Regulation of Wnt/β-catenin signalling by tankyrase-dependent poly(ADP-ribosyl)ation and scaffolding

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Abstract

The Wnt/β-catenin signalling pathway is pivotal for stem cell function and the control of cellular differentiation, both during embryonic development and tissue homeostasis in adults. Its activity is carefully controlled through the concerted interactions of concentration-limited pathway components and a wide range of posttranslational modifications, including phosphorylation, ubiquitylation, sumoylation, poly(ADP-ribosyl)ation (PARylation) and acetylation. Regulation of Wnt/β-catenin signalling by PARylation was discovered relatively recently. The poly(ADP-ribose)polymerase (PARP) tankyrase PARylates AXIN1/2, an essential central scaffolding protein in the β-catenin destruction complex, and targets it for degradation, thereby fine-tuning the responsiveness of cells to the Wnt signal. The past few years have not only seen much progress in our understanding of the molecular mechanisms by which PARylation controls the pathway but also witnessed the successful development of tankyrase inhibitors as tool compounds and promising agents for the therapy of Wnt-dependent dysfunctions, including colorectal cancer. Recent work has hinted at more complex roles of tankyrase in Wnt/β-catenin signalling as well as challenges and opportunities in the development of tankyrase inhibitors. Here we review some of the latest advances in our understanding of tankyrase function in the pathway and efforts to modulate tankyrase activity to re-tune Wnt/β-catenin signalling in colorectal cancer cells.

Abbreviations

ADP, adenosine trisphosphate; AKT, historical name for protein kinase B; APC, adenomatous polyposis coli; ARC, ankyrin repeat cluster; ARTD, Diphtheria-toxin-like ADP-ribosyltransferase; AXIN, axis inhibition protein; β-TRCP, β-transducin repeats-containing protein; B9L, B-cell

CLL/lymphoma 9-like protein; CK1, casein kinase 1; CLEM, correlative light and electron microscopy; CRC, colorectal cancer; DAX, polymerising domain present in <u>D</u>ishevelled and <u>AX</u>IN; DKK1, Dickkopf-related protein 1; DVL, Dishevelled; EGFR, epidermal growth factor receptor; FoxM1, Forkhead box protein M1; FRAP, fluorescence recovery after photobleaching; GSK3, glycogen synthase kinase 3; IC_{50}, inhibitory concentration of 50%; ISC, intestinal stem cell; JAK, Janus kinase; LEF1, lymphoid enhancer-binding factor 1; LRP5/6, low-density lipoprotein receptor-related protein 5/6; MCR, mutation cluster region in APC; NAD^+, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; PAR, poly(ADP-ribose); PARdU, PAR-dependent ubiquitylation; PARP, poly(ADP-ribose)polymerase; PDB, protein data bank; PI3K, phosphatidylinositol-3-kinase; RGS, regulator of G-protein signalling (domain); RING, really interesting new gene (domain); RNF, RING finger; SAM, sterile alpha motif (domain); SAR, structure-activity relationship; SBDD, structure-based drug design; STAT, signal transducer and activator of transcription; TBM, tankyrase-binding motif; TNKS/TNKS2, tankyrase; TNKSi, tankyrase inhibitor; TERF1/TRF1, telomeric repeat-binding factor 1; V5, epitope tag derived from the P and V proteins of paramyxovirus and simian virus 5, respectively; Wg/WNT, Wingless and its vertebrate orthologue; WWE, domain named after a motif containing two conserved Trp (W) residues and one conserved Glu (E)

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Regulation of Wnt/β-catenin signalling by tankyrase-dependent AXIN poly(ADP-ribosyl)ation – an overview

The Wnt/β-catenin signalling pathway plays key roles during embryonic development, tissue homeostasis and regeneration (reviewed by Clevers and Nusse, 2012 and Clevers et al., 2014). Central to the pathway is the β-catenin destruction complex, which tightly controls the levels of nuclear, transcriptionally active β-catenin (reviewed by Stamos and Weis, 2013). Dysregulation of β-catenin destruction complex function underlies the vast majority of colorectal cancers (CRCs) and other conditions such as fibrosis, neurodegeneration and osteoporosis (reviewed by Clevers and Nusse, 2012 and Kahn, 2014). AXIN (AXIN1/AXIN2), the central scaffold of the destruction complex, directly binds all its core components: the scaffolding protein APC, the kinases GSK3 and CK1 and β-catenin (reviewed by Stamos and Weis, 2013) (Figure 1A). (AXIN1 and AXIN2 will be referred to collectively as AXIN where the discussed aspects apply to both.) The complex enables the phosphorylation of β-catenin at a phosphodegron to prime it for ubiquitylation and subsequent degradation by the proteasome (reviewed by Stamos and Weis, 2013). AXIN is thought to be the concentration-limiting component of the complex (Salic et al., 2000; Lee et al., 2003). Therefore, controlling its abundance is an effective way to regulate β-catenin destruction. Wnt/β-catenin signalling is regulated by a wide range of posttranslational modifications (reviewed by Gao et al., 2014). The discovery of a regulatory role of tankyrase in Wnt/β-catenin signalling sparked much excitement given the limited number of known targetable enzymes in the pathway (Huang et al., 2009). Tankyrase, with two human paralogues (TNKS and TNKS2; Tnks and Tnks2 in other species discussed; from here on simply referred to as "tankyrase" where concepts apply to both tankyrases), is a poly(ADP-ribose)polymerase (PARP), and as such catalyses the attachment of poly(ADP-ribose) (PAR) chains onto its substrates (reviewed by Gibson and Kraus, 2012 and Haikarainen et al., 2014a). In addition to its auto-PARylation activity, tankyrase binds and PARylates AXIN. In turn, PARylation activates the PAR-dependent E3 ubiquitin ligase RNF146/Iduna, which then ubiquitylates AXIN, tankyrase and itself, targeting the entire complex for proteasomal degradation (Callow et al., 2011; Zhang et al., 2011) (Figure 1A). This process, known as PAR-dependent ubiquitylation (PARdU) (DaRosa et al., 2015), is thought to occur constitutively and tune the receptiveness of cells to Wnt stimuli by limiting destruction complex formation (Wang et al., 2016b). A tankyrase-associated ubiquitin-specific protease (USP25) can de-ubiquitylate tankyrase, thereby stabilising it and supporting PARdU of AXIN (Xu et al., 2017a). Recent studies point toward another role of tankyrase, namely in promoting the formation of active, membrane-localised Wnt signalosomes upon Wnt stimulation (Yang et al., 2016; Wang et al., 2016a) (Figure 1B). Furthermore, additional tankyrase interactors in the Wnt/β-catenin pathway, other than AXIN, are emerging (Croy et al., 2016). Structure-function studies are providing a

detailed picture of the molecular mechanisms by which tankyrase controls Wnt/β-catenin signalling and are revealing non-catalytic scaffolding roles of tankyrase (Guettler et al., 2011; Morrone et al., 2012; DaRosa et al., 2015; 2016; Eisemann et al., 2016; Mariotti et al., 2016; Riccio et al., 2016; Xu et al., 2017a). Conserved functions of tankyrase in the Wnt/β-catenin pathway are being increasingly appreciated from studies in *Drosophila* and human CRC cell lines (Lau et al., 2013; de la Roche et al., 2014; Yang et al., 2016; Wang et al., 2016b; 2016c). Recently developed tankyrase-specific catalytic inhibitors are serving as tool compounds and promising pre-clinical leads for the treatment of CRC and other Wnt-dependent conditions (Lau et al., 2013; reviewed by Haikarainen et al., 2014a). Here, we discuss a selection of recent insights into the roles of tankyrase in the Wnt/β-catenin signalling pathway. In terms of potential therapeutic applications, we will focus on CRC, given its high incidence and the importance of the Wnt/β-catenin pathway in its emergence.

Tankyrase as a scaffold in Wnt/β-catenin signalling – a structural perspective

TNKS and TNKS2 share a highly similar multi-domain organisation: five N-terminal ankyrin repeat clusters (ARCs) for substrate binding are followed by a polymerising sterile alpha motif (SAM) domain and a C-terminal PARP catalytic domain (Figure 2A). With the exception of ARC3, the ARCs act as discrete substrate recognition domains for degenerate 8-amino-acid peptides of the consensus R-[any]-[any]-[small hydrophobic or G]-[D/E]-G-[no P]-[D/E], termed the tankyrase-binding motif (TBM) (Seimiya, 2002; Seimiya et al., 2004; Guettler et al., 2011). (Briefly, an arginine at position 1 and glycine at position 6 of the TBM are essential. A small, hydrophobic residue or glycine are preferred at position 4, and acidic residues are optimal at positions 5 and 8, while proline is disallowed at position 7.) Crystal structures of human TNKS2 ARC4 with various TBM peptides (Guettler et al., 2011), of murine Tnks ARC2-3 in complex with Axin1 (Morrone et al., 2012), of human TNKS ARC1-3, combined with in-solution structural studies (Eisemann et al., 2016), and of human TNKS ARC5 bound to the TBM of USP25 (Xu et al., 2017a), revealed the architecture of the ARCs and the principles of their substrate recognition. AXIN contains two TBMs in its N-terminus (Morrone et al., 2012), with the second motif bearing an unusual insertion (Figure 2B-E). The Tnks ARC2-3:Axin1 crystal structure shows a dimeric arrangement of ARC2-3 with each TBM peptide bound to one copy of ARC2, respectively (Morrone et al., 2012). AXIN is also able to contact two ARCs in the same Tnks molecule (Eisemann et al., 2016). TNKS ARCs 1-3 adopt a relatively rigid asymmetric U-shape, whereas ARCs 4-5 are more dynamic and flexibly linked to ARC1-3 (Eisemann et al., 2016). Multiple AXIN binding sites in the ARCs and two TBMs in AXIN enable their cooperative interaction, but distance and conformational restraints create a preference for bivalent AXIN to either simultaneously bind ARCs 1 and 2, 4 and 5 or 2 and 5, with a preference for combinations involving ARC2, the strongest AXIN binder (Eisemann et al.,

