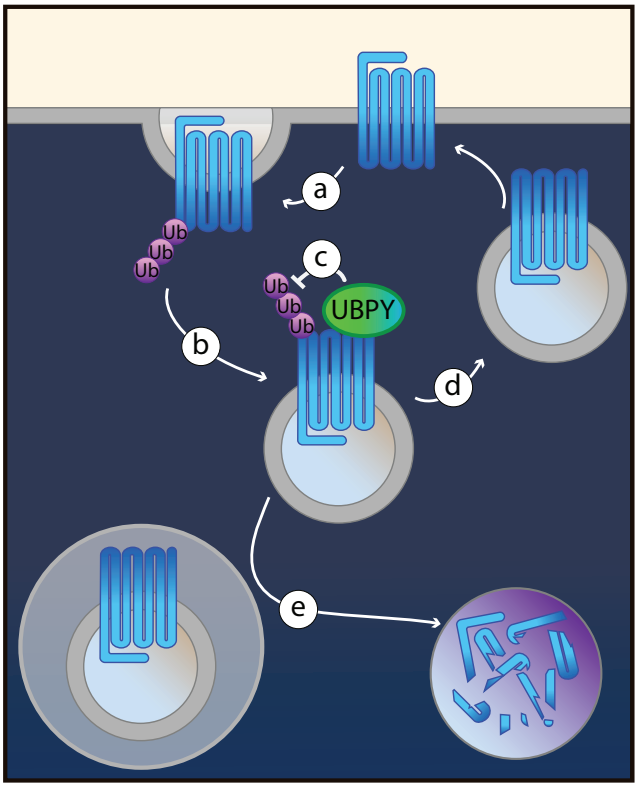

Figure 8. The intracellular trafficking of Frizzled.
At the cell membrane Frizzled is ubiquitylated (**a**), which induces its internalization (**b**). Subsequently, Frizzled is either deubiquitylated by UBPY (**c**) and recycled to the membrane (**d**), or degraded via the late endosome / lysosome pathway (**e**). In this model, the activity of UBPY, as well as the rate of ubiquitylation at the cell membrane, regulate the steady state levels of Frizzled. Figure reconstructed and adapted from Mukai *et al*. (2012).

molecules and their receptors are not within the scope of this review. For more information on these topics, the reader is referred to the following reviews: Lorenowicz and Korswagen (2009); Port and Basler (2010) & Harterink and Korswagen (2012).

4.2 Ubiquitin and the intracellular trafficking of Wnt receptors

As discussed previously, ubiquitin modifications have been studied extensively for their involvement in the internalization and subsequent transport of membrane-bound receptor proteins such as RTKs. In this case, ubiquitin functions as a sorting flag that directs the vesicle-transporting machinery to translocate the receptors to their subcellular destination. These sorting mechanisms have also been implicated in the trafficking of the Wnt receptors, and one example of this is the transport of LRP-6. The export of LRP-6 from the endoplasmic reticulum (ER) depends on its palmitoylation, which is the post-translational covalent modification of a protein with a palmitate group (Abrami *et al.*, 2008). This modification was proposed to tilt the receptor relative to the plane of the ER membrane, which allows for it to escape the ER quality control mechanism. This system assesses the proper folding of endogenous proteins. Upon the recognition of a misfolded target, the protein is retained in the ER and finally degraded (Hebert and Molinari, 2007). When the palmitoylation of LRP-6 is inhibited, the receptor is retained in the ER. However, a palmitoylation-deficient variant of LRP-6 that was mutated at residues that are substrates for ubiquitylation was found to escape the ER quality control system, and to emerge at the cell membrane. Thus, monoubiquitylation regulates the exit of LRP-6 from the ER (Abrami *et al.*, 2008).

In addition to transport towards the cell membrane, the ubiquitylation of Wnt receptors has also been implicated in their internalization. In different cellular pathways, the endocytosis of receptors has been implicated in their positive regulation. For example, the Wnt PCP pathway even requires endocytosis to occur in order to function properly (Gagliardi *et al.*, 2008). Recently, Mukai *et al*. demonstrated that canonical Wnt signalling requires a strict balance in Frizzled ubiquitylation. Increases in Frizzled ubiquitylation were shown to lead to higher rates of internalization and subsequent degradation of Frizzled by its targeting to lysosomes in *Drosophila* and mammalian cells, while a decrease in ubiquitylation had the opposite effect. The responsible modification in this case was suggested to be primarily multiple monoubiquitylation.

In accordance with this model, a DUB for Frizzled has been shown to regulate its intracellular trafficking. Namely, the DUB USP8, also called UBPY, was shown to regulate the deubiquitylation of Frizzled and thereby to influence its endosomal transport (see **Figure 8**). An increase in UBPY activity resulted in the increased recycling of Frizzled to the membrane. The degradation of Frizzled via ubiquitylation was shown to include its targeting to lysosomes, and not the proteasome, as has been the case for most of the Wnt pathway proteins discussed previously. In addition, Frizzled degradation by ubiquitylation differs from that of the RTKs. In the latter case, receptor internalization increases upon the activation of the related signalling pathway after ligand binding (Saksena *et al.*, 2007). In contrast, the ubiquitylation and trafficking of Frizzled were found to be independent of activation by Wnt ligands. Thus, the ubiquitylation of Frizzled was proposed to regulate its steady-state membrane levels, and UBPY was identified as a positive regulator of Frizzled recycling (Mukai *et al.*, 2010, 2012). The E3 ubiquitin ligase that is responsible for the ubiquitylation of Frizzled that is counteracted by UBPY has not been identified.

In a recent study, the trafficking of LRP6 has also been shown to depend on its ubiquitylation. Namely, the inactivation of Rap2, a member of the Ras family that is comprised of small GTP-binding proteins (Bos, 1997), coincides with proteasome and/or lysosome-mediated LRP6 degradation (Park *et al.*, 2013). This downregulation was also shown to be independent of Wnt signalling. Rap2 and LRP6 interact directly, and the destabilizing effect of Rap2 activity is mediated via its downstream effector TRAF2/Nick-interacting kinase (TNIK). These results are intriguing, especially when considering that Wnt-induced LRP6 internalization is needed for β-catenin accumulation, as well as the fact that Rap2 is required for canonical Wnt signalling during *Xenopus* development (Choi and Han, 2005). Park *et al*. proposed that LRP6 is internalized both in response to Wnt signalling as well as constitutively in the absence of Wnt ligand. In this model, Rap2 possibly functions as a switch between the recycling and

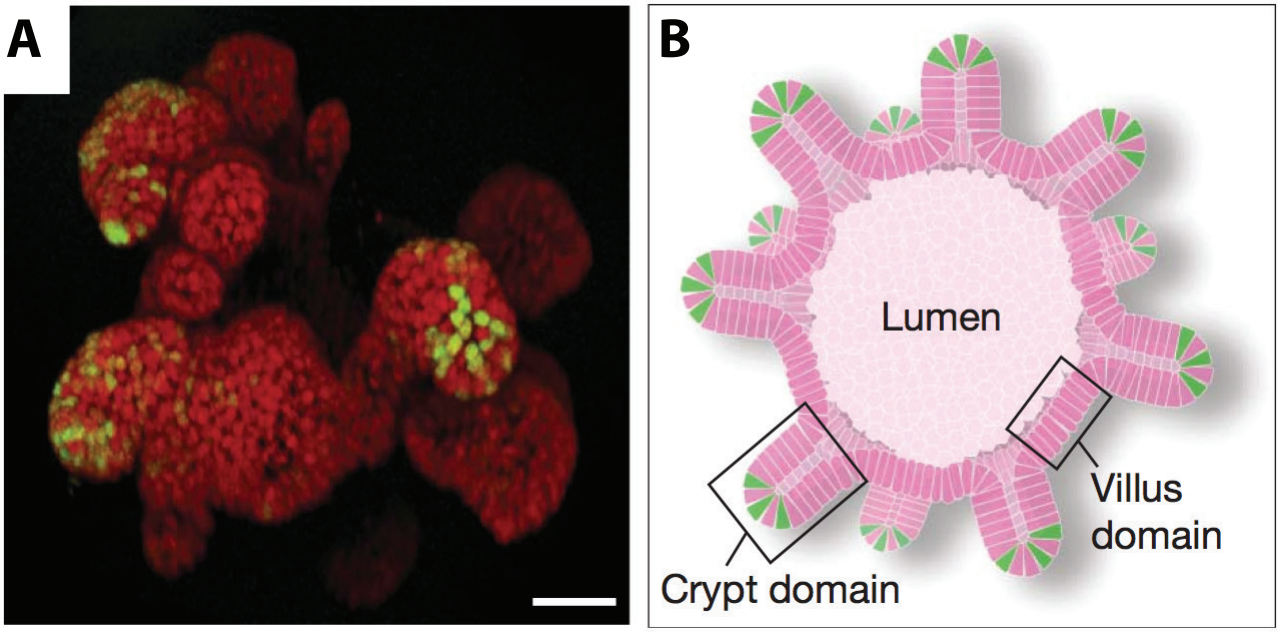

Figure 9. Organoids grown from single LGR-5-positive cells (Sato *et al*., 2009).

(**A**) Multiple confocal images reconstructed to a 3D overview of a single cultured organoid. LGR5-positive cells are labelled in green and DNA is labelled in red. Scale bar length equals 50 µm.

(**B**) Schematic representation of a cultured organoid, which contains a central lumen and multiple interconnected crypt and villus domains.

lysosomal pathway of internalized LRP6-containing endosomes. Similar mechanisms are thought to occur in the trafficking of Frizzled (Mukai *et al.*, 2010; Park *et al.*, 2013). Whether Rap2 plays a role in the trafficking of both receptors remains to be demonstrated.

In addition to Frizzled, the receptor-like tyrosine kinase (RYK) and receptor tyrosine kinase-like orphan receptor 2 (ROR2) proteins are transmembrane receptors capable of binding Wnt ligands. RYK has also been implicated in the activation of canonical Wnt signalling (Lu *et al.*, 2004). However, the significance of both ROR and RYK function in canonical Wnt signalling remains elusive (Angers and Moon, 2009). Berndt *et al.* recently reported that the E3 ubiquitin ligase Mindbomb 1 (MIB1) interacts with RYK. In addition, MIB1 expression promotes the ubiquitylation and subsequent degradation of RYK, thus reducing its stability at the cell membrane. In addition, RYK and MIB1 were shown to cooperate in the induction of canonical Wnt signalling. Whether ubiquitylation by MIB1 induces RYK internalization upon Wnt signalling in a manner similar to other membrane receptors has, however, not been determined. Interestingly, while RYK is required for canonical Wnt signalling, the expression of MIB1 also promotes the activity of this pathway, even though it leads to the downregulation of RYK at the plasma membrane. A possible explanation proposed by Berndt *et al*. is that MIB1 induces the internalization of a complex containing RYK, as well as other Wnt receptors. In agreement with this hypothesis, the knockdown of RYK or MIB1 leads to the decreased phosphorylation of LRP6 and a weaker Wnt signalling response induced by Wnt ligand.

4.3 The R-spondins

The roof plate-specific spondins (R-spondins or RSPOs) are secreted glycoproteins that were initially shown to be required for dorsal neural tube development in mammals and myogenesis in *Xenopus*. The expression of RSPOs depends on Wnt expression, and conversely they are also potent activators of the canonical Wnt signalling pathway (Chen *et al.*, 2002; Kamata *et al.*, 2004; Kazanskaya *et al.*, 2004; Cruciat and Niehrs, 2013). The RSPOs are evolutionarily conserved proteins, and four isoforms (RSPO1-4) have been identified in vertebrates. Their Wnt-stimulating activity requires the presence of Wnt ligands (Binnerts *et al.*, 2007). The exact mechanism of RSPO function has long been debated over, and several receptors have previously been proposed to interact with these signalling molecules.

