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Winding back Wnt signalling: potential therapeutic targets for treating gastric cancers

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Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015a; Alexander et al., 2015b).

Abstract

Gastric cancer persists as a frequent and deadly disease that claims over 700,000 lives annually. Gastric cancer is a multifactorial disease that is genetically, cytologically and architecturally more heterogeneous than other gastrointestinal cancers, making it

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therapeutically challenging. As such, and largely attributed to late-stage diagnosis, gastric cancer patients show only partial response to standard chemo and targeted molecular therapies, highlighting an urgency to develop new targeted therapies for this disease. Wnt signalling has a well-documented history in the genesis of many cancers and is therefore an attractive therapeutic target. As such, drug discovery has focused on developing inhibitors that target multiple nodes of the Wnt signal cascade, some of which have progressed to clinical trials. The collective efforts of patient genomic profiling has uncovered genetic lesions to multiple components of the Wnt pathway in gastric cancer patients, which strongly suggest that Wnt targeted therapies could offer therapeutic benefit for gastric cancer patients. These data have been supported by studies in mouse models of gastric cancer, which identify Wnt signalling as a driver of gastric tumourigenesis. Here we review the current literature regarding Wnt signalling in gastric cancer, and highlight the suitability of each class of Wnt inhibitor for the potential treatment of gastric cancer patients, in relation to the type of Wnt deregulation observed.

Abbreviations

ACh-M_3	Achetylcholine-muscarinic receptor-3
APC	Adenomatous polyposis coli
BCL-9	B-cell lymphoma-9
BRAF	Proto-oncogene B-Raf
CagA	Cytotoxin-associated gene product
CBP	cAMP response element-binding protein
CDH1	Cadherin-1
CDX2	Caudal-related homeobox transcription factor 2
CIN	Chromosomal instable
CK1	Casein kinase 1
CpG	Cytosine-phosphate-Guanine
CRC	Colorectal Cancer
CRD	Cysteine rich domain

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CSC	Cancer stem cell
CTNNB1	β-catenin
CXCR4	C-X-C chemokine receptor type 4
Dkk	Dickkopf
Dvl	Dishevelled
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FAP	Familial Adenomatous Polyposis
FOLFIRI	Folinic Acid, Fluorouracil, Irinotecan
FOXFOL	Folinic Acid, Fluorouracil, Oxaliplatin
FZD	Frizzled
GAPPS	Gastric adenocarcinoma and proximal polyposis of the stomach
GC	Gastric cancer
GEJ	Gastroesophageal junction
GSK3	Glycogen synthase kinase-3
H. pylori	*Helicobacter Pylori*
HER2	Human epidermal growth factor receptor 2
IgG	Immunoglobulin G
IWP-2	Inhibitor of Wnt production-2
LEF1	Lymphoid enhancer binding factor-1
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor-5
LOF	Loss of function
LOH	Loss of heterozygosity
LRP5/6	Low-density lipoprotein receptor-related protein 5 or 6
Mist-1	Muscle, intestine and stomach expression-1
MMTV	Mouse Mammary tumour virus
MSI	Microsatellite instability
MSS	Microsatellite stable
MUC2	Mucin 2
NGF	Nerve growth factor

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p300	Histone acetyltransferase p300
PARP1	Poly (ADP-ribose) polymerase 1
PARsylation	Poly(ADP-ribosyl)ation
PORCN	Porcupine
PUMA	p53 up-regulated modulator of apoptosis
RGS	Regulator of G-protein signalling
RNF43	Ring finger 43
R-Spo	R-Spondin
sFRP	Secreted Frizzled Related Proteins
SPEM	Spasmolytic polypeptide-expressing metaplasia
T4SS	Type IV secretion system
TCF4	T-cell factor-4
TNK1/2	Tankyrase 1/2
TOP	TCF optimal promoter
VEGFR-2	Vascular endothelial growth factor receptor-2
YAP	Yes-associated protein
YY1	Ying Yang-1
ZNRF3	Zinc-ring finger 3