2016). When binding to ARCs 2 and 5, AXIN induces a more compact conformation of the ARCs, which might place the PARP domain into closer proximity to ARC-bound AXIN, in turn promoting AXIN PARylation (Eisemann et al., 2016). Further studies are needed to explore this hypothesis. In the context of polymeric tankyrase, it appears equally likely that AXIN binds separate tankyrase molecules in the same tankyrase filament, with different implications for tankyrase conformation and potentially a further augmentation of cooperativity (Mariotti et al., 2016; Riccio et al., 2016). Both TNKS and TNKS2 polymerise through their SAM domains (De Rycker and Price, 2004; Mariotti et al., 2016; Riccio et al., 2016). Recent crystallographic studies of the SAM domains revealed the electrostatic nature of the head-to-tail SAM-SAM interfaces within the helical filament (Mariotti et al., 2016; Riccio et al., 2016) (Figure 2F), in agreement with a polymer model guided by NMR studies to identify the residues perturbed upon polymerisation (DaRosa et al., 2016). Compatible with the outward-facing N- and C-termini in the filament (DaRosa et al., 2016; Mariotti et al., 2016; Riccio et al., 2016), full-length tankyrase indeed polymerises (De Rycker and Price, 2004; Mariotti et al., 2016; Riccio et al., 2016). TNKS and TNKS2 form cytoplasmic puncta rather than microscopically visible filaments, which may reflect the dynamic nature of the polymers (Mariotti et al., 2016; Riccio et al., 2016). This is consistent with observations made for other proteins containing polymerising SAM domains (Isono et al., 2013) and for polymerising AXIN and DVL/DVL2 (Schwarz-Romond et al., 2007; Fiedler et al., 2011; reviewed by Bienz, 2014). Supporting this view, polymerisation-deficient TNKS and TNKS2 mutants localise diffusely (Mariotti et al., 2016; Riccio et al., 2016). Luciferase reporter assays revealed that scaffolding through the ARCs and SAM domain is essential for tankyrase function in Wnt/β-catenin signalling (Mariotti et al., 2016; Riccio et al., 2016). Surprisingly, tankyrase can drive Wnt/β-catenin activity even in the absence of its catalytic PARP activity, entirely through scaffolding (Mariotti et al., 2016). Tankyrase polymerisation enables productive interactions with the limited pool of AXIN, through avidity effects arising from multivalency and polymerisation in both tankyrase and AXIN (Fiedler et al., 2011; Mariotti et al., 2016) (Figure 2G), a requirement that appears overridden by AXIN overexpression (Riccio et al., 2016 and our unpublished observations). The SAM domain and SAM-domain dependent polymerisation are also required for full tankyrase PARP activity (De Rycker and Price, 2004; Levaot et al., 2011; Mariotti et al., 2016; Riccio et al., 2016). Interestingly, while PARP activity is dispensable for tankyrase-driven Wnt/β-catenin signalling under basal conditions (Mariotti et al., 2016), it is necessary for tankyrase to potentiate Wnt-induced β-catenin activity (Riccio et al., 2016). This may well reflect the recently discovered requirement of AXIN PARylation in the formation of Wnt-induced signalosomes (see below) (Yang et al., 2016; Wang et al., 2016a) (Figure 1B).

A potential role of tankyrase in the formation of β-catenin degradasomes

When AXIN is overexpressed or stabilised in APC-mutant cells by tankyrase inhibition (using the inhibitors JW67, JW74, JW55, XAV939, or G007-LK; see Table 1 and below for a discussion of inhibitors), it accumulates in cytoplasmic puncta, together with other β-catenin destruction complex components, including GSK3β, APC, β-catenin, β-TRCP and tankyrase (Waaler et al., 2011; 2012; de la Roche et al., 2014; Thorvaldsen et al., 2015; Martino-Echarri et al., 2016) (Figure 3). The puncta gradually disappear upon removal of the tankyrase inhibitor (TNKSi), and re-establish with subsequent inhibitor treatment (Thorvaldsen et al., 2015). The puncta, referred to as β-catenin degradasomes, are understood to be "morphological correlates" of β-catenin destruction complexes, which without tankyrase inhibition are not visible by light microscopy due to the normally low AXIN levels (de la Roche et al., 2014; Thorvaldsen et al., 2015; Pedersen et al., 2016). Several features illustrate functionality of these degradasomes. Firstly, they contain phosphorylated β-catenin and also colocalise with ubiquitin and β-TRCP, a component of the E3 ubiquitin ligase responsible for β-catenin ubiquitylation (Thorvaldsen et al., 2015). Secondly, live-cell imaging by fluorescence recovery after photobleaching (FRAP) in SW480 cells showed that β-catenin is rapidly turned over in degradasomes (Thorvaldsen et al., 2015), demonstrating their capacity to degrade β-catenin.

Two recent studies suggest that tankyrase plays a structural role in degradasome formation (Thorvaldsen et al., 2015; Martino-Echarri et al., 2016). Correlative light and electron microscopy (CLEM) suggests that TNKS-GFP-containing degradasomes in TNKSi-treated SW480 cells correspond to electron-dense and possibly filamentous substructures (Thorvaldsen et al., 2015), perhaps reflecting the polymeric nature of both AXIN and tankyrase. Simultaneous silencing of both tankyrases abolishes degradasome formation (Martino-Echarri et al., 2016). Other studies have shown that, like tankyrase inhibition, TNKS/TNKS2 RNAi increases the levels of AXIN1/2 (Huang et al., 2009). Strikingly, despite increased AXIN levels, degradasomes are absent under TNKS/TNKS2-depleted conditions (Martino-Echarri et al., 2016), supporting a direct, structural role for tankyrase in degradasome formation. FRAP studies have shown that TNKS stably resides in degradasomes, similarly to AXIN (Schwarz-Romond et al., 2005; Thorvaldsen et al., 2015), although tankyrase and AXIN dynamics have not yet been studied in the same cell. The multivalent interactions of AXIN with tankyrase and the avidity-enhancing polymerisation of both proteins may underlie a scaffolding function of tankyrase in degradasome formation (Mariotti et al., 2016) (Figure 2G). Tankyrase polymerisation may be promoted by its catalytic inhibition (De Rycker and Price, 2004), which might offer a potential explanation for the TNKSi-induced stabilisation of degradasomes. APC2, which was recently reported to bind tankyrase (Croy et al., 2016), may also

contribute to the avidity-dependent degradasome assembly, given its numerous AXIN and β-catenin binding sites.

AXIN2 protein levels rapidly increase upon tankyrase inhibition in SW480 cells, whereas AXIN1 levels do not change until much later (Pedersen et al., 2016; Thorvaldsen et al., 2017). Knockdown of AXIN2 but not AXIN1 prevents degradasome formation (Thorvaldsen et al., 2017), indicating that AXIN2 is the predominant AXIN scaffold in these cells. The TNKSi-induced accumulation of AXIN2 is dependent on new protein synthesis (Thorvaldsen et al., 2017) and active transcription of the *AXIN2* gene (Pedersen et al., 2016). Proteasome inhibition leads to increased levels of phosphorylated β-catenin in degradasomes (Thorvaldsen et al., 2015), but prolonged proteasome inhibition impairs degradasome formation (Martino-Echarri et al., 2016; Pedersen et al., 2016). Pedersen and colleagues proposed that the transcription factor FoxM1, whose activating phosphorylation is suppressed by proteasome inhibition, controls the *AXIN2* gene (Pedersen et al., 2016). *AXIN2* is also a Wnt/β-catenin target gene as part of a negative feedback loop, and is highly expressed in APC mutated CRC cells (Yan et al., 2001; Jho et al., 2002; Lustig et al., 2002). It is unknown why tankyrase inhibitors induce β-catenin degradasomes in many CRC cells but far less so in cells with an intact Wnt/β-catenin pathway (de la Roche et al., 2014). It is possible that tankyrase inhibition strongly represses the *AXIN2* gene in Wnt/β-catenin wild-type cells. Conversely, APC-mutant cells might still display residual *AXIN2* transcription with ensuing AXIN2 protein accumulation due to blocked PARdU and the formation of large degradasomes. Degradasome assembly depends on the concentrations of their components (Bienz, 2014), and it is likely that fully functional degradasomes also form in Wnt/β-catenin wild-type cells, but that these structures remain small.

The structural basis of PARdU

Once PARylated, AXIN is engaged by the PAR-binding E3 ubiquitin ligase RNF146/Iduna (Callow et al., 2011; Kang et al., 2011; Zhang et al., 2011). RNF146 consists of a RING domain, followed by a PAR-binding WWE domain and an extended C-terminus, which is predicted to be largely unstructured (Figure 4B). The PAR-dependency of the enzyme suggested an allosteric activation mechanism; in addition, PAR may serve as a scaffold to enable increased local concentrations of the enzyme (Callow et al., 2011; Kang et al., 2011; Zhang et al., 2011). A crystal structure of RNF146 (RING-WWE) bound to iso-ADP-ribose, an internal unit of PAR (Figure 4A), and an E2 conjugating enzyme is compatible with the allosteric activation of RNF146 (DaRosa et al., 2015) (Figure 4C). Iso-ADP-ribose not only binds the WWE but also the RING domain (Wang et al., 2012; DaRosa et al., 2015) and appears to induce restructuring of a loop which in the apo form of the RING domain extends into the E2-E3 enzyme contact region, thereby precluding the interaction

(Figure 4C). The restructured loop residues become part of an extended central helix in the RING domain, which no longer obstructs E2 binding (Figure 4C), a model supported by NMR spectroscopy and mutagenesis (DaRosa et al., 2015). The extended C-terminus of RNF146 directly binds tankyrase via five proposed TBMs (DaRosa et al., 2015) (Figure 4B). It is noteworthy that all of these TBMs are atypical in their length or sequence, suggesting that they might be of relatively low individual affinity and may need to act collectively to recruit tankyrase. A model in which the tankyrase:RNF146 complex can still bind other TBM-containing proteins via the multivalent ARCs implies that tankyrase's substrate specificity determines RNF146 substrate specificity (DaRosa et al., 2015). Like many PARPs, tankyrase modifies itself, and not all tankyrase binders are also PARylated (Bae, 2002; Guettler et al., 2011; Bisht et al., 2012). This raises the interesting possibility that non-PARylated tankyrase binders may still be ubiquitylated by RNF146 present in the complex, with RNF146 getting activated by tankyrase auto-PARylation or PAR attachment to different, simultaneously bound substrates.