Recent developments have shed light on the mechanism of RSPO function, and these involve the leucine-rich repeat containing G-protein-coupled receptors (LGRs). LGRs have been identified as markers for stem cells (Barker *et al.*, 2013). For example, LGR5 expression allows for the identification of stem cells in the stomach, small intestine, colon and hair follicle, and LGR6 marks stem cells in the skin (Jaks *et al.*, 2008; Barker *et al.*, 2010; Snippert *et al.*, 2010). Interestingly, the compartments in which these cells are located were all found to be dependent on Wnt signalling, and the same goes for the expression of LGR5. The stem cell identity of the LGR5-positive cells has been demonstrated elegantly by, for example, the culturing of single LGR5-positive cells taken from mouse intestinal crypts. These single stem cells can establish tissues containing both crypts and villus-like structures *in vitro* (see **Figures 9A & B**). Interestingly, these organoids contain all of the differentiated cell types that are found in the mouse intestinal tract *in vivo* (Sato *et al.*, 2009).

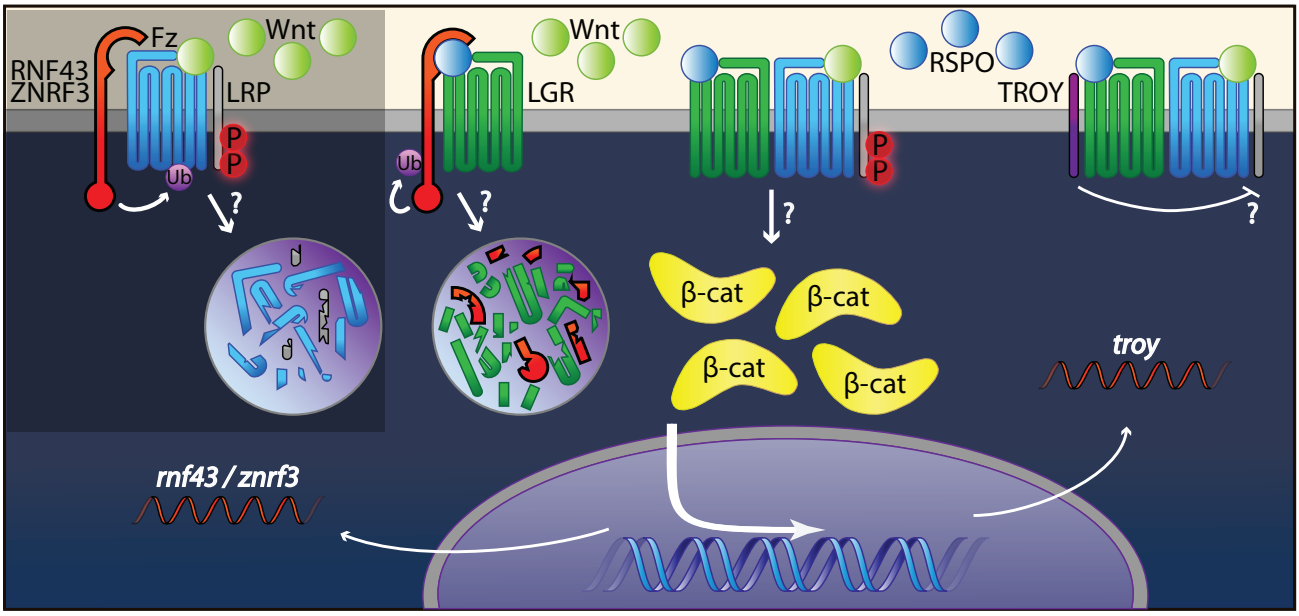

Figure 10. Schematic representation of Wnt receptor regulation at the cell membrane.

(**Shaded area**) In the absence of RSPO, Frizzled is ubiquitylated by RNF43/ZNRF3. This leads to the downregulation of Frizzled and LRP6 at the cell membrane, possibly via lysosomal degradation. (**Light area**) In the presence of both Wnt and RSPO, LGR4 forms a ternary complex with RNF43/ZNRF3 and RSPO. This leads to the autoubiquitylation of ZNRF3 (and possibly RNF43) and its downregulation at the cell membrane, which could occur via lysosomal degradation. In addition, LGR4/5 might be involved in the direct amplification of signalling induced by Wnt-Frizzled by associating with the Frizzled-LRP receptor complex. The RSPO-induced enhancement of Wnt signalling is attenuated by TROY, possibly via its interaction with LGR5 and the inhibition of LRP phosphorylation. Finally, increases in β-catenin levels promote the expression of negative regulators of Wnt signalling such as *rnf43*/*znrf3* and *troy*, thus constituting a negative feedback loop.

In addition, these crypts maintain the same structure as normal intestinal crypts, in which the LGR5-positive cells are located at the bottom. The culture media for such organoids are supplied with different growth factors, one of which is RSPO1.

Interestingly, the LGRs have recently been identified as the receptors for RSPOs. Namely, RSPO1-4 were demonstrated to bind to LGR4, LGR5 and LGR6 (de Lau *et al.*, 2011; Wang *et al.*, 2013). The knockdown of all three of these receptors abrogated RSPO-induced enhancement of Wnt signalling, and the re-introduction of any of the three LGRs reconstituted the RSPO effect. In addition, the knockdown of LGR4 and LGR5 mimicked the loss of RSPO1, and this phenotype can be rescued by the activation of Wnt signalling (de Lau *et al.*, 2011). Receptor internalization was proposed to play a role in the mechanism of RSPO action, as canonical Wnt signalling requires clathrin expression, while Wnt/PCP signalling requires caveolin expression in mammals and *Xenopus* (Glinka *et al.*, 2011). LGR4 and LGR5 were found to activate Wnt signalling in response to RSPOs via the phosphorylation of LRP6. However, no mediators of intracellular signalling, such as G-proteins, Ca^{2+} mobilization, cAMP production or β-arrestin were identified in connection to these LGRs. Interestingly, LGR4 and LGR5 were internalized constitutively into large intracellular bodies, even in the non-RSPO-bound state (Carmon *et al.*, 2011). With the requirement of Frizzled co-receptor internalization for Wnt signalling in mind, it was subsequently speculated that the LGR-RSPO complexes might have an influence on the recycling of the Wnt receptor complex.

Recently, two papers published in *Nature* have shed light on the mechanism of RSPO function (Hao *et al.*, 2012; Koo *et al.*, 2012). In these papers, Ring Finger Protein 43 (RNF43) and Zink and Ring Finger 3 (ZNRF3) are identified as negative regulators of the Wnt signalling pathway. These proteins are related transmembrane RING finger proteins that show E3 ubiquitin ligase activity. Interestingly, their coding genes were discovered by different approaches in the two publications. Koo *et al.* assessed the genes the expression of which was enriched in LGR5-positive crypt cells by microarray experiments. Hao *et al.*, on the other hand, searched for additional negative regulators of Wnt signalling by assessing the expression of genes that correlated with the expression of the *AXIN2* mRNA. Interestingly, both papers identified RNF43 and ZNRF3 as negative regulators of Wnt signalling, and Hao *et al.* also implicated ZNRF3 in the inhibition of both Wnt/β-catenin and Wnt/PCP signalling. Both of the transmembrane E3 ubiquitin ligases were found to regulate the turnover of Frizzled and its co-receptors. This downregulation occurs by the ubiquitylation-dependent promotion of Frizzled and LRP-5/6 internalization and the subsequent targeting of these receptors to lysosomes, which notably was not dependent on the proteasome system (Koo *et al.*, 2012). Interestingly, both RNF43 and ZNRF3 were previously identified as Wnt target genes (Yagyu *et al.*, 2004; Van der Flier *et al.*, 2007; van der Flier *et al.*, 2009). Thus, these ubiquitin ligases form another potential negative feedback loop for Wnt signalling (see **Figure 10**) (Koo *et al.*, 2012).

In addition, RSPOs were shown to be negative regulators

of ZNRF3 function. Namely, RSPO binds to the extracellular domain of ZNRF3, which induces its interaction with LGR4. The formation of such a ternary complex subsequently leads to ZNRF3 internalisation (Hao *et al.*, 2012). Thus, the inhibition of ZNRF3 by RSPO and LGR function leads to increased levels of Frizzled and its co-receptors at the cell membrane, which potentiates Wnt signalling. Recently, the structural interface of the interaction of RSPO1 with both LGR5 and RNF43 has also been mapped (Chen *et al.*, 2013; Peng *et al.*, 2013). In addition, cultured organoids derived from LGR5-positive crypt cells in which RNF43 and ZNRF3 were knocked down showed increased growth rates and RSPO1-independence. These organoids were, however, strongly dependent on supplied Wnt ligands. These experiments illustrate that RSPOs enhance Wnt signalling by increasing membrane Wnt receptor levels, via the binding of LGRs and the downregulation of RNF43 and ZNRF3 (Koo *et al.*, 2012).

Finally, a recent study that focused on the identification of Wnt target genes in colorectal cancers cells identified tumour necrosis factor receptor family, member 19 (TNFRSF19 or TROY). Canonical Wnt signalling stimulates the expression of this protein, as was shown by the knockdown of APC and the stimulation of cells with Wnt ligand. In accordance with these results, the knockdown of TROY stimulates the phosphorylation of LRP6. Both in colorectal tumour-derived cells as well as wild type LGR5-positive stem cells TROY expression is markedly upregulated. In addition, TROY interacts with LGR5, and it is capable of inhibiting the stimulation of Wnt signalling that is induced by RSPOs (see **Figure 10**). The latter was shown by the in vitro growth of LGR5-positive stem cell-based organoids, which could be maintained at significantly lower RSPO concentrations when TROY was inactivated, compared to wild type organoids (Fafilek *et al.*, 2013). These experiments are reminiscent of the previously discussed effects of RNF43 and ZNRF3 knockdown on such organoids. Thus, TROY could possibly be used as yet another reliable marker for the identification of Wnt-dependent (intestinal) stem cells. However, the way in which TROY inhibits the RSPO signal, possibly via its interaction with LGR5 or even via RNF43 and/or ZNRF3 remains to be determined.

5. The roles of Wnt signalling and ubiquitylation in human disease.

Ever since its initial discovery, the Wnt signalling pathway has often been implicated in the development of human diseases. Accordingly, inappropriate activation of the canonical Wnt pathway is one of the hallmarks leading to the unbalanced division, growth and differentiation of cells. In many tissues the hyperactivation of canonical Wnt signalling, which often converges on the accumulation of β-catenin, has been linked to the development of different types of cancer and neurodegenerative diseases. In addition, a lot of today's research focuses on understanding and manipulating the Wnt pathway to develop possible cures for these diseases. As such, many reviews are available on the progress of such studies, including Clevers and Nusse (2012) & Holland *et al.*, (2013).

In this review, the main focus has been the role of ubiquitin modifications on canonical Wnt pathway behaviour. Notably, these modifications have been implicated in many aspects of the behaviour of proteins, such as their signalling capabilities, turnover rates and subcellular localization. Thus, it comes as no surprise that ubiquitin modifications have often been implicated in both the direct and indirect regulation of β-catenin steady-state levels. Logically, errors in the ubiquitylation process have thus been implicated in the deregulation of the canonical Wnt signalling pathway. In this section, keynote examples of such deregulation and their mechanistic relation to human pathogenesis will be discussed.

5.1 The roles of RNF43 and ZNRF3 in carcinogenesis.

The ZNRF3 and RNF43 transmembrane E3 ubiquitin ligases, which were discussed previously, have been implicated in carcinogenesis. Namely, two cancerous cell lines derived from the colon that show hyperactivation of β-catenin contain mutations in the *RNF43* gene (Ivanov *et al.*, 2007). These cells are heterozygous for an activating β-catenin mutation, which leads to increased activation of the Wnt pathway. In addition, these mutations can cause the Wnt pathway to be hyperactivated by increased extracellular Wnt ligand concentrations. The HCT116 cell line is also homozygous for inactivating *RNF43* mutations, thus decreasing its ability to lower Wnt responsiveness by downregulation of the Wnt receptors. When RNF43 is reintroduced into these cells, this inhibitory mechanism is restored. Thus, the inactivation of RNF43 can increase the carcinogenic effects of activating β-catenin mutations (Koo *et al.*, 2012). Mechanistically, RNF43 and ZNRF3 are thought to balance the proliferation and differentiation zones in stem cell compartments like those that are found in the crypts of Liberkühn in the colon. Inside these compartments, a small stem cell zone is maintained at the bottom of the crypt. This is where the crypt base columnar (CBC) stem cells and the Paneth cells, which supply growth factors to the stem cells, reside (Clevers, 2013). Lgr5-positive CBC cells in this zone continually cycle through round of mitosis, producing undifferentiated daughter cells. These cells migrate out of the stem cell zone, towards the tip of the villi, and they start differentiating in transit (Clevers, 2013). ZNRF3 and RNF43 were proposed to help maintain the adequate size of the stem cell zone in such compartments. Namely, the inactivation of these proteins leads to an expansion of the proliferative stem cell zone similar to the consequences of deleting APC in this compartment (el Marjou *et al.*, 2004; Koo *et al.*, 2012). A similar Wnt-dependent stem cell zone is found in, for example, the hair follicle (Jaks *et al.*, 2008; Snippert *et al.*, 2010).