While tankyrase RNAi stabilises AXIN and reduces β-catenin-dependent transcription in certain CRC cell lines (Huang et al., 2009; Callow et al., 2011), silencing of RNF146 fails to increase AXIN levels in HCT-15 or SW480 CRC cells, both of which bear APC truncations, and does not inhibit Wnt/β-catenin signalling in HCT-15 cells (Callow et al., 2011). This suggests that there are alternative pathways for the degradation of PARylated AXIN in these cells (Callow et al., 2011). The existence of numerous RING-type E3 ubiquitin ligases with PAR-binding WWE domains suggests that functional redundancies may exist (Wang et al., 2012).

Tankyrase and APC set a threshold for Wnt responsiveness by limiting AXIN abundance

To assess the maximum AXIN level still allowing productive Wnt/β-catenin signalling, Wang *et al.* engineered flies overexpressing C-terminally V5 epitope-tagged Axin (Axin-V5) (Wang et al., 2016b). (Note that *Drosophila* has a single Axin paralogue; see Figure 2C.) Despite an up to four-fold Axin-V5 overexpression, flies develop normally with only a mild defect attributable to inhibited Wg signalling (Wang et al., 2016b), in agreement with previous studies (Peterson-Nedry et al., 2008). This suggested that Axin-V5 is still subject to physiological regulation at this level. Likewise, loss of tankyrase (of which there is also only a single paralogue in *Drosophila*) results in a mild (two- to three-fold) increase in endogenous Axin abundance in larvae, without measurable developmental impact (Feng et al., 2014; Wang et al., 2016b; 2016c). However, developmental defects and loss of Wg/Armadillo target gene expression are observed when Axin-V5 is expressed in wing imaginal discs in a tankyrase null background, which results in a further three-fold increase of Axin-V5 levels, positioning the inhibitory threshold for Axin three- to nine-fold above endogenous regulated levels (Wang et al., 2016b). This illustrates tankyrase's strong capacity to

buffer negative regulation of Wg signalling by Axin, a phenomenon also seen in mammalian cells (Mariotti et al., 2016). These observations are compatible with the previous finding that knockdown of tankyrase in the developing wing only leads to a Wg phenotype if Axin is simultaneously overexpressed (Feng et al., 2014).

Besides their role in promoting β-catenin/Armadillo degradation, Apc/Apc2 also play a positive role in regulating Wg/Armadillo signalling by posttranscriptionally limiting Axin levels (Takacs et al., 2008; Wang et al., 2016b). Accordingly, Axin-V5 accumulates in imaginal discs lacking Apc1 and Apc2 (Wang et al., 2016b). The regulation of Axin levels by Apc is strictly dependent on the Apc-binding RGS domain of Axin, suggesting that a physical interaction between the two proteins is required (Wang et al., 2016b). Surprisingly, while the ability of tankyrase to destabilise Axin is independent of Apc, the TBM of Axin is necessary for Axin destabilisation by Apc, suggesting that tankyrase binding is required (Wang et al., 2016b). Alternatively, the Axin N-terminus may play a tankyrase-independent role in Axin regulation by Apc. The mechanism by which Apc destabilises Axin remains unclear, and it will be interesting to decipher whether PARdU is involved. A recent report suggests the existence of a TBM in Apc2 (Croy et al., 2016). Tankyrase may bind to both Apc2 and Axin, thereby providing an additional scaffolding role. The existence of partially separable degradation pathways for Axin, through tankyrase and Apc/Apc2, may explain the mild Tnks null phenotype in flies (Wang et al., 2016b). By analogy, Wang *et al.* propose that if AXIN regulation by APC is lost in APC-mutant CRC cells, then these cells might be particularly susceptible to tankyrase inhibition, in contrast to APC wild-type cells, opening the possibility for selectively targeting APC-mutant cells (Wang et al., 2016b). This is an interesting idea for further exploration.

Tankyrase mouse models

The physiological role of tankyrase in Wnt/β-catenin signalling is still far from being fully understood. In mice, loss of both tankyrases gives rise to embryonic lethality, without indication that lethality is attributable to defective Wnt/β-catenin signalling (Chiang et al., 2008). Individual knockout of either Tnks or Tnks2 results in non-pathogenic phenotypes, but again there is no sign of a dysregulated Wnt pathway (Chiang et al., 2006; Hsiao et al., 2006; Chiang et al., 2008). One may speculate that embryonic lethality in the double knockout arises from another essential role of tankyrase in development or pleiotropy due to the complex involvement of tankyrase in many biological processes, masking a function in Wnt/β-catenin signalling. Moreover, these observations point toward a substantial functional redundancy between the two tankyrases. In the absence of conditional Tnks/Tnks2 knockout mice, the role of tankyrase in Wnt-dependent physiology is difficult to assess. However, an Axin2 V26D mutation, which maps to the stronger, N-terminal

TBM of murine Axin2 (Figure 2C), also results in embryonic lethality, but with an identifiable Wnt phenotype (Qian et al., 2011). The mutation is expected to abrogate tankyrase binding (Guettler et al., 2011), compatible with increased Axin2 levels and reduced Wnt/β-catenin signalling in most tissues (Qian et al., 2011). However, increased rather than decreased Wnt/β-catenin signalling is observed in the late primitive streak, a structure that marks the beginning of gastrulation and the definition of body axes, and consequential formation of ectopic tails (Qian et al., 2011), an observation that points to complex functions of the tankyrase-Axin interaction. The Axin2 mutation exposes a physiological role of tankyrase in Wnt/β-catenin signalling that was likely masked in the Tnks/Tnks2 double-knockout mice.

Tankyrase controls adult intestinal stem cell homeostasis in *Drosophila*

A more detailed analysis of Tnks mutant flies revealed a sharp drop in viability upon nutrient limitation, paralleled by an accumulation of Axin and hyperproliferation of intestinal stem cells (ISCs) in the midgut (Wang et al., 2016c). The Drosophila midgut can be subdivided into several morphologically and physiologically distinct domains. It displays high levels of Wg target gene transcription close to the inter-domain boundaries, which may represent source areas for the Wg ligand (Buchon et al., 2013; Tian et al., 2016). Loss of tankyrase distant from the midgut-hindgut boundary, where β-catenin/Armadillo activity is low, completely abolishes the activation of Wg reporters in this region (Wang et al., 2016c). This is not the case in areas close to the midgut-hindgut boundary, where Wg signalling is high. The authors suggested that by counteracting Axin, tankyrase amplifies β-catenin/Armadillo activity in compartments of otherwise low pathway activity. They hypothesized that tankyrase's context-specific roles may explain seemingly contradicting *in-vivo* functions of tankyrase in flies and zebrafish (Huang et al., 2009; Feng et al., 2014) and that similar mechanisms may be in place where Wnt gradients are observed in vertebrates, for example in the gut (Wang et al., 2016c). (See below for a further discussion of tankyrase's roles in the gastrointestinal tract.) Interestingly, hyperproliferation of ISCs is non-cell-autonomous: it does not result from tankyrase loss or abrogated Wg/Armadillo signalling in ISCs but in enterocytes (Tian et al., 2016; Wang et al., 2016c). The authors suggested that decreased Wg signalling in enterocytes upon loss of tankyrase promotes JAK/STAT signalling in ISCs, which in turn drives their proliferation during homeostasis.

A role of AXIN PARylation in Wnt signalosome assembly

While tankyrase's role in PARdU of AXIN is well established, two recent studies point to a novel function in Wnt signalosome assembly (Yang et al., 2016; Wang et al., 2016a) (Figure 1B). During *Drosophila* embryonic development, the abundance of weakly expressed Axin-V5 changes in a

biphasic manner (Yang et al., 2016). Initially distributed uniformly in the ectoderm, Axin-V5 first accumulates in segmental stripes marked by Wg induction. Secondly, with progressing development, Axin levels drop specifically in the Wg stripes, an observation analogous to delayed AXIN destabilisation in mammalian cells (Li et al., 2012 and references therein). In embryos expressing an Axin-V5 mutant variant lacking the TBM, or in embryos lacking tankyrase, Axin-V5 levels are uniformly high. Early Axin-V5 stripes fail to form, with a concomitant loss of normal Wg target gene expression. Importantly, this is not merely due to increased Axin-V5 levels under these circumstances since strong Axin-V5 overexpression does not copy this phenotype (Yang et al., 2016). The authors hence propose a role of tankyrase in regulating Axin function rather than levels (Figure 1B). Studies in *Drosophila* and work with *Drosophila* cells and HEK293T cells showed that Wg/Wnt stimulation results in accumulation of PARylated Axin-V5/AXIN1 with an enhanced formation of AXIN- and LRP6/Arrow-containing Wnt/Wg signalosomes (Yang et al., 2016). Capitalising on the ability to detect endogenous *Drosophila* Axin, a second study suggests that Axin is present in both the cytoplasm and at the membrane, even under basal conditions. Wg stimulation results in Axin accumulation in both compartments, with an enrichment of PARylated Axin at the membrane (Wang et al., 2016a). Forcing Axin to the membrane under basal conditions increases its PARylation and destabilisation, presumably by PARdU, suggesting that tankyrase acts at the membrane or Axin's susceptibility to PARylation is augmented upon membrane recruitment (Wang et al., 2016a). Phosphorylation by GSK3 (Kim et al., 2013) or binding of the small molecule HLY78 (Wang et al., 2013) was reported to induce an open AXIN conformation for productive interaction with LRP6. AXIN PARylation may control the assembly of the Wnt signalosome complex in a similar manner. Alternatively, PAR itself may act as a molecular glue to recruit AXIN to Wnt signalosomes. While the intact LRP5 C-terminus is required for AXIN binding (Mao et al., 2001), it is presently not clear whether AXIN and LRP5/6 interact directly (Mao et al., 2001; Kim et al., 2013; Wang et al., 2013), but there are indications that this may be the case (MacDonald et al., 2011). Future studies will unravel the mechanisms behind the Wnt/Wg-induced accumulation of PARylated Axin and the role of PARylation in signalosome assembly. Yang *et al*. propose that the PAR-assisted signalosome formation enables a rapid response to Wnt signals while the subsequent downregulation of AXIN limits the re-assembly of β-catenin destruction complexes, thereby conferring both responsiveness and robustness to the pathway (Yang et al., 2016).

Tankyrase inhibitors

In this section, we provide an overview of tankyrase inhibitors; for more comprehensive discussions, we refer the reader to other recent reviews (Lehtiö et al., 2013; Steffen et al., 2013; Haikarainen et al., 2014a). All small-molecule tankyrase inhibitors developed to date are mimetics

of β-NAD$^+$ or its adenosine or nicotinamide portions. The first potent toolbox tankyrase inhibitors, XAV939 (Huang et al., 2009), IWR-1 and IWR-2 (Chen et al., 2009; Gunaydin et al., 2012), were discovered in phenotypic screens designed to identify antagonists of the Wnt/β-catenin pathway, as were the inhibitors JW74 (Waaler et al., 2011), JW55 (Waaler et al., 2012), WIKI4 (James et al., 2012) and K-756 (Okada-Iwasaki et al., 2016). Numerous additional inhibitors were established through diverse approaches (Zhan et al., 2014), including screening for compounds that rescue tankyrase-induced lethality of yeast cells (Yashiroda et al., 2010) or induce a mitotic spindle defect (Johannes et al., 2015b), fragment screening (Larsson et al., 2013; de Vicente et al., 2015), proteomics (Thomson et al., 2017), *in-silico* screening or substructure searching, followed by compound optimisation (Bregman et al., 2013b; Elliott et al., 2015), screening of a DNA-encoded library (Samain et al., 2015), and extensive structure-activity relationship (SAR) studies, assisted by the structural analysis of tankyrase/PARP:inhibitor complexes (Hua et al., 2013; Shultz et al., 2013; Voronkov et al., 2013; Narwal et al., 2013a; Liscio et al., 2014; Qiu et al., 2014; Haikarainen et al., 2014b; Kumpan et al., 2015; Nkizinkiko et al., 2015; Paine et al., 2015; Haikarainen et al., 2016; Thomson et al., 2017). Numerous more drug-like molecules, with optimised pharmacological properties, are now available, for example G007-LK (Lau et al., 2013; Voronkov et al., 2013) and NVP-TNKS656 (Shultz et al., 2013). Table 1 gives examples of published tankyrase inhibitors.

Inhibitor binding sites on the tankyrase PARP domain
The tankyrase PARP domain shares strong homology with the catalytic domains of the 17 human Diphtheria-toxin-like ADP-ribosyltransferases (ARTDs) (reviewed by Hottiger et al., 2010). Among all ARTDs known to synthesise PAR (Vyas et al., 2014), the TNKS/TNKS2 PARP domains are unique in that they coordinate zinc, lack an N-terminal helical regulatory subdomain and also lack a loop distal to the active site (Lehtiö et al., 2008). In all other respects, they share the typical structural elements with other ARTD catalytic domains (Figure 5A). A so-called donor site coordinates the co-substrate NAD$^+$ while the acceptor site accommodates either the peptide for the priming modification or the growing PAR chain poised for extension (Figure 5A). The donor site is lined by three loops: the donor site or D-loop, a glycine-rich G-loop and a phenylalanine-containing F-loop (Figure 5A). Tankyrase contains a catalytic H-Y-E triad required for PAR synthesis (Figure 5A). All tankyrase inhibitors developed to date target the NAD$^+$-binding donor site, but can broadly be classified by three binding modes, according to our structural understanding of NAD$^+$ binding to Diphtheria toxin (Bell and Eisenberg, 1996). Inhibitors either primarily engage the nicotinamide subsite (e.g., XAV939) (Karlberg et al., 2010) the adenosine subsite (e.g., IWR-1 and G007-LK) (Narwal et al., 2012; Voronkov et al., 2013) or both sites in the case of the more recently developed dual-site inhibitors (Bregman et al., 2013b) (Figure 5A; Table 1). In some instances, a phosphate

site between the two subsites is specified as well (reviewed by Steffen et al., 2013). Crystal structures of tankyrase catalytic domains without inhibitor show closed D-loop conformations, albeit different ones in apo-TNKS and apo-TNKS2 with indications of flexibility (Lehtiö et al., 2008; Karlberg et al., 2010). Adenosine site binders appear to induce their own pocket, conferring disorder or conformational changes to the D-loop (Lehtiö et al., 2008; Gunaydin et al., 2012; Shultz et al., 2012). Hence, the adenosine subsite appears highly adaptable and is sometimes referred to as the "induced pocket".

Achieving selectivity

PARP inhibitor profiling against ARTDs revealed a remarkable promiscuity for a number of PARP inhibitors (Wahlberg et al., 2012; Thorsell et al., 2017). For example, XAV939 inhibits TNKS, TNKS2, PARP1 and PARP2 with comparable potency (e.g., IC_{50} values of 95, 5, 74, 27 nM in a direct comparison using the catalytic domains of TNKS and TNKS2 and full-length PARP1 and 2, respectively), while IWR-1 is more specific for the tankyrases (no measurable IC_{50} for full-length PARPs 1 and 2) (Thorsell et al., 2017). High-resolution PARP domain:TNKSi co-crystal structures have (1) rationalised TNKSi selectivity and (2) enabled structure-based drug design of more selective and potent tankyrase binders (reviewed by Lehtiö et al., 2013; Steffen et al., 2013; Haikarainen et al., 2014a). Specific examples for the former include G007-LK, which was optimised from JW74 (Lau et al., 2013; Voronkov et al., 2013), and WIKI4 (James et al., 2012; Haikarainen et al., 2013). Examples for the latter include NVP-TNKS656, developed from XAV939 (Shultz et al., 2013), and dual-site inhibitors (Hua et al., 2013) (Table 1). These studies demonstrated that the unique structural features of the tankyrase catalytic domain can be exploited to gain selectivity. For example, WIKI4 in the adenosine subsite would sterically clash with the helical subdomain in PARPs 1-3 (Haikarainen et al., 2013). More subtle differences can also be harnessed. Compared to other PARPs, the D-loop of tankyrases is 3 amino acids shorter, more flexible and often disordered in crystal structures of the domain with inhibitors, due to the absence of three proline residues, and characterised by large hydrophobic amino acids, which confer a narrower, more hydrophobic donor site pocket (Lehtiö et al., 2008; Wahlberg et al., 2012). Selectivity and potency can be gained by "growing" compounds toward this narrow pocket, as seen in the optimisation of quinazolinones (Nathubhai et al., 2013), tetrahydro-1,6-naphtyridin-5-ones (Kumpan et al., 2015) and the XAV939 core to NVP-TNKS656 (Shultz et al., 2013) (Table 1). Of note, although selectivity over other ARTDs can be achieved, many other enzymes also use NAD^+ as a co-substrate, and so inhibitors designed to target the NAD^+ donor site may have unknown off-target effects at high concentrations. However, the example of sirtuins shows that this potential challenge can be overcome (Ekblad and Schüler, 2016).

Future developments

While further optimisation of catalytic tankyrase inhibitors is progressing, non-catalytic scaffolding roles of tankyrase in Wnt/β-catenin signalling are emerging (Mariotti et al., 2016), and these may be augmented when prolonged TNKSi treatment results in tankyrase stabilisation by blocked PARdU (Huang et al., 2009). Furthermore, overexpression of tankyrase in several tumour types has been reported (Matsutani et al., 2001; Gelmini et al., 2004; 2006; 2007; Shervington et al., 2007; Shebzukhov et al., 2008; Zhao et al., 2009; Gao et al., 2011; Tang et al., 2012; Busch et al., 2013) and may accentuate tankyrase's concentration-dependent scaffolding functions, contributing to TNKSi resistance (Mariotti et al., 2016). Therefore, blocking tankyrase's ARC- and SAM-dependent scaffolding functions holds considerable potential. Importantly, the ARCs and SAM domain are highly conserved between the two tankyrases (Figure 2A) but unique among the ARTD family, therefore offering the opportunity for target selectivity of potential compounds over other ARTDs, in addition to the potential benefits of inhibiting non-catalytic scaffolding functions. Moreover, potential interference with other NAD^+-dependent enzymes could be circumvented by this approach. Whereas blocking SAM-domain-dependent polymerisation appears challenging due to the relatively shallow polymerisation interface, targeting the deeper TBM-binding pocket on the ARCs is more promising (Guettler et al., 2011; Morrone et al., 2012). This binding pocket is not conserved across ankyrin repeat proteins in general and appears to be unique to tankyrase. Given the presence of four substrate-binding ARCs, abrogation of each of these substrate/ligand binding sites would likely be required, in both TNKS and TNKS2. However, this appears feasible given the conservation of the TBM-binding pocket across the TNKS and TNKS2 ARCs (Guettler et al., 2011). Xu *et al.* have recently shown that a stapled TBM peptide, based on a previously reported optimised TBM sequence (Guettler et al., 2011) and fused to a cell-permeability conferring peptide, can compete with AXIN and abrogate Wnt signalling (Xu et al., 2017b) (Figure 5B). This proof-of-concept study will encourage further development of tankyrase substrate binding antagonists. While a recent tankyrase interactome study is in agreement with the notion that TNKS and TNKS2 are largely functionally redundant (Li et al., 2017), there may be benefit to selectively targeting either TNKS or TNKS2, should unique functions emerge in the future, which may be TNKS/TNKS2-intrinsic or result from other sources such as differential expression or regulation. One inhibitor study (see Table 1, compound **3**) suggests that, in principle, a certain degree of such selectivity can be achieved (Larsson et al., 2013).