After the initial description of ZNRF3 and RNF43, these proteins have been related to non-intestinal types of cancer as well. For example, Jiang *et al.* reported that inactivating RNF43 mutations have been found in pancreatic cancer cells. In addition, pancreatic cancer cell lines that carry these mutations are strongly Wnt-dependent, similar to the colon cancer cells described above. While the inhibition of Wnt secretion, β-catenin depletion and the reintroduction of wild type RNF43 were able to inhibit the proliferation of cell carrying inactivating RNF43 mutations, this was not the case in tumour cells that

carry wild type RNF43. Thus, the presence of RNF43 mutations in cancers could be used as a potent biomarker when selecting patients for Wnt-inhibitory drugs (Jiang *et al.*, 2013). In addition, RNF43 mutations have been identified in intraductal papillary mucinous neoplasm and cholangiocarcinoma (Wu *et al.*, 2011; March *et al.*, 2012; Ong *et al.*, 2012), and one study focusing on gene expression in mucinous ovarian tumours identified RNF43 as one of the most frequently mutated genes in this type of cancer (Ryland *et al.*, 2013). In another recent publication, ZNRF3 was shown to be a tumour suppressor in gastric adenocarcinoma. In this case, the reintroduction of wild type ZNRF3 in a gastric cancer cell line suppressed proliferation and promoted apoptosis in these cells (Zhou *et al.*, 2013). Thus, both the RNF43 and ZNRF3 E3 ubiquitin ligases have been identified as potent targets for the development novel therapies that focus on Wnt-dependent types of cancer.

5.2 The regulation of E3 ubiquitin ligases by Wnt pathway components and their involvement in carcinogenesis.

In addition to being targets for (de-)ubiquitylation, some of the central Wnt pathway components can regulate the activity of E3 ubiquitin ligases (Tauriello and Maurice, 2010). Furthermore, there is cross talk between these proteins and signalling components of other cellular cascades. For example, the RING-type E3 ubiquitin ligase siah-1 is involved in modifying β-catenin with atypical lysine 11-linked ubiquitin chains in response to p53 expression, which leads to β-catenin degradation and thus the inhibition of Wnt signalling (Liu *et al.*, 2001; Matsuzawa and Reed, 2001). Naturally, this interchange of information between signalling pathways could lead to strong pleiotropic effects when the function of a protein is altered by mutation. Another interesting example of such cross talk is mediated by the scaffold protein Axin. In addition to its role as a component of the β-catenin destruction complex, Axin was found to be involved in the regulation of c-Myc turnover. c-Myc is a notorious transcription factor that regulates up to 15% of the human genes. Unsurprisingly, c-Myc is also one of the genes that are most often found to be hyperactivated in a great variety of cancer types (Grandori *et al.*, 2000; Fernandez *et al.*, 2003; Patel *et al.*, 2004).

In an APC-independent manner, Axin has been implicated in the ubiquitylation and degradation of c-Myc. Both Axin and GSK3 have been found to form a complex with c-Myc, and this complex mediates phosphorylation of the c-Myc threonine 58 and serine 62 residues (Arnold *et al.*, 2009). Interestingly, these residues are required for c-Myc degradation and they are often mutated in cancer cells. In addition to its role in the degradation of β-catenin, the $SCF^{\beta\text{-}TrCP}$ complex targets c-Myc and mediates its heterotypic polyubiquitylation, which leads to the increased stability of this oncogene. In contrast, SCF^{Fbw7} is responsible for the lysine 48-linked polyubiquitylation of c-Myc, which mediates its degradation (Popov *et al.*, 2010). This route of c-Myc degradation was shown to be dependent on its phosphorylation on the two residues targeted by the Axin-GSK3 complex, while $SCF^{\beta\text{-}TrCP}$ targets different residues in the c-Myc N-terminus (Yada *et al.*, 2004; Welcker *et al.*, 2006; Kitagawa *et al.*, 2009). A number of cancer cell lines in which the hyperactivity of c-Myc was reported have also been found to harbour inactivating mutations in components of the Axin-GSK3 c-Myc destruction complex (Arnold *et al.*, 2009). For example, mutations in the GSK3-binding moiety of Axin were identified in leukaemia cell lines with increased c-Myc activity (Malempati *et al.*, 2006). In addition, inactivating Axin mutations were identified in both primary and cultured breast cancer cells, and these were demonstrated to cause an increase in c-Myc stability (Zhang *et al.*, 2011b). Thus, Axin and GSK3 are involved in regulating the stability of multiple major regulators of stem cell behaviour via their ubiquitylation.

In addition to its role in c-Myc stabilization, GSK3β has been implicated in the stabilization of Ras (Liu *et al.*, 2008). This well-known oncogene activates the mitogen-activated protein kinase (MAPK) pathway *inter alia*, which is involved in both the growth and differentiation of cells. Similarly to c-Myc, mutations that alter the activity or stability of Ras have been implicated in a variety of cancers. Notably, up to one third of all genotyped human cancers have been shown to carry oncogenic mutations for the *Ras* gene (Pylayeva-Gupta *et al.*, 2011). One of the three Ras isoforms, H-Ras, is both mono- and di-ubiquitylated, and these modifications affect its subcellular localization and activity (Jura *et al.*, 2006). In addition, $SCF^{\beta\text{-}TrCP}$ was shown to mediate the polyubiquitylation and subsequent degradation of H-Ras, which is stimulated by Axin/APC expression and inhibited by the activation of Wnt signalling (Kim *et al.*, 2009). Recently, GSK3β was shown to phosphorylate Ras at its threonine 144 and threonine 148 residues. These modifications are subsequently recognized by $SCF^{\beta\text{-}TrCP}$, which polyubiquitylates Ras, leading to its proteasome-mediated degradation. Wnt signalling inhibits, and Axin/APC activity promotes, this phosphorylation of Ras by GSK3β (see **Figure 11**) (Jeong *et al.*, 2012). Thus, Wnt signalling regulates the stability of both β-catenin and Ras in the same manner. In addition, the same study by Jeong *et al*. demonstrates that this connection between the Wnt and MAPK pathways influences carcinogenesis *in vivo*. Namely, the stabilization of Ras through canonical Wnt signalling was shown to contribute to colorectal tumorigenesis (Jeong *et al.*, 2012). In conclusion, Wnt signalling is involved in multiple cellular signalling pathways via the regulation of protein ubiquitylation, and mutations that counteract this regulation are involved in multiple types of cancer.

6. Future perspectives

In summary, this review has constructed an overview of the currently known roles that ubiquitin modifications play in the canonical Wnt signalling pathway. When focussing on the regulation of β-catenin stability, it has become overly clear that ubiquitylation is involved in regulating most, if not all, of the active components in this pathway. Ubiquitin modifications have been demonstrated to be diverse in structure, and to affect the stability, sub-cellular localization and signalling capability of proteins *inter alia*. In addition, a large variety of E3 ubiquitin ligases and DUBs determine the balance of these protein modifications, and many of those have already been characterized in different model systems. However, in most

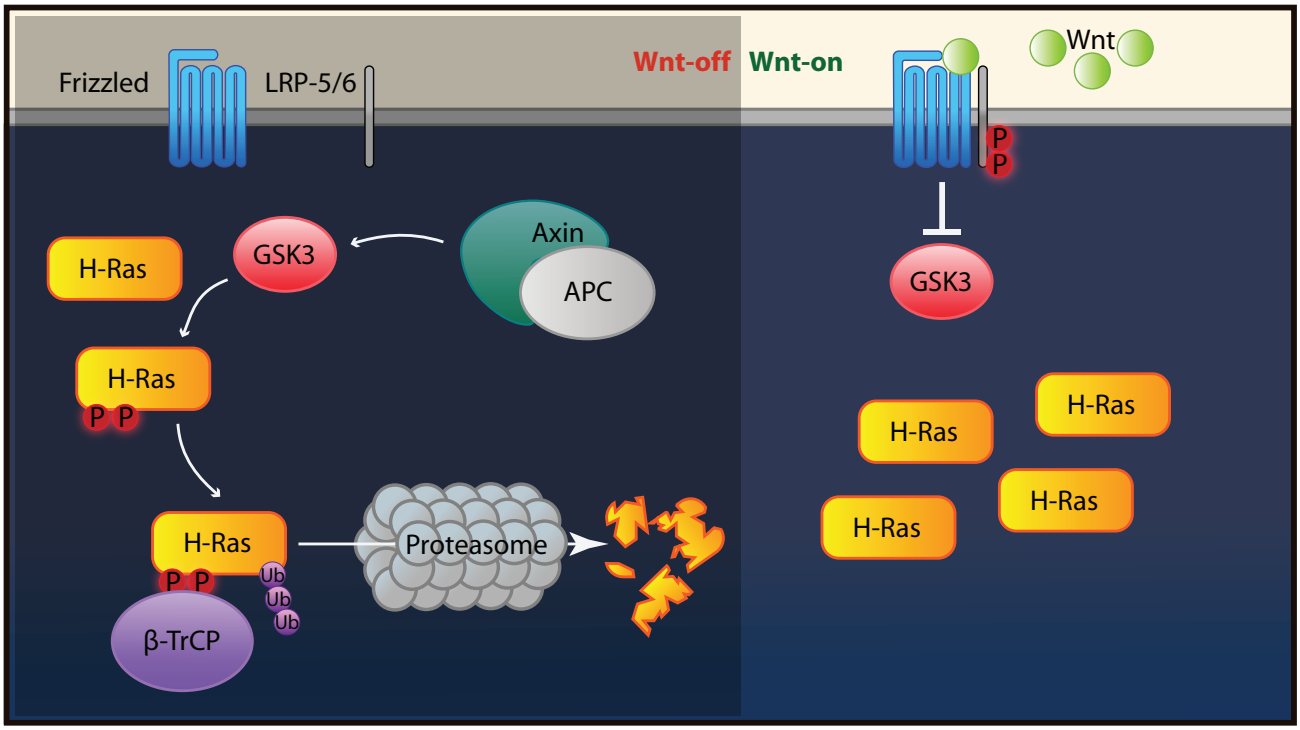

Figure 11. Schematic representation of H-Ras turnover regulation by Wnt signalling.
During the Wnt-off phase, H-Ras phosphorylation by GSK3β on its threonine 144 and threonine 148 residues is stimulated by Axin & APC. These modifications are recognized by β-TrCP, which mediates the subsequent polyubiquitylation and proteasome-mediated degradation of H-Ras. During the Wnt-on phase, GSK3β is inhibited and H-Ras, in a manner similar to β-catenin, is no longer targeted for degradation by β-TrCP. Figure based on the model described in Jeong *et al*. (2013).

cases the counteracting or collaborating pairs of E3 ubiquitin ligases and DUBs remain to be identified. In addition, the exact regulatory mechanisms that control these E3 ligases and DUBs remain elusive. Thus, at present it is too early to construct a full-scale interaction map of the ubiquitin regulators that influence the Wnt signalling pathway. A number of small-scale models that focus on specific nodes in the Wnt signalling pathway have been proposed (Tauriello and Maurice, 2010), but these often contain but a few of the known synergizing DUBs and E3 ubiquitin ligases.