Functional and pre-clinical studies of tankyrase inhibitors in CRC

Differential sensitivity of CRC cell lines to tankyrase inhibition

Most CRC tumours ($\approx$ 80%) are hemizygous for C-terminal truncations of APC (reviewed by Bodmer, 2006), focussed at a hotspot area known as the mutation cluster region (MCR) (Miyoshi et al., 1992; Kohler et al., 2008; reviewed by Minde et al., 2011) (Figure 6A). Following the N-terminal Armadillo repeat domain, APC contains three 15-amino-acid β-catenin-binding repeats, seven 20-amino-acid β-catenin-binding repeats and three interspersed AXIN-binding SAMP repeats, among other elements not required for APC's function in Wnt/β-catenin signalling (reviewed by Stamos and Weis, 2013). Dependent on the position of the truncating mutation, a variable number of these motifs is lost (Figure 6A). In APC-truncated CRC cells, an inability to assemble a functional β-catenin destruction complex underlies the accumulation of transcriptionally active β-catenin. A range of studies has investigated the responsiveness of model CRC cell lines to tankyrase inhibition (Lau et al., 2013; de la Roche et al., 2014; Tanaka et al., 2017). While it would have been conceivable that the assembly and function of the β-catenin destruction complex cannot be sufficiently rescued by tankyrase inhibition in the absence of fully functional APC, there clearly are cases in which dysregulated Wnt/β-catenin signalling can be curbed. In SW403 and COLO-320DM cells, both of which bear extensive C-terminal APC truncations (Figure 6A), tankyrase inhibition (by G007-LK, IWR-1 or XAV939) gives rise to AXIN2 stabilisation, the formation of β-catenin degradasomes (in COLO-320DM cells), a robust reduction in active (non-phosphorylated) β-catenin and prominently attenuated β-catenin-dependent transcription, both in reporter assays and at the level of endogenous Wnt/β-catenin target genes (Lau et al., 2013; de la Roche et al., 2014; Tanaka et al., 2017). Importantly, tankyrase inhibition limits the proliferation of these cells in cell culture (for G007-LK and IWR-1) and xenograft (for G007-LK) models (Lau et al., 2013; Tanaka et al., 2017). A similar cell response to tankyrase inhibition is observed in DLD-1 and HCT-15 cells, although the levels of active β-catenin are reduced less robustly and the transcriptional effect is more subtle (Lau et al., 2013; de la Roche et al., 2014; Tanaka et al., 2017). Conversely, in SW480 and SW620 cells, although tankyrase inhibition results in the stabilisation of AXIN2, degradasome formation and a strong decrease in active β-catenin levels, it fails to block Wnt/β-catenin target genes or the TOPFlash reporter (Lau et al., 2013; de la Roche et al., 2014). In yet another group of CRC cells (COLO-205, HT-29, HCC2998 and LS-411N), tankyrase inhibition increases AXIN1/2 levels but with only a modest or no decrease in active β-catenin levels (Lau et al., 2013; Tanaka et al., 2017). For KM12 cells, no effect on the levels of AXIN1/2 or active β-catenin levels and β-catenin-dependent transcription was observed upon tankyrase inhibition (Tanaka et al., 2017). As expected, Wnt/β-catenin signalling in CRC cells with oncogenic mutations in β-catenin (LS174T, HCT116) or β-catenin-independent CRC cells (RKO) are not sensitive to tankyrase inhibition (Lau et al., 2013; Tanaka et al., 2017).

Tankyrase inhibition can therefore restore, at least partially, β-catenin destruction complex function in a subset of APC-mutant CRC cells (Figure 6A). Why another subset of CRC cell lines is TNKSi-resistant is being investigated. It has been proposed that high levels of β-catenin in SW480 cells account for TNKSi resistance (Lau et al., 2013), but β-catenin levels in the TNKSi-sensitive COLO-320DM cells appear comparable (de la Roche et al., 2014). Compared to SW480, COLO-320DM cells appear to have higher levels of AXIN1, TNKS and phospho-β-catenin after tankyrase inhibition (de la Roche et al., 2014), suggesting that β-catenin may be sequestered in stalled destruction complexes, thereby limiting the availability of active β-catenin.

A potential correlation between the site of APC truncation and tankyrase inhibitor sensitivity has been explored, both in CRC cell lines and tumour-derived cells from patients (Tanaka et al., 2017). APC truncations removing all β-catenin-binding 20-amino-acid repeats (as in COLO-320DM and SW403 cells; Figure 6A) were proposed to render cells TNKSi-responsive at the level of cell proliferation and might serve as a predictive biomarker (Tanaka et al., 2017). Another distinguishing feature of these cells is their particularly strong Wnt/β-catenin pathway activity (Tanaka et al., 2017). The authors suggest that the longer APC variants of other cell lines act as hypomorphs maintaining a higher residual level of β-catenin regulation: their silencing further stabilises β-catenin. Tankyrase inhibitors (G007-LK, IWR-1) can revert this accumulation, but not reduce β-catenin abundance below its cell-characteristic elevated levels (Tanaka et al., 2017). In turn, cell proliferation remains unresponsive to tankyrase inhibition (Tanaka et al., 2017). The genetic background of additional cell lines and tumour samples will need to be explored to confirm the suitability of APC truncations as predictive biomarkers for TNKSi sensitivity. In line with a requirement of AXIN2 for degradasome formation (see above), tankyrase inhibitors depend on AXIN2 to reduce active β-catenin levels (Tanaka et al., 2017). Large APC truncations, eliciting high β-catenin activity and thus *AXIN2* gene transcription, may be required for the TNKSi-induced accumulation of sufficient amounts of AXIN2. Indeed, absolute *AXIN2* mRNA levels are high in COLO-320DM cells, and upon tankyrase inhibition remain higher than in many other CRC cell lines (Tanaka et al., 2017).

Upon prolonged Wnt stimulation, sequestration of β-catenin in nuclear transcriptional complexes may shield β-catenin from the β-catenin destruction complex and account for TNKSi (XAV939) resistance (de la Roche et al., 2014). High expression levels of LEF1 and B9L and a CRC environment providing sustained Wnt levels are potential predictors of tankyrase inhibitor resistance (de la Roche et al., 2014). Moreover, the acquisition of APC mutations is considered an early event in the emergence of CRC, and secondary mutations in genes such as *KRAS*, *P53* and *SMAD4* contribute to driving carcinogenesis (Drost et al., 2015); such mutations may modulate the TNKSi response, although this remains speculative. Of note, DLD-1 colony formation can be

inhibited with XAV939 under low- but not high-serum conditions (Huang et al., 2009; Bao et al., 2012; Lau et al., 2013), and similarly DLD-1 and HCT-15 colony formation does not respond to G007-LK at high serum, while that of COLO-320DM and SW403 cells does (Lau et al., 2013). This points to additional sensitivity determinants outside the APC and β-catenin mutational landscape, although simple compound sequestration by serum components may in some cases also contribute. In support of a more complex determination of sensitivity, Mashima *et al*. generated a TNKSi-resistant COLO-320DM line, showing decreased Wnt/β-catenin signalling and upregulated mTOR signalling (Mashima et al., 2017). These cells were generated to tolerate IWR-1 but also displayed considerable resistance to G007-LK. The authors showed that the mTOR pathway determines TNKSi resistance in these cells. In conclusion, we need to better understand how the genetic and signalling profile of CRC cells and tumours affects TNKSi responsiveness. Furthermore, a much deeper analysis of the β-catenin destruction complex and the Wnt signalosome is required to appreciate the mechanisms by which tankyrase inhibition affects the molecular events underlying Wnt/β-catenin signalling, both in the context of wild-type and mutant APC.

Tankyrase inhibitors in murine models

In-vivo preclinical studies have demonstrated the anti-tumour activity of various tankyrase inhibitors (Waaler et al., 2011; 2012; Lau et al., 2013). JW74 was studied in both xenograft and the Apc^{Min} mouse models and reported to be well tolerated while reducing both the total tumour load in the small intestine and the tumour number in the colon (Waaler et al., 2011). Similar observations were made for JW55 in mice with a conditional Apc truncation in the ISC compartment (Waaler et al., 2012). An improved derivative of JW74, G007-LK, which inhibits tankyrase with double-digit nanomolar IC$_{50}$ values and good specificity (Table 1) (Voronkov et al., 2013), decreases the tumour area in the small intestine of these mice by approximately two thirds and shows significant inhibition of tumour growth in various xenograft models with APC-mutant human cell lines (SW480, COLO-320DM and SW403) (Lau et al., 2013).

Intestinal toxicity remains a major challenge for many Wnt/β-catenin pathway inhibitors (reviewed by Kahn, 2014). A more careful analysis of the effects of G007-LK at dose-limiting levels revealed reduced cell proliferation in crypt bases of the small intestine, inflammation, necrosis, disrupted epithelial architecture, with ensuing weight loss and morbidity (Lau et al., 2013). Whether the observed toxicity is reversible has not been explored. Conversely, in a study investigating the role of tankyrase in glucose metabolism, the long-term (six-month) treatment of mice with G007-LK at a lower dose did not result in detectable toxicity despite the observed stabilisation of Axin1 (Zhong et al., 2016a). Since the expression of the Wnt pathway antagonist Dkk1 (Dickkopf-related protein 1) in the gut epithelium gives rise to similar toxicity (Pinto et al., 2003; Kuhnert et al., 2004; Lau et

al., 2013), it is likely that TNKSi toxicity is an on-target, Wnt/β-catenin pathway-specific effect. A mouse xenograft study evaluating the tankyrase inhibitor G-631 (patent by Feng et al., 2013) also revealed considerable intestinal toxicity, even at sub-therapeutic doses (Zhong et al., 2016b). Importantly, ablation of Wnt signalling by either adenoviral Dkk1 expression or treatment with G-631 is reversible (Kuhnert et al., 2004; Zhong et al., 2016b).