The construction of large-scale models is further complicated by the fact that the functions of the different types of ubiquitin modifications remain largely elusive. For example, lysine 48-linked polyubiquitin chains are often linked to the targeting of substrates for proteasomal degradation, whereas lysine 63-linked chains are commonly associated with protein activation and complex formation (Ikeda and Dikic, 2008). However, homotypic chains linked through the other 5 lysine residues present on the ubiquitin protein have been explored in far less detail. The same goes for other types of polyubiquitin modifications such as those consisting of mixed-chains, in which multiple types of linkage are assimilated, and heterologous chains, in which both ubiquitin and ubiquitin-like moieties such as the earlier discussed SUMO protein are intertwined (Ikeda and Dikic, 2008). Although these atypical polyubiquitin chains are gradually being implicated in different regulatory functions, most of their influences on protein behaviour remain to be discovered (Kulathu and Komander, 2012). Naturally, the identification and characterization of more UBDs that recognize these different types of ubiquitin modifications, and the discovery of factors that regulate the proteins containing these domains, will be crucial for this endeavour.

As discussed previously, factors that are mediators or targets of protein ubiquitylation are frequently implicated in human pathogenesis. For example, the aberrant stability or expression of β–catenin, the transmembrane E3 ubiquitin ligases RNF43 and ZNRF3, the DUB CYLD, the signalling hub Dvl and the scaffold proteins APC and Axin have all been linked to tumorigenesis. In line with these findings, it will be important to find out how these proteins are regulated by the Wnt signalling pathway. The further identification of their E3 ubiquitin ligases, DUBs and other UBD-containing interactors will shed light on how their deregulation contributes to tumorigenesis. In addition, such factors could be of invaluable worth as markers for stem and/or cancer cells, as is already the case for proteins such as LGR5 and β-catenin, respectively.

Identifying promising targets for therapeutic purposes is complicated by the fact that pathways often cross-communicate, as was discussed previously. The Wnt signalling pathway is no exception due to its ubiquitin-dependent interaction with major regulators of cell behaviour such as c-Myc, Ras and components of the non-canonical Wnt signalling pathways. A major challenge for future research will be to integrate canonical Wnt signalling molecules into these different pathways, which is crucial for avoiding pleiotropic effects when developing therapies that interfere with the dynamics of these proteins.

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References

Abrami, L., Kunz, B., Iacovache, I., and van der Goot, G. (2008). Palmitoylation and ubiquitination regulate exit of the Wnt signaling protein LRP6 from the endoplasmic reticulum. Proc. Natl. Acad. Sci. U. S. A. *105*, 5384–5389.

Amit, S., Hatzubai, A., Birman, Y., Andersen, J. S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y., and Alkalay, I. (2002). Axin-mediated CKI phosphorylation of β-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev. *16*, 1066–1076.

Angers, S., and Moon, R. T. (2009). Proximal events in Wnt signal transduction. Nat. Rev. Mol. Cell Biol. *10*, 468–477.

Angers, S., Thorpe, C. J., Biechele, T. L., Goldenberg, S. J., Zheng, N., MacCoss, M. J., and Moon, R. T. (2006). The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-β-catenin pathway by targeting Dishevelled for degradation. Nat. Cell Biol. *8*, 348–357.

Arnold, H. K., Zhang, X., Daniel, C. J., Tibbitts, D., Escamilla-Powers, J., Farrell, A., Tokarz, S., Morgan, C., and Sears, R. C. (2009). The Axin1 scaffold protein promotes formation of a degradation complex for c-Myc. EMBO J. *28*, 500–512.

Axelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T., and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. Genes Dev. *12*, 2610–2622.

Baig-Lewis, S., Peterson-Nedry, W., and Wehrli, M. (2008). Wingless/Wnt signal transduction requires distinct initiation and amplification steps that both depend on Arrow/LRP. Dev. Biol. *306*, 94–111.

Barford, D. (2011). Structural insights into anaphase-promoting complex function and mechanism. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *366*, 3605–3624.

Barker, N. *et al.* (2010). Lgr5^{+ve} stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell Stem Cell *6*, 25–36.

Barker, N., Tan, S., and Clevers, H. (2013). Lgr proteins in epithelial stem cell biology. Development *140*, 2484–2494.

Barth, a I., Pollack, a L., Altschuler, Y., Mostov, K. E., and Nelson, W. J. (1997). NH2-terminal deletion of beta-catenin results in stable colocalization of mutant beta-catenin with adenomatous polyposis coli protein and altered MDCK cell adhesion. J. Cell Biol. *136*, 693–706.

Beelman, C. A., Stevens, A., Caponigro, G., LaGrandeur, T. E., Hatfield, L., Fortner, D. M., and Parker, R. (1996). Functional interaction of β-catenin with the transcription factor LEF-1. Nature *382*, 638–642.

Bernassola, F., Karin, M., Ciechanover, A., and Melino, G. (2008). The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell *14*, 10–21.

Bhanot, P., Brink, M., Samon, C. H., Hsieh, J.-C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the *frizzled* family from *Drosophila* functions as a Wingless receptor. Nature *382*, 225–230.

Bignell, G. R. *et al.* (2000). Identification of the familial cylindromatosis tumour-suppressor gene. Nat. Genet. *25*, 160–165.

Bilic, J., Huang, Y.-L., Davidson, G., Zimmermann, T., Cruciat, C.-M., Bienz, M., and Niehrs, C. (2007). Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. Science *316*, 1619–1622.

Binnerts, M. E. *et al.* (2007). R-Spondin1 regulates Wnt signaling by inhibiting internalization of LRP6. Proc. Natl. Acad. Sci. U. S. A. *104*, 14700–14705.

Borgal, L., Habbig, S., Hatzold, J., Liebau, M. C., Dafinger, C., Sacarea, I., Hammerschmidt, M., Benzing, T., and Schermer, B. (2012). The ciliary protein nephrocystin-4 translocates the canonical Wnt regulator Jade-1 to the nucleus to negatively regulate β-catenin signaling. J. Biol. Chem. *287*, 25370–25380.

Bos, J. L. (1997). Ras-like GTPases. Biochim. Biophys. Acta *1333*, M19–M31.

Bourhis, E., Tam, C., Franke, Y., Bazan, J. F., Ernst, J., Hwang, J., Costa, M., Cochran, A. G., and Hannoush, R. N. (2010). Reconstitution of a frizzled8-Wnt3a-LRP6 signaling complex reveals multiple Wnt and Dkk1 binding sites on LRP6. J. Biol. Chem. *285*, 9172–9179.

Bremm, A., and Komander, D. (2011). Emerging roles for Lys11-linked polyubiquitin in cellular regulation. Trends Biochem. Sci. *36*, 355–363.

Brooks, C. L. (2004). Monoubiquitination - The Signal for p53 Nuclear Export? Cell Cycle *3*, 436–438.

Cadigan, K. M. (2012). TCFs and Wnt/β-catenin signaling: more than one way to throw the switch. Curr. Top. Dev. Biol. *98*, 1–34.

Callow, M. G. *et al.* (2011). Ubiquitin ligase RNF146 regulates tankyrase and Axin to promote Wnt signaling. PLoS One *6*, 1-14.

Carmon, K., Gong, X., Lin, Q., Thomas, A., and Liu, Q. (2011). R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling. Proc. Natl. Acad. Sci. U. S. A. *108*, 11452–11457.

Chai, Y., Berke, S. S., Cohen, R. E., and Paulson, H. L. (2004). Poly-ubiquitin binding by the polyglutamine disease protein ataxin-3 links its normal function to protein surveillance pathways. J. Biol. Chem. *279*, 3605–3611.

Chan, D. W., Chan, C.-Y., Yam, J. W. P., Ching, Y.-P., and Ng, I. O. L. (2006). Prickle-1 negatively regulates Wnt/beta-catenin pathway by promoting Dishevelled ubiquitination/degradation in liver cancer. Gastroenterology *131*, 1218–1227.

Chano, T., Kontani, K., Teramoto, K., Okabe, H., and Ikegawa, S. (2002). Truncating mutations of RB1CC1 in human breast cancer. Nat. Genet. *31*, 285–288.

Chen, B. *et al.* (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat. Chem. Biol. *5*, 100–107.

Chen, J.-Z., Wang, S., Tang, R., Yanh, Q.-S., Zhao, E., Chao, Y., Ying, K., Xie, Y., and Mao, Y.-M. (2002). Cloning and identification of a cDNA that encodes a novel human protein with thrombospondin type I repeat domain, hPWTSR. Mol. Biol. Rep. *29*, 287–292.

Chen, P.-H., Chen, X., Lin, Z., Fang, D., and He, X. (2013). The structural basis of R-spondin recognition by LGR5 and RNF43. Genes Dev. *27*, 1345–1350.

Chen, S. *et al.* (2011). Structural and functional studies of LRP6 ectodomain reveal a platform for Wnt signaling. Dev. Cell *21*, 848–861.

Chen, W., ten Berge, D., Brown, J., Ahn, S., Hu, L. a, Miller, W. E., Caron, M. G., Barak, L. S., Nusse, R., and Lefkowitz, R. J. (2003). Dishevelled 2 recruits beta-arrestin 2 to mediate Wnt5A-stimulated

endocytosis of Frizzled 4. Science *301*, 1391–1394.

Chen, Z. J., and Sun, L. J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. Mol. Cell *33*, 275–286.

Chitalia, V. C., Foy, R. L., Bachschmid, M. M., Zeng, L., Maria, V., Zhou, M. I., Bharti, A., Seldin, D. C., and Lecker, S. H. (2008). Jade-1 inhibits Wnt signaling by ubiquitinating β-catenin and mediates Wnt pathway inhibition by pVHL. Nat. Cell Biol. *10*, 1208–1216.

Chitalia, V., Shivanna, S., Martorell, J., Meyer, R., Edelman, E., and Rahimi, N. (2013). c-Cbl, a ubiquitin E3 ligase that targets active β-catenin - A novel layer of Wnt regulation. J. Biol. Chem. *288*, 23505–23517.

Choi, J. D., Ryu, M., Ae Park, M., Jeong, G., and Lee, J.-S. (2013). FIP200 inhibits β-catenin-mediated transcription by promoting APC-independent β-catenin ubiquitination. Oncogene *32*, 2421–2432.

Choi, J., Park, S. Y., Costantini, F., Jho, E.-H., and Joo, C.-K. (2004). Adenomatous polyposis coli is down-regulated by the ubiquitin-proteasome pathway in a process facilitated by Axin. J. Biol. Chem. *279*, 49188–49198.

Choi, S.-C., and Han, J.-K. (2005). Rap2 is required for Wnt/β-catenin signaling pathway in *Xenopus* early development. EMBO J. *24*, 985–996.

Chopra, V. L. (1976). Effect of the Wingless Development (wg1) Mutation on Wing and Haltere in *Drosophila melanogaster*. *465*, 461–465.

Clark, C. E. J., Nourse, C. C., and Cooper, H. M. (2012). The tangled web of non-canonical Wnt signalling in neural migration. Neurosignals. *20*, 202–220.

Clevers, H. (2006). Wnt/β-catenin signaling in development and disease. Cell *127*, 469–480.

Clevers, H. (2013). The intestinal crypt, a prototype stem cell compartment. Cell *154*, 274–284.

Clevers, H., and Nusse, R. (2012). Wnt/β-catenin signaling and disease. Cell *149*, 1192–1205.

Cong, F., Schweizer, L., and Varmus, H. (2011). Casein Kinase Iε Modulates the Signaling Specificities of Dishevelled. Mol. Cell. Biol. *24*, 2000–2011.

Cruciat, C.-M., and Niehrs, C. (2013). Secreted and transmembrane wnt inhibitors and activators. Cold Spring Harb. Perspect. Biol. *5*, 1–26.

Cselenyi, C. S., Jernigan, K. K., Tahinci, E., Thorne, C. A., Lee, L. A., and Lee, E. (2008). LRP6 transduces a canonical Wnt signal independently of Axin degradation by inhibiting GSK3's phosphorylation of β-catenin. PNAS *105*.