While toxicity poses challenges in continued efforts to explore the therapeutic potential of tankyrase inhibitors, it is no reason to be discouraged. Does TNKSi toxicity indeed reflect on-target or off-target action of the inhibitors? What is the tissue distribution of the compounds? For example, do they accumulate in the gastrointestinal tract, aggravating toxicity? Do chemically distinct tankyrase inhibitors display similar signs of toxicity? The fact that SW480 cell xenograft growth can be contained by tankyrase inhibition despite the incomplete penetrance on β-catenin dependent transcription (see above) suggests that full inhibition of signalling might not be required for a therapeutic effect; so what degree of β-catenin inhibition translates into a biological effect? Moreover, different dosage and timing regimes could be implemented to manage known toxicities (reviewed by Meric-Bernstam and Mills, 2012). Encouragingly, inhibitors of the acyl transferase Porcupine, which palimitoylates Wnt during its biogenesis, show limited intestinal toxicity at effective doses, suggesting that substantial therapeutic windows can be achieved by targeting the Wnt/β-catenin pathway (Liu et al., 2013; Proffitt et al., 2013; reviewed by Madan and Virshup, 2015). The point of intervention in the pathway, drug specificity and potency, pharmacokinetics, functional redundancy of targeted pathway components and the genetic background of the tumour cells may all define the therapeutic window. Combination with inhibitors targeting additional cancer dependencies (e.g., EGFR and PI3K-AKT) provides another possible strategy for increasing the effectiveness of tankyrase inhibitors while ensuring their safety (Casas-Selves et al., 2012; Tenbaum et al., 2012; Arques et al., 2016).

A deeper knowledge of the responses and toxicities elicited by Wnt/β-catenin pathway modulators in different species is much needed to exploit β-catenin dependencies in cancer. With the vast range of known and putative tankyrase targets (Guettler et al., 2011; Li et al., 2017), it would be surprising if TNKSi effects and toxicities were entirely due to Wnt/β-catenin pathway inhibition. Another prominent system highly relevant to the human stem cell compartment is telomere length homeostasis, which is also regulated by tankyrase, as is sister telomere resolution in mitosis (Smith et al., 1998; Smith and de Lange, 2000; Canudas et al., 2007; Kulak et al., 2015). Telomeric functions require tankyrase to bind TRF1/TERF1 (telomeric repeat-binding factor 1). Importantly, telomere regulation by tankyrase is not conserved in mice since murine Trf1/Terf1 lacks the TBM (Figure 6B) and does not bind tankyrase (Muramatsu et al., 2007; reviewed by Hsiao and Smith, 2008). Consistent with an absent role of telomeric tankyrase functions in mice, Tnks and Tnks2

knockout mice do not display any telomere phenotype (Hsiao et al., 2006; Chiang et al., 2008), but a definitive answer will be obtained from comparing murine and human cells deficient in both tankyrases. Therefore, pre-clinical studies of tankyrase inhibitors in mice and other species lacking the Terf1 TBM (e.g., zebrafish, Figure 6B) are unlikely to predict the full extent of biological effects and toxicity in humans. Conversely, rabbits or Chinese hamsters, for example, both display a functional TBM in Terf1 and might be more suitable models for studying the *in-vivo* consequences of tankyrase inhibition, at least with regards to Wnt/β-catenin signalling and telomere maintenance. In rats, the somewhat stronger deviation of the TBM will require a prior validation of a telomeric role for tankyrase (Muramatsu et al., 2007). Telomere maintenance in *Drosophila* occurs via a transposon-mediated mechanism rather than telomerase (Villasante et al., 2008), and a telomeric tankyrase link in flies is therefore unlikely. While Wg/Armadillo pathway regulation by tankyrase is clearly evident in *Drosophila*, some mechanistic aspects may be different, for example given that *Drosophila* Axin only bears a single TBM (Figure 2C).

Outstanding Questions

The past few years have seen a rapid progress in our understanding of how Wnt/β-catenin signalling is regulated by PARylation and tankyrase. Tankyrase is now an established core component of the Wnt/β-catenin network. Nonetheless, we are still far from fully understanding the complex roles tankyrase plays in the pathway. How does tankyrase promote Wnt/β-catenin signalling non-catalytically, and do these mechanisms contribute to TNKSi resistance? Does scaffolding through tankyrase directly control β-catenin degradasome assembly, and how is this process regulated? How does AXIN PARylation promote the function of the Wnt signalosome? It will be interesting to explore the consequences of AXIN PARylation on both its conformation and interactions with components of both the signalosome and degradasome complexes. Given early indications of a role for tankyrase in APC-regulated destabilisation of AXIN, additional work is needed to decipher how APC limits AXIN abundance. Tankyrase inhibitors have now reached a remarkable specificity. The continued exploration of their pharmacodynamics, the identification of potential biomarkers for their therapeutic implementation and the in-depth analysis of emerging resistance and toxicity mechanisms are important avenues for future research. The latter will require a careful choice of model systems. The development of alternative tankyrase inhibition strategies through interfering with tankyrase's non-catalytic scaffolding functions, in particular substrate binding, will undoubtedly offer new and exciting opportunities to understand tankyrase function and explore alternative therapeutic strategies.

Nomenclature of Targets and Ligands

Acknowledgements

Conflicts of interest

The authors declare that they have no conflicts of interest.

Figure Legends

Figure 1: Roles of tankyrase-dependent poly(ADP-ribosyl)ation in Wnt/β-catenin signalling.
(A) Under basal Wnt/β-catenin signalling conditions, PARylation by tankyrase limits the levels of AXIN. Following PARylation, AXIN is ubiquitylated by RNF146 and targeted for proteasomal degradation. **(B)** Upon Wnt stimulation, PARylated AXIN is stabilised. PARylation facilitates AXIN interaction with LRP5/6 in Wnt signalosomes. Note that AXIN, Dishevelled and tankyrase polymerise and APC dimerises, and that this is a mechanistically important aspect of the dynamic signalling complexes (Fiedler et al., 2011; Kunttas-Tatli et al., 2014; Mariotti et al., 2016). For simplicity, proteins are shown as monomers; higher-order stoichiometry and multivalency are not reflected in the cartoons, and nomenclature does not consider multiple paralogues of pathway components.

Figure 2: Scaffolding functions of tankyrase.
(A) Domain organisation of human TNKS and TNKS2. HPS, N-terminal extension containing homopolymeric stretches of His, Pro and Ser; ARCs, ankyrin repeat clusters; SAM, sterile alpha motif domain; PARP, catalytic domain. The percentage identity of amino acids between TNKS and TNKS2 is specified for the indicated functional domains. **(B)** Domain organisation of AXIN1. TBM, tankyrase-binding motif; RGS, regulator of G-protein signalling domain; DAX, polymerising

domain present in <u>D</u>ishevelled and <u>AX</u>IN. Binding sites for other β-catenin destruction complex components are indicated. **(C)** Multiple sequence alignment of AXIN orthologues/paralogues from the indicated species, coloured by percentage identity. The TBMs are indicated. Note that *Drosophila* Axin lacks the second TBM. The red asterisk denotes a V26D mutation identified in murine Axin2 (Qian et al., 2011). **(D)** Structural (surface and cartoon) representation of murine Tnks ARC2-3, bound to the murine Axin1 N-terminus with two TBMs, shown in stick representation (PDB code 3UTM) (Morrone et al., 2012). In the crystal, ARC2-3 forms a dimer in which both copies of ARC2 are bound by one of the two TBMs of Axin1, respectively. **(E)** Detailed structural representation of the Axin1 TBMs (with indicated amino acid positions) on Tnks ARC2. The figure was generated by superimposing both ARC2-3 copies onto each other and displaying ARC2 bound to TBM1. Despite the N-terminal insertion in TBM2, the arginine (typically at position 1) occupies the same sub-pocket on the ARC, resulting in a looping out of the intervening residues. **(F)** Structural (transparent surface and cartoon) representation of a TNKS SAM polymer observed by X-ray crystallography (PDB code 5JU5) (Mariotti et al., 2016). **(G)** Avidity model for the interaction of AXIN and tankyrase, modified from Mariotti *et al.*, 2016. Multivalency and polymerisation of both tankyrase and AXIN enable avidity contributions in the interaction between both proteins. Note that tankyrase polymerisation also promotes its PARP activity (Mariotti et al., 2016; Riccio et al., 2016).

Figure 3: β-catenin degradasomes induced by tankyrase inhibitors.

SW480 CRC cells were treated with the tankyrase inhibitor XAV939 and immunostained for AXIN2 (red) and transiently expressed epitope-tagged wild-type TNKS2 (TNKS2 WT) or a TNKS2 mutant variant deficient in substrate binding (through site-directed mutation of ARCs 1, 2, 4 and 5) and polymerisation (TNKS2 xx3xx VY903/920WA) (green). Yellow arrowheads indicate colocalisation of TNKS2 and AXIN2 in β-catenin degradasomes; red arrowheads indicate absence of colocalisation for the scaffolding-defective mutant variant of TNKS2. The figure was modified from Mariotti et al., 2016.

Figure 4: Allosteric regulation of RNF146/Iduna by PAR binding.

(A) Structure of a linear PAR chain, here attached to Asp/Glu. The O-glycosidic bonds linking ADP-ribose units are highlighted. The green box indicates iso-ADP-ribose. Tankyrase is thought to generate linear PAR chains (Rippmann et al., 2002); PAR branches are therefore omitted. **(B)** Domain organisation of human RNF146. Potential tankyrase-binding motifs (TBMs) are indicated (DaRosa et al., 2015). The boxed area, which includes the isolated RING domain, corresponds to (C). **(C)** Structural representation of RNF146 bound to iso-ADP-ribose (PDB code 4QPL) and the

E2 enzyme UbcH5a (DaRosa et al., 2015). The isolated RING domain of RNF146 (PDB code 2D8T, one representative of the solution structure ensemble) is superimposed (DaRosa et al., 2015). Domains and corresponding Zn^{2+} ions are colour-coded as in (B). Key residues involved in PAR coordination and the allosteric switch are shown in stick representation. Note the clash occurring between the RING domain (yellow) and the E2 enzyme (blue) in the absence of the PAR ligand, and the conformational change upon PAR binding, resulting in a reorientation of Trp65.