Cullinan, S. B., Gordan, J. D., Jin, J., Wade, J., Diehl, J. A., and Harper, J. W. (2004). The Keap1-BTB Protein Is an Adaptor That Bridges Nrf2 to a Cul3-Based E3 Ligase: Oxidative Stress Sensing by a Cul3-Keap1 Ligase. Mol. Cell. Biol. *24*, 8477–8486.

Dajani, R., Fraser, E., Roe, S. M., Yeo, M., Good, V. M., Thompson, V., Dale, T. C., and Pearl, L. H. (2003). Structural basis for recruitment of glycogen synthase kinase 3β to the axin-APC scaffold complex. EMBO J. *22*, 494–501.

Dao, K.-H. T., Rotelli, M. D., Petersen, C. L., Kaech, S., Nelson, W. D., Yates, J. E., Hanlon Newell, A. E., Olson, S. B., Druker, B. J., and Bagby, G. C. (2012). FANCL ubiquitinates β-catenin and enhances its nuclear function. Blood *120*, 323–334.

Dashwood, R. H., Suzui, M., Nakagama, H., Sugimura, T., and Nagao, M. (1998). Advances in Brief High Frequency of β-Catenin (Ctnnb1) Mutations in the Colon Tumors Induced by Two Heterocyclic Amines in the F344 Rat1. Cancer Res. *58*, 1127–1129.

De, A. (2011). Wnt/Ca^{2+} signaling pathway: a brief overview. Acta Biochim Biophys Sin *43*, 745–756.

Deshaies, R. J., and Joazeiro, C. a P. (2009). RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. *78*, 399–434.

Dikic, I., Wakatsuki, S., and Walters, K. J. (2009). Ubiquitin-binding domains - from structures to functions. Nat. Rev. Mol. Cell Biol. *10*, 659–671.

Ding, Y., Zhang, Y., Xu, C., Tao, Q.-H., and Chen, Y.-G. (2013). HECT domain-containing E3 ubiquitin ligase NEDD4L negatively regulates Wnt signaling by targeting dishevelled for proteasomal degradation. J. Biol. Chem. *288*, 8289–8298.

Elbert, M., Cohen, D., and Mu, A. (2006). PAR1b Promotes Cell–Cell Adhesion and Inhibits Dishevelled-mediated Transformation of Madin-Darby Canine Kidney Cells. Mol. Cell. Biol. *17*, 3345–3355.

Fafilek, B. *et al.* (2013). Troy, a tumor necrosis factor receptor family member, interacts with lgr5 to inhibit wnt signaling in intestinal stem cells. Gastroenterology *144*, 381–391.

Faux, M. C., Coates, J. L., Catimel, B., Cody, S., Clayton, a H. a, Layton, M. J., and Burgess, a W. (2008). Recruitment of adenomatous polyposis coli and β-catenin to axin-puncta. Oncogene *27*, 5808–5820.

Fernandez, P. C., Frank, S. R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003). Genomic targets of the human c-Myc protein. Genes Dev. *17*, 1115–1129.

Van der Flier, L. G. *et al.* (2007). The Intestinal Wnt/TCF Signature. Gastroenterology *132*, 628–632.

Van der Flier, L. G. *et al.* (2009). Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell *136*, 903–912.

Forde, J. E., and Dale, T. C. (2007). Glycogen synthase kinase 3: a key regulator of cellular fate. Cell. Mol. Life Sci. *64*, 1930–1944.

Fuchs, S. Y., Spiegelman, V. S., and Kumar, K. G. S. (2004). The many faces of β-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. Oncogene *23*, 2028–2036.

Furukawa, M., He, Y. J., Borchers, C., and Xiong, Y. (2003). Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases. Nat. Cell Biol. *5*, 1001–1007.

Furukawa, M., and Xiong, Y. (2005). BTB Protein Keap1 Targets Antioxidant Transcription Factor Nrf2 for Ubiquitination by the Cullin 3-Roc1 Ligase. Mol. Cell. Biol. *25*, 162–171.

Gagliardi, M., Piddini, E., and Vincent, J.-P. (2008). Endocytosis: a positive or a negative influence on Wnt signalling? Traffic *9*, 1–9.

Ganner, A. *et al.* (2009). Regulation of ciliary polarity by the APC/C. PNAS *106*, 17799–17804.

Gao, C. *et al.* (2010). Autophagy negatively regulates Wnt signalling by promoting Dishevelled degradation. Nat. Cell Biol. *12*, 781–790.

Gao, Z.-H., Seeling, J. M., Hill, V., Yochum, A., and Virshup, D. M. (2002). Casein kinase I phosphorylates and destabilizes the β-catenin degradation complex. Proc. Natl. Acad. Sci. U. S. A. *99*, 1182–1187.

Glinka, A., Dolde, C., Kirsch, N., Huang, Y.-L., Kazanskaya, O., Ingelfinger, D., Boutros, M., Cruciat, C.-M., and Niehrs, C. (2011). LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signalling. EMBO Rep. *12*, 1055–1061.

Goldstein, G., Scheid, M., Hammerling, U., Schlesinger, D. H., Niall, H. D., and Boyse, E. A. (1975). Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proc. Natl. Acad. Sci. U. S. A. *72*, 11–15.

Gönczy, P. (2004). Myosin assembly: the power of multiubiquitylation. Cell *118*, 272–274.

González-sancho, J. M., Brennan, K. R., Leslie, A., Brown, A. M. C., and Castelo-soccio, L. A. (2004). Wnt Proteins Induce Dishevelled Independent Mechanism, Irrespective of Their Ability To Stabilize β -Catenin.

Gounari, F., Chang, R., Cowan, J., Guo, Z., Dose, M., Gounaris, E., and Khazaie, K. (2005). Loss of adenomatous polyposis coli gene function disrupts thymic development. Nat. Immunol. *6*, 800–809.

Grandori, C., Cowley, S. M., James, L. P., and Eisenman, R. N. (2000). The Myc/Max/Mad Network and the Transcriptional Control of Cell Behavior. Annu. Rev. Cell Dev. Biol. *16*, 653–699.

Grossman, S. R., Deato, M. E., Brignone, C., Chan, H. M., Kung, A. L., Tagami, H., Nakatani, Y., and Livingston, D. M. (2003). Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science *300*, 342–344.

Haglund, K., and Dikic, I. (2005). Ubiquitylation and cell signaling. EMBO J. *24*, 3353–3359.

Haglund, K., Di Fiore, P. P., and Dikic, I. (2003a). Distinct monoubiquitin signals in receptor endocytosis. Trends Biochem. Sci. *28*, 598–603.

Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003b). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat. Cell Biol. *5*, 461–466.

Haigis, K. M., Hoff, P. D., White, A., Shoemaker, A. R., Halberg, R. B., and Dove, W. F. (2004). Tumor regionality in the mouse intestine reflects the mechanism of loss of Apc function. Proc. Natl. Acad. Sci. U. S. A. *101*, 9769–9773.

Hämmerlein, a, Weiske, J., and Huber, O. (2005). A second protein kinase CK1-mediated step negatively regulates Wnt signalling by disrupting the lymphocyte enhancer factor-1/β-catenin complex. Cell. Mol. Life Sci. *62*, 606–618.

Hao, H.-X. *et al.* (2012). ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. Nature *485*, 195–200.

Harada, N., Miyoshi, H., and Murai, N. (2002). Lack of Tumorigenesis in the Mouse Liver after Adenovirus-mediated Expression of a Dominant Stable Mutant of β-Catenin. Cancer Res. *62*, 1971–1977.

Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B., and Polakis, P. (1998). Downregulation of β-catenin by human Axin and its association with the APC tumor suppressor, β-catenin and GSK3β. Curr. Biol. *8*, 573–581.

Harterink, M., and Korswagen, H. C. (2012). Dissecting the Wnt secretion pathway: key questions on the modification and intracellular trafficking of Wnt proteins. Acta Physiol. *204*, 8–16.

Hatzis, P. *et al.* (2008). Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. Mol. Cell. Biol. *28*, 2732–2744.

Hay, R. T. (2005). SUMO: a history of modification. Mol. Cell *18*, 1–12.

Hay-Koren, A., Caspi, M., Zilberberg, A., and Rosin-Arbesfeld, R. (2011). The EDD E3 ubiquitin ligase ubiquitinates and up-regulates β-catenin. Mol. Biol. Cell *22*, 399–411.

He, X., Semenov, M., Tamai, K., and Zeng, X. (2004). LDL receptor-related proteins 5 and 6 in Wnt/β-catenin signaling: arrows point the way. Development *131*, 1663–1677.

Hebert, D. N., and Molinari, M. (2007). In and Out of the ER: Protein Folding, Quality Control, Degradation, and Related Human Diseases. Physiol. Rev. *87*, 1377–1408.

Hernández, A. R., Klein, A. M., and Kirschner, M. W. (2012). Kinetic responses of β-catenin specify the sites of Wnt control. Science *338*, 1337–1340.

Hernández-Muñoz, I., Lund, A. H., van der Stoop, P., Boutsma, E., Muijrers, I., Verhoeven, E., Nusinow, D. A., Panning, B., Marahrens, Y., and van Lohuizen, M. (2005). Stable X chromosome inactivation involves the PRC1 Polycomb complex and requires histone MACROH2A1 and the CULLIN3/SPOP ubiquitin E3 ligase. Proc. Natl. Acad. Sci. U. S. A. *102*, 7635–7640.

Hicke, L. (2001). Protein regulation by monoubiquitin. Nat. Rev. Mol. Cell Biol. *2*, 195–201.

Hicke, L., and Dunn, R. (2003). Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. *19*, 141–172.

Hoege, C., Pfander, B., Moldovan, G.-L., Pyrowolakis, G., and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature *419*, 135–141.

Holland, J. D., Klaus, A., Garratt, A. N., and Birchmeier, W. (2013). Wnt signaling in stem and cancer stem cells. Curr. Opin. Cell Biol. *25*, 254–264.

Hoppe, T. (2005). Multiubiquitylation by E4 enzymes: "one size" doesn't fit all. Trends Biochem. Sci. *30*, 183–187.

Hoppe, T., Cassata, G., Barral, J. M., Springer, W., Hutagalung, A. H., Epstein, H. F., and Baumeister, R. (2004). Regulation of the myosin-directed chaperone UNC-45 by a novel E3/E4-multiubiquitylation complex in *C. elegans*. Cell *118*, 337–349.

Hsieh, J.-C., Rattner, A., Smallwood, P. M., and Nathans, J. (1999). Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein. Proc. Natl. Acad. Sci. U. S. A. *96*, 3546–3551.

Hu, T., Li, C., Cao, Z., Van Raay, T. J., Smith, J. G., Willert, K., Solnica-Krezel, L., and Coffey, R. J. (2010). Myristoylated Naked2 antagonizes Wnt-β-catenin activity by degrading Dishevelled-1 at the plasma membrane. J. Biol. Chem. *285*, 13561–13568.

Huang, S.-M. A. *et al.* (2009a). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature *461*, 614–620.

Huang, X., Langelotz, C., Hetfeld-Pechoc, B. K. J., Schwenk, W., and Dubiel, W. (2009b). The COP9 signalosome mediates β-catenin degradation by deneddylation and blocks adenomatous polyposis coli destruction via USP15. J. Mol. Biol. *391*, 691–702.

Ikeda, F., and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. EMBO Rep. *9*, 536–542.

Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin. EMBO J. *17*, 1371–1384.

Ivanov, I., Lo, K. C., Hawthorn, L., Cowell, J. K., and Ionov, Y. (2007). Identifying candidate colon cancer tumor suppressor genes using inhibition of nonsense-mediated mRNA decay in colon cancer cells. Oncogene *26*, 2873–2884.

Iwai, K., and Tokunaga, F. (2009). Linear polyubiquitination: a new regulator of NF-κB activation. EMBO Rep. *10*, 706–713.

Iwao, K., Nakamori, S., Kameyama, M., Imaoka, S., Kinoshita, M., Fukui, T., Ishiguro, S., Nakamura, Y., and Miyoshi, Y. (1998). Activation of the β-Catenin Gene by Interstitial Deletions Involving Exon 3 in Primary Colorectal Carcinomas without Adenomatous Polyposis Coli Mutations. Cancer Res. *58*, 1021–1026.