Figure 5: Binding modes of catalytic tankyrase inhibitors and alternative inhibition strategy.
(A) Structural representation of TNKS/TNKS2 PARP domain:inhibitor complexes. Residues of the catalytic H-Y-E triad are indicated. Adenosine and nicotinamide sites are highlighted with blue and orange dashed circles, respectively, in the left and central panels, respectively. Left, structure of the TNKS2 PARP domain with the adenosine site binder G007-LK (shown in blue) (PDB code 4HYF) (Voronkov et al., 2013). Loops lining the inhibitor binding site are indicated. Centre, structure of the TNKS PARP domain with the nicotinamide site binder XAV939 (shown in orange) (PDB code 3UH4) (Kirby et al., 2012). Right, structure of the TNKS PARP domain with a dual site binder (shown in green) (PDB code 4I9I) (Bregman et al., 2013b). The donor and acceptor sites are highlighted in magenta. **(B)** Structural representation of TNKS2 ARC4 (in surface representation) bound to two macrocyclised TBM peptides (PDB codes 5BXO and 5BXU) (Xu et al., 2017b). The peptide sequence is shown on the right with the position of the two different peptide staples, whose structures are shown. Amino acid positions of the TBM are indicated.
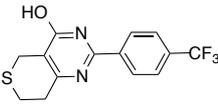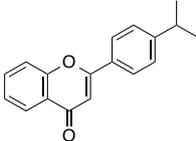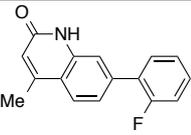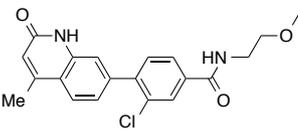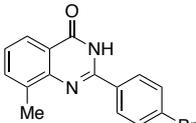
Figure 6: Potential determinants of tankyrase inhibitor responses: APC mutation status and telomeric roles of tankyrase.
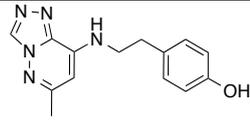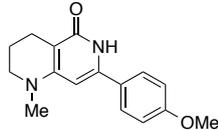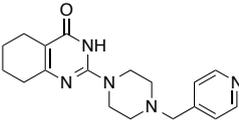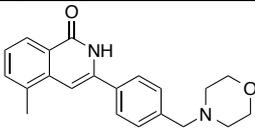**(A)** Schematic representation of APC with domains and motifs drawn to scale (reviewed by Stamos and Weis, 2013). 15- and 20-amino-acid-repeats (15R and 20R) bind β-cateni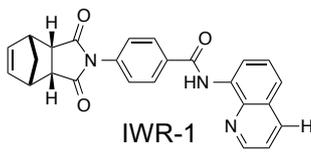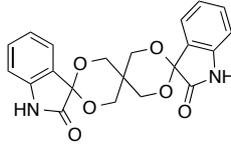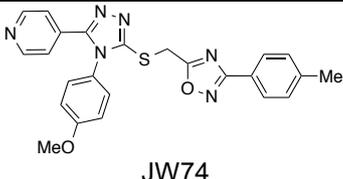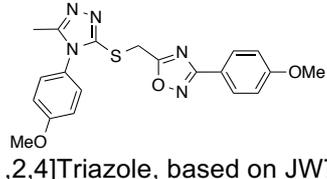n (except for 20R2), with the affinity of the 20Rs for β-catenin being enhanced by their phosphorylation (Eklof Spink et al., 2001; Ha et al., 2004; Xing et al., 2004). SAMP repeats bind to AXIN1/2 (Spink et al., 2000). 20R2 and the catenin interaction domain (CID) / region B are required for β-catenin ubiquitylation but do not bind β-catenin; instead, CID / region B regulate AXIN1/2 binding to APC and are proposed to bind α-catenin (Liu et al., 2006; Kohler et al., 2008; Choi et al., 2013; Pronobis et al., 2015). The mutation cluster region (MCR), a mutation hotspot in CRC (Kohler et al., 2008), is indicated in magenta. APC truncations observed in commonly used CRC cell lines are indicated by the arrows (Rowan et al., 2000; Ikediobi et al., 2006); labels are colour-coded according to the indicated effects of tankyrase inhibitors on AXIN and non-phospho (active) β-catenin levels and β-catenin-dependent transcription. *Note that the classification of DLD-1 and HCT-15 cells as

TNKSi-sensitive or -resistant varies between studies, given an 'intermediate' response (Huang et al., 2009; Lau et al., 2013; de la Roche et al., 2014; Tanaka et al., 2017). Very low AXIN1/2 levels in KM12 cells (Tanaka et al., 2017) may be responsible for non-detectable AXIN accumulation upon tankyrase inhibition. **(B)** Multiple sequence alignment of the N-termini of TERF1/TRF1 (telomeric repeat binding factor 1) orthologues from the indicated species, coloured by percentage identity. The amino acid numbering refers to human TERF1. The 8-amino-acid TBM is boxed in red. The murine Terf1 orthologue sequence is boxed in yellow and shows no conservation of the TBM.

Table 1: Tankyrase inhibitors.

The structures and properties of reported tankyrase-selective inhibitors are shown, grouped according to binding site. Dual site binders occupy both the nicotinamide and adenosine subsites. One example structure is given if numerous similar compounds in a series were detailed in the literature. Biochemical IC_{50} values (i.e., corresponding to inhibition of PARP activity) for TNKS, TNKS2, PARP1 and PARP2 and IC_{50} values in Wnt/β-catenin reporter assays are listed if reported. "-" indicates that values have not been reported. Note that NVP-TNKS656 is reported as a "triple site binder", also interacting with a hydrophobic 'nook' adjacent to the phosphate-binding groove (Shultz et al., 2012; 2013). *Although the IC_{50} for PARP1/2 was not determined for compound **9** (Elliott et al., 2015), other compounds in the same series displayed IC_{50} values for PARP1/2 of at least one order of magnitude above those for TNKS (Elliott et al., 2015; Paine et al., 2015).

No.	Tankyrase Inhibitor	TNKS IC_{50} (nM)	TNKS2 IC_{50} (nM)	PARP1 [PARP2] IC_{50} (nM)	IC_{50} in Wnt reporter assay (nM)	Binding Site	References
1	XAV939	11	4	PARP domain: 2194 [114] full-length: 74 [27]	78 (HEK293, Wnt-3a)	Nicotinamide	(Huang et al., 2009; Karlberg et al., 2010; Kirby et al., 2012; Shultz et al., 2012; Thorsell et al., 2017)
2	Flavone-based	6	72	19,100 [34,900]	-	Nicotinamide	(Narwal et al., 2013a; 2013b)
3	Quinolinyl-phenyl-based	860	52	>10,000 [>10,000]	-	Nicotinamide	(Larsson et al., 2013)
4	Quinolinyl-benzamide	-	9	-	-	Nicotinamide	(Larsson et al., 2013)
5	2-arylquinazolin-4-one	32	9	>5,000	-	Nicotinamide	(Nathubhai et al., 2013; 2016)