Jaks, V., Barker, N., Kasper, M., van Es, J. H., Snippert, H. J., Clevers, H., and Toftgård, R. (2008). Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat. Genet. *40*, 1291–1299.

Jeong, W.-J., Yoon, J., Park, J.-C., Lee, S.-H., Lee, S.-H., Kaduwal, S., Kim, H., Yoon, J.-B., and Choi, K.-Y. (2012). Ras stabilization through aberrant activation of Wnt/β-catenin signaling promotes intestinal tumorigenesis. Sci. Signal. *5*, 1–13.

Jernigan, K. K. *et al.* (2011). Gβγ Activates GSK3 to Promote LRP6-Mediated β-Catenin Transcriptional Activity. Sci. Signal. *3*, 1–19.

Jiang, X. *et al.* (2013). Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. Proc. Natl. Acad. Sci. U. S. A. *110*, 12649–12654.

Jung, H. *et al.* (2013). Deubiquitination of Dishevelled by Usp14 is required for Wnt signaling. Oncogenesis *2*, 1–11.

Jura, N., Scotto-Lavino, E., Sobczyk, A., and Bar-Sagi, D. (2006). Differential modification of Ras proteins by ubiquitination. Mol. Cell *21*, 679–687.

Kamata, Y., Katsube, K., Michikawa, M., Yamada, M., Takada, S., and Mizusawa, H. (2004). R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnts mutants. Biochim. Biophys. Acta *1676*, 51–62.

Katanaev, V. L., Ponzielli, R., Sémériva, M., and Tomlinson, A. (2005). Trimeric G protein-dependent frizzled signaling in *Drosophila*. Cell *120*, 111–122.

Kazanskaya, O., Glinka, A., del Barco Barrantes, I., Stannek, P., Niehrs, C., and Wu, W. (2004). R-Spondin2 is a secreted activator of Wnt/β-catenin signaling and is required for *Xenopus* myogenesis. Dev. Cell *7*, 525–534.

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu. Rev. Cell Dev. Biol. *22*, 159–180.

Kim, I., Mi, K., and Rao, H. (2004). Multiple Interactions of Rad23 Suggest a Mechanism for Ubiquitylated Substrate Delivery Important in Proteolysis. Mol. Biol. Cell *15*, 3357–3365.

Kim, M. J., Chia, I. V, and Costantini, F. (2008). SUMOylation target sites at the C terminus protect Axin from ubiquitination and confer protein stability. FASEB J. *22*, 3785–3794.

Kim, S., and Jho, E. (2010). The protein stability of Axin, a negative regulator of Wnt signaling, is regulated by Smad ubiquitination regulatory factor 2 (Smurf2). J. Biol. Chem. *285*, 36420–36426.

Kim, S.-E., Yoon, J.-Y., Jeong, W.-J., Jeon, S.-H., Park, Y., Yoon, J.-B., Park, Y. N., Kim, H., and Choi, K.-Y. (2009). H-Ras is degraded by Wnt/β-catenin signaling via β-TrCP-mediated polyubiquitylation. J. Cell Sci. *122*, 842–848.

Kimura, Y., and Tanaka, K. (2010). Regulatory mechanisms involved in the control of ubiquitin homeostasis. J. Biochem. *147*, 793–798.

Kinzler, K. W., Nilbert, M. C., Su, L. K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, a C., Hedge, P., and McKechnie, D. (1991). Identification of FAP locus genes from chromosome 5q21. Science *253*, 661–665.

Kirisako, T., Kamei, K., Murata, S., Kato, M., Fukumoto, H., Kanie, M., Sano, S., Tokunaga, F., Tanaka, K., and Iwai, K. (2006). A ubiquitin ligase complex assembles linear polyubiquitin chains. EMBO J. *25*, 4877–4887.

Kishida, M., Hino Si, Michiue, T., Yamamoto, H., Kishida, S., Fuki, A., Asashima, M., and Kikuchi, A. (2001). Synergistic activation of the Wnt signaling pathway by Dvl and casein kinase Iε. J. Biol. Chem. *276*, 33147–33155.

Kitagawa, K., Hiramatsu, Y., Uchida, C., Isobe, T., Hattori, T., Oda, T., Shibata, K., Nakamura, S., Kikuchi, a, and Kitagawa, M. (2009). Fbw7 promotes ubiquitin-dependent degradation of c-Myb: involvement of GSK3-mediated phosphorylation of Thr-572 in mouse c-Myb. Oncogene *28*, 2393–2405.

Klingensmith, J., Nusse, R., and Perrimon, N. (1994). The *Drosophila* segment polarity gene dishevelled encodes a novel protein required for response to the wingless signal. Genes Dev. *8*, 118–130.

Knippschild, U., Gocht, A., Wolff, S., Huber, N., Löhler, J., and Stöter, M. (2005). The casein kinase 1 family: participation in multiple cellular processes in eukaryotes. Cell. Signal. *17*, 675–689.

Kobayashi, A., Kang, M., Okawa, H., Zenke, Y., Chiba, T., Igarashi, K., and Ohtsuji, M. (2004). Oxidative Stress Sensor Keap1 Functions as an Adaptor for Cul3-Based E3 Ligase To Regulate Proteasomal Degradation of Nrf2. Mol. Cell. Biol. *24*, 7130–7139.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635–644.

Kofron, M., Birsoy, B., Houston, D., Tao, Q., Wylie, C., and Heasman, J. (2007). Wnt11/β-catenin signaling in both oocytes and early embryos acts through LRP6-mediated regulation of axin. Development *134*, 503–513.

Koinuma, D. *et al.* (2011). RB1CC1 protein positively regulates transforming growth factor-β signaling through the modulation of Arkadia E3 ubiquitin ligase activity. J. Biol. Chem. *286*, 32502–32512.

Komander, D., Reyes-Turcu, F., Licchesi, J. D. F., Odenwaelder, P., Wilkinson, K. D., and Barford, D. (2009). Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. EMBO Rep. *10*, 466–473.

Koo, B.-K. *et al.* (2012). Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. Nature *488*, 665–669.

Krek, W. (2003). BTB proteins as henchmen of Cul3-based ubiquitin ligases. Nat. Cell Biol. *5*, 950–951.

Kulathu, Y., and Komander, D. (2012). Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. Nat. Rev. Mol. Cell Biol. *13*, 508–523.

Kwan, H. T., Chan, D. W., Cai, P. C. H., Mak, C. S. L., Yung, M. M. H., Leung, T. H. Y., Wong, O. G. W., Cheung, A. N. Y., and Ngan, H. Y. S. (2013). AMPK activators suppress cervical cancer cell growth through inhibition of DVL3 mediated Wnt/β-catenin signaling activity. PLoS One *8*, 1–10.

De La Coste, A. *et al.* (1998). Somatic mutations of the β-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proc. Natl. Acad. Sci. U. S. A. *95*, 8847–8851.

Lai, S.-L., Chien, A. J., and Moon, R. T. (2009). Wnt/Fz signaling and the cytoskeleton: potential roles in tumorigenesis. Cell Res. *19*, 532–545.

De Lau, W. *et al.* (2011). Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature *476*, 293–297.

Lee, E., Salic, A., and Kirschner, M. W. (2001). Physiological regulation of β-catenin stability by Tcf3 and CK1ε. J. Cell Biol. *154*, 983–993.

Lee, E., Salic, A., Krüger, R., Heinrich, R., and Kirschner, M. W. (2003). The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. PLoS Biol. *1*, 116–132.

Lee, Y.-N., Gao, Y., and Wang, H.-Y. (2008). Differential mediation of the Wnt canonical pathway by mammalian Dishevelleds-1, -2, and -3. Cell. Signal. *20*, 443–452.

Li, V. S. W. *et al.* (2012). Wnt signaling through inhibition of β-catenin degradation in an intact Axin1 complex. Cell *149*, 1245–1256.

Li, W., Bengtson, M. H., Ulbrich, A., Matsuda, A., Reddy, V. A.,

Orth, A., Chanda, S. K., and Batalov, S. (2008). Genome-Wide and Functional Annotation of Human E3 Ubiquitin Ligases Identifies MULAN, a Mitochondrial E3 that Regulates the Organelle's Dynamics and Signaling. PLoS One *1*, 1–14.

Licchesi, J. D. F. *et al.* (2012). An ankyrin-repeat ubiquitin-binding domain determines TRABID's specificity for atypical ubiquitin chains. Nat. Struct. Mol. Biol. *19*, 62–71.

Lipkowitz, S., and Weissman, A. M. (2011). RINGs of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. Nat. Rev. Cancer *11*, 629–643.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., Zhang, Z., Lin, X., and He, X. (2002). Control of β-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell *108*, 837–847.

Liu, J., Stevens, J., Rote, C. a, Yost, H. J., Hu, Y., Neufeld, K. L., White, R. L., and Matsunami, N. (2001). Siah-1 mediates a novel β-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. Mol. Cell *7*, 927–936.

Liu, S. *et al.* (2008). Homozygous deletion of glycogen synthase kinase 3β bypasses senescence allowing Ras transformation of primary murine fibroblasts. Proc. Natl. Acad. Sci. U. S. A. *105*, 5248–5253.

Liu, X., Rubin, J. S., and Kimmel, A. R. (2005). Rapid, Wnt-induced changes in GSK3β associations that regulate β-catenin stabilization are mediated by Gα proteins. Curr. Biol. *15*, 1989–1997.

Lorenowicz, M. J., and Korswagen, H. C. (2009). Sailing with the Wnt: charting the Wnt processing and secretion route. Exp. Cell Res. *315*, 2683–2689.

Lu, W., Yamamoto, V., Ortega, B., and Baltimore, D. (2004). Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. Cell *119*, 97–108.

Lui, T. T. H., Lacroix, C., Ahmed, S. M., Goldenberg, S. J., Leach, C. A., Daulat, A. M., and Angers, S. (2011). The ubiquitin-specific protease USP34 regulates axin stability and Wnt/β-catenin signaling. Mol. Cell. Biol. *31*, 2053–2065.

MacDonald, B. T., Tamai, K., and He, X. (2009). Wnt/β-catenin signaling: components, mechanisms, and diseases. Dev. Cell *17*, 9–26.

Madshus, I. H., and Stang, E. (2009). Internalization and intracellular sorting of the EGF receptor: a model for understanding the mechanisms of receptor trafficking. J. Cell Sci. *122*, 3433–3439.

Malempati, S., Tibbitts, D., Cunningham, M., Akkari, Y., Olson, S., Fan, G., and Sears, R. C. (2006). Aberrant stabilization of c-Myc protein in some lymphoblastic leukemias. Leukemia *20*, 1572–1581.

Malynn, B. a, and Ma, A. (2009). A20 takes on tumors: tumor suppression by an ubiquitin-editing enzyme. J. Exp. Med. *206*, 977–980.

Mao, J. *et al.* (2001). Low-Density Lipoprotein Receptor-Related Protein-5 Binds to Axin and Regulates the Canonical Wnt Signaling Pathway. Mol. Cell *7*, 801–809.

March, H. N. *et al.* (2012). Europe PMC Funders Group Insertional mutagenesis identifies multiple networks of co-operating genes driving intestinal tumorigenesis. Nat. Genet. *43*, 1202–1209.

El Marjou, F., Janssen, K.-P., Chang, B. H.-J., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., and Robine, S. (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis *39*, 186–193.

Matsuzawa, S. I., and Reed, J. C. (2001). Siah-1, SIP, and Ebi collaborate in a novel pathway for β-catenin degradation linked to p53 responses. Mol. Cell *7*, 915–926.

Melkoumian, Z. K., Peng, X., Gan, B., Wu, X., and Guan, J.-L. (2005). Mechanism of cell cycle regulation by FIP200 in human breast cancer cells. Cancer Res. *65*, 6676–6684.

Metcalfe, C., Mendoza-Topaz, C., Mieszczanek, J., and Bienz, M. (2010). Stability elements in the LRP6 cytoplasmic tail confer efficient signalling upon DIX-dependent polymerization. J. Cell Sci. *123*, 1588–1599.