#	Structure						
6	Triazolo piridazine	12	200	>10,000 [>10,000]	-	Nicotinamide	(Liscio et al., 2014)
7	Tetrahydro-1,6-naphthyridinone	1.7	1.1	3,400	-	Nicotinamide	(Kumpan et al., 2015)
8	Tetrahydroquinazolinone-based	370	71	5,900 [4,200]	-	Nicotinamide	(Nkizinkiko et al., 2015)
9	Isoquinolin-1-one	12	-	- *	25 (DLD-1)	Nicotinamide	(Elliott et al., 2015; Paine et al., 2015)
10	Tetrazoloquinoxaline	32	-	>1,995	32 (HEK293, Wnt-3a)	Nicotinamide	(Thomson et al., 2017)
11	G-631	8	24	>10,000	8 (HEK293, Wnt-3a)	Nicotinamide	(patent by Feng et al., 2013; Zhong et al., 2016b)
12	IWR-1	131	56	>18,750 [>18,750]	180 (L cells, Wnt-3a)	Adenosine	(Chen et al., 2009; Lu et al., 2009; Huang et al., 2009; Gunaydin et al., 2012)
13	JW67	-	-	-	1,170 (HEK293, Wnt-3a)	Unknown	(Waaler et al., 2011)
14	JW74	2,550	650	-	790 (HEK293, Wnt-3a)	Adenosine	(Waaler et al., 2011; Shultz et al., 2012; Voronkov et al., 2013)
15	[1,2,4]Triazole, based on JW74	-	33	>19,000 [>19,000]	215 (HEK293, Wnt-3a)	Adenosine	(Shultz et al., 2012)
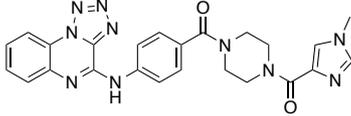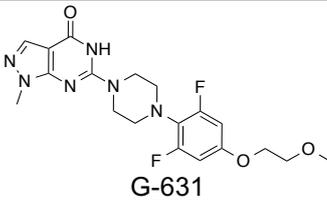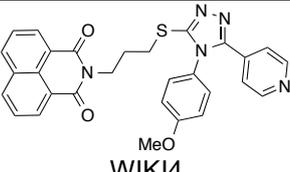
16	 WIKI4	26	15	>10,000 [>10,000]	-	Adenosine	(James et al., 2012; Haikarainen et al., 2013)
17	 JW55	1,800	2,010	-	1,230 (HEK293, Wnt-3a)	Adenosine	(Waaler et al., 2012; Haikarainen et al., 2016)
18	 G007-LK	46	25	>10,000 [>10,000]	50 (HEK293, Wnt-3a)	Adenosine	(Lau et al., 2013; Voronkov et al., 2013)
19	 Oxazolidinone	1	-	>85,000 [>170,000]	-	Adenosine	(Bregman et al., 2013a)
20	 Oxazolidinone-based	2	2	>85,000 [>170,000]	12 (DLD-1)	Adenosine	(Huang et al., 2013)
21	 K-756	31	36	-	110 (DLD-1)	Adenosine	(Okada-Iwasaki et al., 2016)
22	 Long quinazolinone	8	2	[931]	36,000 (HEK293, Wnt-3a)	Dual site	(Bregman et al., 2013b)
23	 Long quinazolinone-based	0.2	2.5	-	1.3 (DLD-1)	Dual site	(Hua et al., 2013)
24	 Long quinazolinone-based	5.1	0.1	6,500 [11,600]	37 (HEK293, Wnt-3a)	Dual site	(Nathubhai et al., 2017)
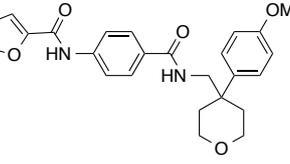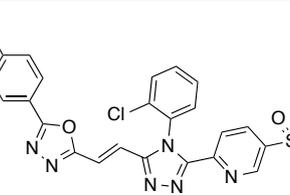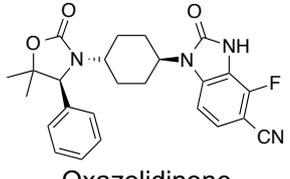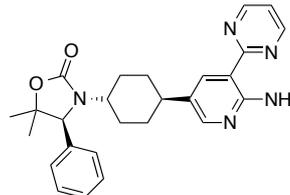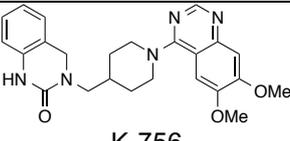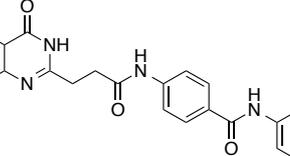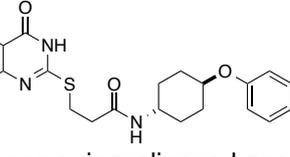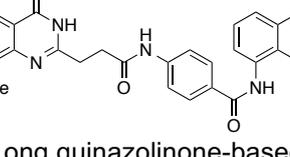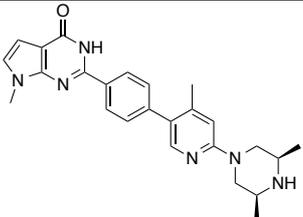
#	Structure						
25	 Pyrimidinone	3	1	2.0 [0.5]	5 (DLD-1)	Dual site	(Johannes et al., 2015a)
26	 NVP-TNKS656	-	6	>19,000 [>30,000]	3.5 (HEK293, Wnt-3a)	Dual site	(Shultz et al., 2013)
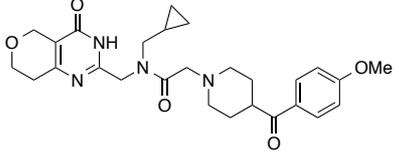
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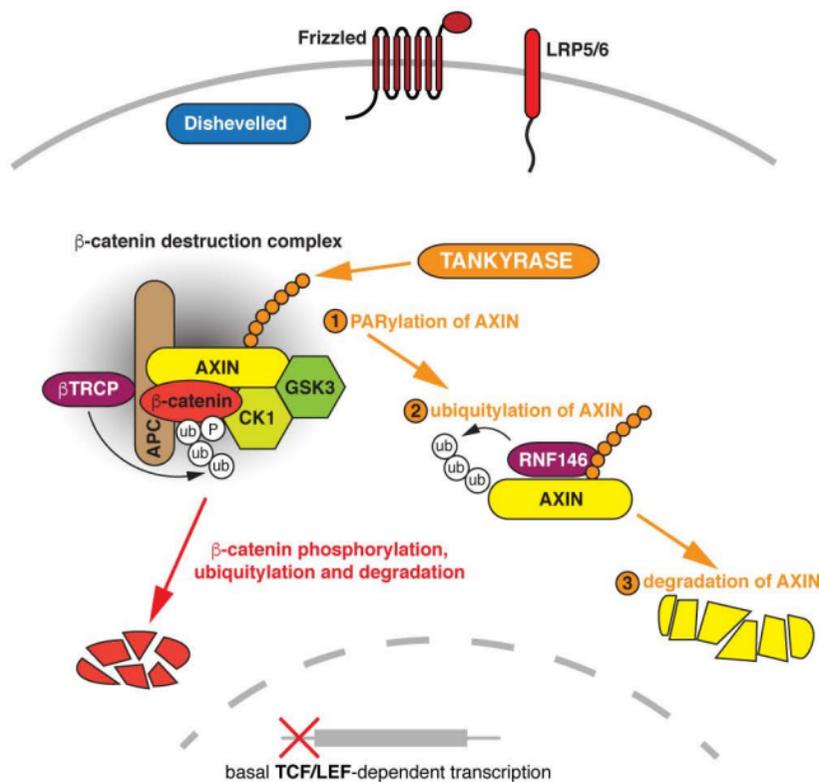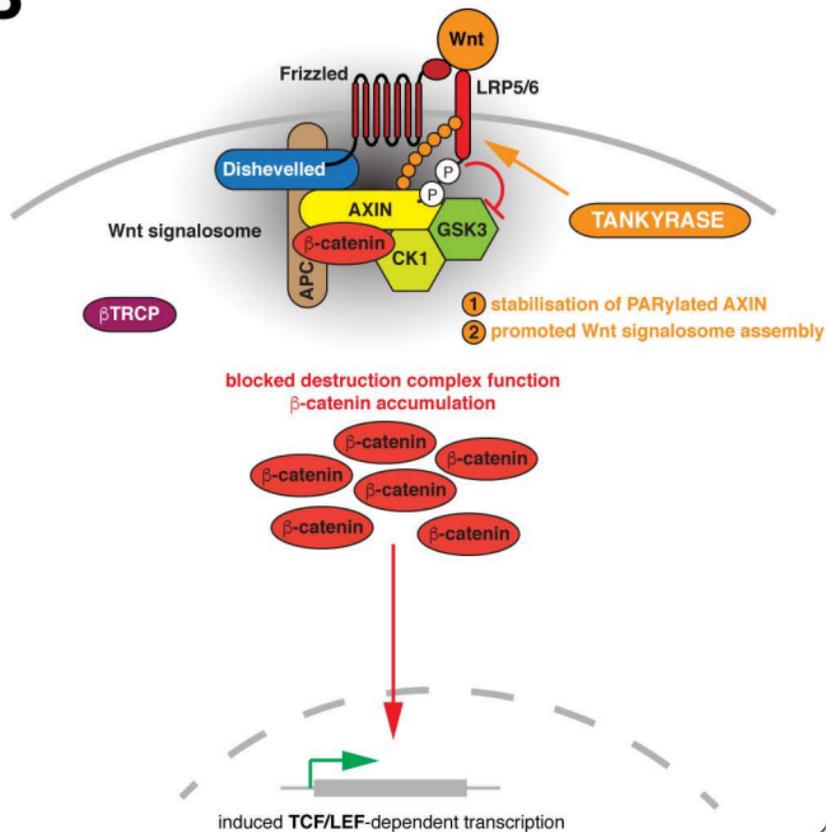
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basal state

A

Frizzled

LRP5/6

Dishevelled

β-catenin destruction complex

TANKYRASE

① PARylation of AXIN

AXIN

βTRCP

β-catenin

GSK3

APC

CK1

ub P

ub

ub

② ubiquitylation of AXIN

ub

ub

ub

RNF146

AXIN

β-catenin phosphorylation, ubiquitylation and degradation

③ degradation of AXIN

basal **TCF/LEF**-dependent transcription

B

upon Wnt stimulation

Wnt

Frizzled

LRP5/6

Dishevelled

Wnt signalosome

AXIN

β-catenin

APC

GSK3

CK1

P

P

TANKYRASE

βTRCP

① stabilisation of PARylated AXIN
② promoted Wnt signalosome assembly

blocked destruction complex function
β-catenin accumulation

β-catenin

β-catenin

β-catenin

β-catenin

β-catenin

β-catenin

β-catenin

β-catenin

induced **TCF/LEF**-dependent transcription

Guettler **Figure 1**

Guettler **Figure 2**

TNKS2   AXIN2   TNKS2 AXIN2 DAPI

TNKS2 WT

xx3xx
VY902/920WA

10 μm

Guettler **Figure 3**

A

iso-ADP-ribose

Asp/Glu

Protein

B

RNF146 1

RING

RING

WWE

potential TBMs

359

C

UbcH5a (E2)

WWE

iso-ADP-ribose

W65

K61

W65

Zn²⁺

RING

RNF146 RING only

Guettler **Figure 4**

A

adenosine site binder
G007-LK

D-loop Y1060

E1138

H1031 TNKS2
F-loop G-loop

nicotinamide site binder
XAV939

Y1213

E1291

H1184 TNKS

dual site binder
quinazolinone compound

acceptor site

donor site TNKS

B

TNKS2 ARC4

6 5
7
4 macrocyclic TBM peptides
C
8
3
2
1 N
staple

n=4
m=3/5

RE X GDG X E
1 2 3 4 5 6 7 8

Guettler **Figure 5**

A

APC 1 ... 2843

15-amino-acid repeats
20-amino-acid repeats
SAMP repeats
CID (β-catenin inhibitory domain) / region B

+ + + - stabilised AXIN1 or AXIN2
+ + - - decreased non-phospho-β-catenin
+ - - - decreased β-catenin-dependent transcription

B

TERF1 Orthologues

TBM

Hs (Human)
Pt (Chimpanzee)
Ggg (Gorilla)
Mm (Rhesus macaque)
Pa (Olive baboon)
Cj (White-tufted-ear marmoset)
Cs (Green monkey)
Bt (Cow)
Mr (Reeves's muntjac)
Eq (Horse)
Sc (Wild pig)
Clf (Dog)
Fsc (Domestic cat)
Am (Giant panda)
Oc (Rabbit)
Cg (Chinese hamster)
Rn (Brown rat)
Cp (Guinea pig)
Mm (House mouse)
Ml (Little brown bat)
Gg (Chicken)
Xl (African clawed frog)
Xt (Western clawed frog)
Ac (Carolina anole)
Oc (Duckbill platypus)
Dr (Zebrafish)

Guettler **Figure 6**