Mi, K., and Johnson, G. V. W. (2005). Role of the intracellular domains of LRP5 and LRP6 in activating the Wnt canonical pathway. J. Cell. Biochem. *95*, 328–338.

Miyazaki, K. *et al.* (2004). NEDL1, a novel ubiquitin-protein isopeptide ligase for dishevelled-1, targets mutant superoxide dismutase-1. J. Biol. Chem. *279*, 11327–11335.

Moldovan, G.-L., and D'Andrea, A. D. (2009). How the fanconi anemia pathway guards the genome. Annu. Rev. Genet. *43*, 223–249.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destrée, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates β-catenin-induced axis formation in Xenopus embryos. Cell *86*, 391–399.

Morgan, D., Eley, L., Sayer, J., Strachan, T., Yates, L. M., Craighead, A. S., and Goodship, J. A. (2002). Expression analyses and interaction with the anaphase promoting complex protein Apc2 suggest a role for inversin in primary cilia and involvement in the cell cycle. Hum. Mol. Genet. *11*, 3345–3350.

Mukai, A., Yamamoto-Hino, M., Awano, W., Watanabe, W., Komada, M., and Goto, S. (2010). Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt. EMBO J. *29*, 2114–2125.

Mukai, A., Yamamoto-Hino, M., Komada, M., Okano, H., and Goto, S. (2012). Balanced ubiquitination determines cellular responsiveness to extracellular stimuli. Cell. Mol. Life Sci., 4007–4016.

Mukhopadhyay, D., and Riezman, H. (2007). Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science *315*, 201–205.

Munemitsu, S., Albert, I., Rubinfeld, B., and Polakis, P. (1996). Deletion of an amino-terminal sequence β-catenin in vivo and promotes hyperphosporylation of the adenomatous polyposis coli tumor suppressor protein. Mol. Cell. Biol. *16*, 4088–4094.

Nethe, M. *et al.* (2012). Rac1 acts in conjunction with Nedd4 and dishevelled-1 to promote maturation of cell-cell contacts. J. Cell Sci. *125*, 3430–3442.

Niehrs, C. (2010). On growth and form: a Cartesian coordinate system of Wnt and BMP signaling specifies bilaterian body axes. Development *137*, 845–857.

Niehrs, C. (2012). The complex world of WNT receptor signalling. Nat. Rev. Mol. Cell Biol. *13*, 767–779.

Nijman, S. M. B., Luna-Vargas, M. P. A., Velds, A., Brummelkamp, T. R., Dirac, A. M. G., Sixma, T. K., and Bernards, R. (2005). A genomic and functional inventory of deubiquitinating enzymes. Cell *123*, 773–786.

Nishisho, I. *et al.* (2013). Mutations of Chromosome 5q21 Genes in FAP and Colorectal Cancer Patients. Science (80-.). *253*, 665–669.

Nusse, R., and Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell *31*, 99–109.

Nüsslein-volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. Nature *287*, 795–801.

Ohshima, R., Ohta, T., Wu, W., Koike, A., Iwatani, T., Henderson, M., Watts, C. K. W., and Otsubo, T. (2007). Putative tumor suppressor EDD

interacts with and up-regulates APC. Genes Cells *12*, 1339–1345.

Ong, C. K. *et al.* (2012). Exome sequencing of liver fluke-associated cholangiocarcinoma. Nat. Genet. *44*, 690–693.

Otto, E. A. *et al.* (2003). Mutations in INVS encoding inversin cause nephronophthisis type 2, linking renal cystic disease to the function of primary cilia and left-right axis determination. *34*, 413–420.

Pai, L. M., Orsulic, S., Bejsovec, A., and Peifer, M. (1997). Negative regulation of Armadillo, a Wingless effector in *Drosophila*. Development *124*, 2255–2266.

Pan, W. *et al.* (2008). Wnt3a-mediated formation of phosphatidylinositol 4,5-bisphosphate regulates LRP6 phosphorylation. Science *321*, 1350–1353.

Park, D.-S., Seo, J.-H., Hong, M., and Choi, S.-C. (2013). Role of the Rap2/TNIK kinase pathway in regulation of LRP6 stability for Wnt signaling. Biochem. Biophys. Res. Commun. *436*, 338–343.

Patel, J. H., Loboda, A. P., Showe, M. K., Showe, L. C., and McMahon, S. B. (2004). Analysis of genomic targets reveals complex functions of MYC. Nat. Rev. Cancer *4*, 562–568.

Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003). A proteomics approach to understanding protein ubiquitination. Nat. Biotechnol. *21*, 921–926.

Peng, W. C., de Lau, W., Forneris, F., Granneman, J. C. M., Huch, M., Clevers, H., and Gros, P. (2013). Structure of stem cell growth factor R-spondin 1 in complex with the ectodomain of its receptor LGR5. Cell Rep. *3*, 1885–1892.

Peters, J. M., McKay, R. M., McKay, J. P., and Graff, J. M. (1999). Casein kinase I transduces Wnt signals. Nature *401*, 345–350.

Peters, J.-M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat. Rev. Mol. Cell Biol. *7*, 644–656.

Petroski, M. D., and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. Nat. Rev. Mol. Cell Biol. *6*, 9–20.

Phillips, B. T., and Kimble, J. (2009). A new look at TCF and β-catenin through the lens of a divergent *C. elegans* Wnt pathway. Dev. Cell *17*, 27–34.

Pickart, C. M., and Eddins, M. J. (2004). Ubiquitin: structures, functions, mechanisms. Biochim. Biophys. Acta *1695*, 55–72.

Pinson, K. I., Brennman, J., Monkley, S., Avery, B. J., and Skarnes, W. C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. Nature *407*, 535–538.

Pintard, L. *et al.* (2003). The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. Nature *425*, 311–316.

Polakis, P. (1999). The oncogenic activation of β-catenin. Curr. Opin. Genet. Dev. *9*, 15–21.

Polakis, P. (2012). Drugging Wnt signalling in cancer. EMBO J. *31*, 2737–2746.

Popov, N., Schülein, C., Jaenicke, L. a, and Eilers, M. (2010). Ubiquitylation of the amino terminus of Myc by SCF(β-TrCP) antagonizes SCF(Fbw7)-mediated turnover. Nat. Cell Biol. *12*, 973–981.

Port, F., and Basler, K. (2010). Wnt trafficking: new insights into Wnt maturation, secretion and spreading. Traffic *11*, 1265–1271.

Price, M. A. (2006). CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. Genes Dev. *20*, 399–410.

Pylayeva-Gupta, Y., Grabocka, E., and Bar-Sagi, D. (2011). RAS oncogenes: weaving a tumorigenic web. Nat. Rev. Cancer *11*, 761–774.

Van Raay, T. J., Fortino, N. J., Miller, B. W., Ma, H., Lau, G., Li, C., Franklin, J. L., Attisano, L., Solnica-Krezel, L., and Coffey, R. J. (2011). Naked1 antagonizes Wnt signaling by preventing nuclear accumulation of β-catenin. PLoS One *6*, 1–12.

Reyes-Turcu, F. E., Ventii, K. H., and Wilkinson, K. D. (2009). Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu. Rev. Biochem. *78*, 363–397.

Riggleman, B., Wieschaus, E., and Schedl, P. (1989). Molecular analysis of the *armadillo* locus: uniformly distributed transcripts and a protein with novel internal repeats are associated with a *Drosophila* segment polarity gene. Genes Dev. *3*, 96–113.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., and Nusse, R. (1987). The *Drosophila* homolog of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. Cell *50*, 649–657.

Rivkin, E. *et al.* (2013). The linear ubiquitin-specific deubiquitinase gumby regulates angiogenesis. Nature *498*, 318–324.

Rothbächer, U., Laurent, M. N., Deardorff, M. A., Klein, P. S., Cho, K. W., and Fraser, S. E. (2000). Dishevelled phosphorylation, subcellular localization and multimerization regulate its role in early embryogenesis. EMBO J. *19*, 1010–1022.

Rotin, D., and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nat. Rev. Mol. Cell Biol. *10*, 398–409.

Rubinfeld, B. (1997). Stabilization of β-Catenin by Genetic Defects in Melanoma Cell Lines. Science *275*, 1790–1792.

Ryland, G. L., Hunter, S. M., Doyle, M. a, Rowley, S. M., Christie, M., Allan, P. E., Bowtell, D. D. L., Gorringe, K. L., and Campbell, I. G. (2013). RNF43 is a tumour suppressor gene mutated in mucinous tumours of the ovary. J. Pathol. *229*, 469–476.

Ryu, K.-Y., Maehr, R., Gilchrist, C. a, Long, M. a, Bouley, D. M., Mueller, B., Ploegh, H. L., and Kopito, R. R. (2007). The mouse polyubiquitin gene UbC is essential for fetal liver development, cell-cycle progression and stress tolerance. EMBO J. *26*, 2693–2706.

Saito-Diaz, K., Chen, T. W., Wang, X., Thorne, C. A., Wallace, H. A., Page-McCaw, A., and Lee, E. (2013). The way Wnt works: components and mechanism. Growth Factors *31*, 1–31.

Sakanaka, C., Leong, P., Xu, L., Harrison, S. D., and Williams, L. T. (1999). Casein kinase Iε in the wnt pathway: regulation of β-catenin function. Proc. Natl. Acad. Sci. U. S. A. *96*, 12548–12552.

Saksena, S., Sun, J., Chu, T., and Emr, S. D. (2007). ESCRTing proteins in the endocytic pathway. Trends Biochem. Sci. *32*, 561–573.

Samowitz, W. S., Powers, M. D., Spirio, L. N., Nollet, F., Roy, F. Van, and Slattery, M. L. (1999). β-Catenin Mutations Are More Frequent in Small Colorectal Adenomas Than in Larger Adenomas and Invasive Carcinomas. Cancer Res. *59*, 1442–1444.

Sato, T. *et al.* (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature *459*, 262–265.

Schneider, I., Schneider, P. N., Derry, S. W., Lin, S., Barton, L. J., Westfall, T., and Slusarski, D. C. (2010). Zebrafish Nkd1 promotes Dvl degradation and is required for left-right patterning. Dev. Biol. *348*, 22–33.

Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P. J. G., Kikuchi, A., Higuchi, Y., and Bienz, M. (2007). The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. Nat. Struct. Mol. Biol. *14*, 484–492.

Semënov, M. V, and Snyder, M. (1997). Human *dishevelled* genes constitute a DHR-containing multigene family. Genomics *42*, 302–310.

Shao, L., Oshima, S., Duong, B., Advincula, R., Barrera, J., Malynn, B. A., and Ma, A. (2013). A20 restricts wnt signaling in intestinal

epithelial cells and suppresses colon carcinogenesis. PLoS One *8*, 1–7.

Shao, M., Liu, Z.-Z., Wang, C.-D., Li, H.-Y., Carron, C., Zhang, H.-W., and Shi, D.-L. (2009). Down syndrome critical region protein 5 regulates membrane localization of Wnt receptors, Dishevelled stability and convergent extension in vertebrate embryos. Development *136*, 2121–2131.

Sharma, J., Mulherkar, S., Mukherjee, D., and Jana, N. R. (2012). Malin regulates Wnt signaling pathway through degradation of dishevelled2. J. Biol. Chem. *287*, 6830–6839.

Shi, D., Pop, M. S., Kulikov, R., Love, I. M., Kung, A. L., Kung, A., and Grossman, S. R. (2009). CBP and p300 are cytoplasmic E4 polyubiquitin ligases for p53. Proc. Natl. Acad. Sci. U. S. A. *106*, 16275–16280.

Siegfried, E., Chou, T. B., and Perrimon, N. (1992). *wingless* signaling acts through *zeste-white 3*, the *Drosophila* homolog of *glycogen synthase kinase-3*, to regulate engrailed and establish cell fate. Cell *71*, 1167–1179.

Simons, M. *et al.* (2005). Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways. Nat. Genet. *37*, 537–543.

Simons, M. *et al.* (2009). Electrochemical cues regulate assembly of the Frizzled/Dishevelled complex at the plasma membrane during planar epithelial polarization. Nat. Cell Biol. *11*, 286–294.

Simons, M., and Mlodzik, M. (2008). Planar cell polarity signaling: from fly development to human disease. Annu. Rev. Genet. *42*, 517–540.

Sims, J. J., and Cohen, R. E. (2009). Linkage-specific avidity defines the lysine 63-linked polyubiquitin-binding preference of rap80. Mol. Cell *33*, 775–783.

Snippert, H. J. *et al.* (2010). Lgr6 Marks Stem Cells in the Hair Follicle That Generate All Cell Lineages of the Skin. Science *327*, 1385–1389.

Sparks, A. B., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Mutational Analysis of the APC/β-Catenin/Tcf Pathway in Colorectal Cancer. Cancer Res., 1130–1134.

Spence, J., Sadis, S., Haas, A. L., Finley, D., Spence, J., Sadis, S., and Haas, A. L. (1995). A Ubiquitin Mutant with Specific Defects in DNA Repair and Multiubiquitination. Mol. Cell. Biol. *15*, 1265–1273.

Spink, K. E., Polakis, P., and Weis, W. I. (2000). Structural basis of the Axin-adenomatous polyposis coli interaction. EMBO J. *19*, 2270–2279.

Su, Y., Fu, C., Ishikawa, S., Stella, A., Kojima, M., Shitoh, K., Schreiber, E. M., Day, B. W., and Liu, B. (2008). APC is essential for targeting phosphorylated β-catenin to the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase. Mol. Cell *32*, 652–661.

Sussman, D. J., Klingensmith, J., Salinas, P., Adams, P. S., Nusse, R., and Perrimon, N. (1994). Isolation and Characterization of a Mouse Homolog of the *Drosophila* Segment Polarity Gene dishevelled. Dev. Biol. *166*, 638–642.

Swiatek, W., Tsai, I.-C., Klimowski, L., Pepler, A., Barnette, J., Yost, H. J., and Virshup, D. M. (2004). Regulation of casein kinase Iε activity by Wnt signaling. J. Biol. Chem. *279*, 13011–13017.

Takahashi, M., Sugimura, T., and Wakabayashi, K. (1998). β-Catenin Is Frequently Mutated and Demonstrates Altered Cellular Location in Azoxymethane-induced Rat Colon Tumors. Cancer Res. *58*, 42–46.

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J.-P., and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. Nature *407*, 530–535.

Tauriello, D. V. F. *et al.* (2012). Wnt/β-catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. Proc. Natl. Acad. Sci. U. S. A. *109*, 812–820.

Tauriello, D. V. F., Haegebarth, A., Kuper, I., Edelmann, M. J., Henraat, M., Canninga-van Dijk, M. R., Kessler, B. M., Clevers, H., and Maurice, M. M. (2010). Loss of the tumor suppressor CYLD enhances Wnt/β-catenin signaling through K63-linked ubiquitination of Dvl. Mol. Cell *37*, 607–619.

Tauriello, D. V. F., and Maurice, M. M. (2010). The various roles of ubiquitin in Wnt pathway regulation. Cell Cycle *9*, 3700–3709.

Tolwinski, N. S., Wehrli, M., Rives, A., Erdeniz, N., Dinardo, S., and Wieschaus, E. (2003). Wg/Wnt Signal Can Be Transmitted through Arrow/LRP5,6 and Axin Independently of Zw3/Gsk3β Activity. Dev. Cell *4*, 407–418.

Tran, H. *et al.* (2013). HectD1 E3 ligase modifies adenomatous polyposis coli (APC) with polyubiquitin to promote the APC-axin interaction. J. Biol. Chem. *288*, 3753–3767.

Tran, H., Hamada, F., Schwarz-Romond, T., and Bienz, M. (2008). Trabid, a new positive regulator of Wnt-induced transcription with preference for binding and cleaving K63-linked ubiquitin chains. Genes Dev. *22*, 528–542.

Tran, H., and Polakis, P. (2012). Reversible modification of adenomatous polyposis coli (APC) with K63-linked polyubiquitin regulates the assembly and activity of the β-catenin destruction complex. J. Biol. Chem. *287*, 28552–28563.

Umbhauer, M., Djiane, A., Goisset, C., Penzo-Méndez, a, Riou, J. F., Boucaut, J. C., and Shi, D. L. (2000). The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/β-catenin signalling. EMBO J. *19*, 4944–4954.

Varshavsky, A. (2006). The early history of the ubiquitin field. Protein Sci. *15*, 647–654.

Verma, R., Oania, R., Graumann, J., and Deshaies, R. J. (2004). Multiubiquitin Chain Receptors Define a Layer of Substrate Selectivity in the Ubiquitin-Proteasome System. Cell *118*, 99–110.

Wang, D., Huang, B., Zhang, S., Yu, X., Wu, W., and Wang, X. (2013). Structural basis for R-spondin recognition by LGR4/5/6 receptors. Genes Dev. *27*, 1339–1344.

Watanabe, T., Sato, K., and Kaibuchi, K. (2009). Cadherin-mediated intercellular adhesion and signaling cascades involving small GTPases. Cold Spring Harb. Perspect. Biol. *1*, 1–13.

Wehrli, M., Dougan, S. T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, a, and DiNardo, S. (2000). *arrow* encodes an LDL-receptor-related protein essential for Wingless signalling. Nature *407*, 527–530.

Wei, W., Li, M., Wang, J., Nie, F., and Li, L. (2012). The E3 ubiquitin ligase ITCH negatively regulates canonical Wnt signaling by targeting dishevelled protein. Mol. Cell. Biol. *32*, 3903–3912.

Welchman, R. L., Gordon, C., and Mayer, R. J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. Nat. Rev. Mol. Cell Biol. *6*, 599–609.

Welcker, M., Orian, A., Grim, J. E., Harper, J. W., Eisenman, R. N., and Clurman, B. E. (2006). The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. PNAS *103*, 17253–17254.

Wertz, I. E., Rourke, K. M. O., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., and Dixit, V. M. (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kB signalling. Nature *430*, 694–699.

Williamson, A., Werner, A., and Rape, M. (2013). The Colossus of

ubiquitylation: decrypting a cellular code. Mol. Cell *49*, 591–600.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999). The SCFβ-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and β-catenin and stimulates IκBα ubiquitination in vitro. Genes Dev. *13*, 270–283.

Wong, H. C., Mao, J., Nguyen, J. T., Srinivas, S., Zhang, W., Liu, B., Li, L., Wu, D., and Zheng, J. (2000). Structural basis of the recognition of the dishevelled DEP domain in the Wnt signaling pathway. Nat. Struct. Biol. *7*, 1178–1184.

Wong, H.-C., Bourdelas, A., Krauss, A., Lee, H.-J., Shao, Y., Wu, D., Mlodzik, M., Shi, D.-L., and Zheng, J. (2003). Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. Mol. Cell *12*, 1251–1260.

Wray, J., and Hartmann, C. (2012). WNTing embryonic stem cells. Trends Cell Biol. *22*, 159–168.

Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., and Pavletich, N. P. (2003). Structure of a β-TrCP1-Skp1-β-catenin complex: destruction motif binding and lysine specificity of the $SCF^{\beta\text{-}TrCP1}$ ubiquitin ligase. Mol. Cell *11*, 1445–1456.

Wu, J. *et al.* (2011). Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. Proc. Natl. Acad. Sci. U. S. A. *108*, 21188–21193.

Wu, Q., Huang, J. H., Sampson, E. R., Kim, K., Zuscik, M. J., Keefe, R. J. O., Chen, D., and Rosier, R. N. (2009). Smurf2 Induces Degradation of GSK-3β and Upregulates β- Catenin in Chondrocytes: A Potential Mechanism for Smurf2- Induced Degeneration of Articular Cartilage. Exp. Cell Res. *315*, 2386–2398.

Xing, Y., Clements, W. K., Kimelman, D., and Xu, W. (2003). Crystal structure of a β-catenin/axin complex suggests a mechanism for the β-catenin destruction complex. Genes Dev. *17*, 2753–2764.

Xu, L., Wei, Y., Reboul, J., Vaglio, P., Shin, T.-H., Vidal, M., Elledge, S. J., and Harper, J. W. (2003). BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. Nature *425*, 316–321.

Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D., and Peng, J. (2009). Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. Cell *137*, 133–145.

Yada, M., Hatakeyama, S., Kamura, T., Nishiyama, M., Tsunematsu, R., Imaki, H., Ishida, N., Okumura, F., Nakayama, K., and Nakayama, K. I. (2004). Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. EMBO J. *23*, 2116–2125.

Yagyu, R., Furukawa, Y., Lin, Y.-M., Shimokawa, T., Yamamura, T., and Nakamura, Y. (2004). A novel oncoprotein RNF43 functions in autocrine manner in colorectal cancer. Int. J. Oncol. *25*, 1343–1348.

Yamamoto, H. (1999). Phosphorylation of Axin, a Wnt Signal Negative Regulator, by Glycogen Synthase Kinase-3β Regulates Its Stability. J. Biol. Chem. *274*, 10681–10684.

Yamamoto, H., Komekado, H., and Kikuchi, A. (2006). Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of β-catenin. Dev. Cell *11*, 213–223.

Yanagawa, S., van Leeuwen, F., Wodarz, A., Klingensmith, J., and Nusse, R. (1995). The dishevelled protein is modified by wingless signaling in *Drosophila*. Genes Dev. *9*, 1087–1097.

Yanagawa, S., Matsuda, Y., Lee, J.-S., Matsubayashi, H., Sese, S., Kadowaki, T., and Ishimoto, A. (2002). Casein kinase I phosphorylates the Armadillo protein and induces its degradation in *Drosophila*. EMBO J. *21*, 1733–1742.

Yang, Y., Lijam, N., Sussman, D. J., and Tsang, M. (1996). Genomic organization of mouse Dishevelled genes. Gene *180*, 121–123.

Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. J., and Moon, R. T. (1996). A frizzled homolog functions in a vertebrate Wnt signaling pathway. Curr. Biol. *6*, 1302–1306.

Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D., and Moon, R. T. (1996). The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes Dev. *10*, 1443–1454.

Yu, A., Rual, J.-F., Tamai, K., Harada, Y., Vidal, M., He, X., and Kirchhausen, T. (2007). Association of Dishevelled with the clathrin AP-2 adaptor is required for Frizzled endocytosis and planar cell polarity signaling. Dev. Cell *12*, 129–141.

Zeng, X. *et al.* (2008). Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. Development *135*, 367–375.

Zhang, D. D., Lo, S., Cross, J. V, Dennis, J., Hannink, M., and Templeton, D. J. (2004). Keap1 Is a Redox-Regulated Substrate Adaptor Protein for a Cul3-Dependent Ubiquitin Ligase Complex. Mol. Cel. Biol. *24*, 10941-10953.

Zhang, L., Jia, J., Wang, B., Amanai, K., Wharton, K. A., and Jiang, J. (2006). Regulation of wingless signaling by the CKI family in *Drosophila* limb development. Dev. Biol. *299*, 221–237.

Zhang, S., Wang, Y., Dai, S.-D., and Wang, E.-H. (2011a). Down-regulation of NKD1 increases the invasive potential of non-small-cell lung cancer and correlates with a poor prognosis. BMC Cancer *11*, 186.

Zhang, X. *et al.* (2011b). Mechanistic insight into Myc stabilization in breast cancer involving aberrant Axin1 expression. PNAS *109*, 2790–2795.

Zhang, Y. *et al.* (2011c). RNF146 is a poly(ADP-ribose)-directed E3 ligase that regulates axin degradation and Wnt signalling. Nat. Cell Biol. *13*, 623–629.

Zhou, M. I., Wang, H., Ross, J. J., Kuzmin, I., Xu, C., and Cohen, H. T. (2002). The von Hippel-Lindau tumor suppressor stabilizes novel plant homeodomain protein Jade-1. J. Biol. Chem. *277*, 39887–39898.

Zhou, Y., Lan, J., Wang, W., Shi, Q., Lan, Y., Cheng, Z., and Guan, H. (2013). ZNRF3 acts as a tumour suppressor by the Wnt signalling pathway in human gastric adenocarcinoma. J. Mol. Histol. *44*, 555–563.