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Introduction

Gastric cancer (GC) is a frequent malignancy and is the third most common form of cancer related death world-wide (Guggenheim & Shah, 2013). Approximately 1,000,000 new cases of GC are diagnosed annually, with a large proportion of cases reported within East Asia, South America and Eastern Europe (Rahman, Asombang & Ibdah, 2014). Wnt signalling regulates many cell functions, including proliferation, migration and cell death, and although it is essential for development and homeostasis of several tissues, it is also deregulated in many cancers. The link between aberrant Wnt signalling and cancer is well characterised in several cancers such as the intestine, breast and liver, however its importance in the stomach is less well understood. This review will describe the current position of the field regarding the

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role of Wnt signalling in gastric cancer and how we might target the Wnt pathway to treat gastric cancer patients.

Homeostasis of the stomach

The mammalian stomach is divided into two anatomically distinct regions; the corpus and the antrum. The corpus is responsible for the main digestive action of the stomach, releasing a cocktail of acids, enzymes and hormones whereas the antrum produces large amounts of mucus and gastric hormones (Figure 1A). The stomach is lined by a simple columnar epithelium that is constantly renewed, a process driven by resident adult stem cells (Barker et al., 2010; Hoffmann, 2008). The gastric epithelium is organised into numerous mucosal invaginations called gastric units. Each gastric unit houses stem cells as well as the various differentiated cell types that perform distinct functions; mucus cells that secrete protective mucus, parietal cells responsible for secreting hydrochloric acid, chief cells that release active pepsin, and several types of endocrine cells that secrete an array of hormones that aid and regulate digestion and absorption (Kim & Shivdasani, 2016). The precise architecture, cellular heterogeneity and turnover rate of the gastric units varies markedly between the antrum and corpus (Figures 1B and C) (Barker, Bartfeld & Clevers, 2010; Karam & Leblond, 1993). There are many facets that help regulate gastric epithelial homeostasis including key developmental pathways and interactions from underlying stromal and nerve cells (Hayakawa et al., 2015; Kim & Shivdasani, 2016). High Wnt signalling is observed transiently in the developing forestomach (Kim, Buchner, Miletich, Sharpe & Shivdasani, 2005), whilst in the adult stomach expression is highest in the antrum where the Wnt target gene Leucine-rich repeat-containing G-protein coupled receptor (*Lgr5*) marks a population of stem cells (Barker et al., 2010). Further functional experiments will be required to elucidate the full requirement for Wnt signalling during gastric homeostasis, as was recently shown for Notch signalling (Demitrack et al., 2015; Kim & Shivdasani, 2011), but its critical inclusion in the culture medium of gastric organoids advocates its importance (Barker et al., 2010).

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Histopathology of gastric cancer

Gastric cancer can be divided pathologically into broad classes; intestinal-type and diffuse-type as classified by Lauren (Lauren, 1965). Of note, each of these classes can be further broken down into sub-classes based on histopathological, anatomical and genomic characteristics. Proximal intestinal-type gastric, also referred to as proximal non-diffuse gastric cancer, is defined by tumours located in the gastric cardia, which may extend into the gastroesophageal junction (GEJ) (Figure 1A). Histological analysis of proximal intestinal-type gastric cancer reveals glandular dysplasia, which can be accompanied by chronic inflammation (Shah et al., 2011). However, unlike the chronic inflammation associated with distal intestinal-type gastric cancer, which is often connected to *Helicobacter Pylori (H. pylori)*, carcinogenic inflammation of proximal intestinal-type gastric cancer is often linked to gastric acid reflux (Crew & Neugut, 2006). Distal intestinal-type (non-diffuse) gastric cancers are primarily located in the antrum, but can occasionally be found in the body of the stomach (Wong & Yau, 2013) (Figure 1A). Distal intestinal-type gastric cancers are well-differentiated and are composed of neoplastic gland-forming cells. Furthermore, this type of gastric tumour is often associated with *H. pylori* infection, the histopathology of which has been well characterised (Wong, 2016). Finally, diffuse gastric cancer, which is characterised by a diffuse pattern of cell infiltrate and poorly differentiated signet-ring cell clusters, is considered to arise *de novo* and is associated with loss of cell-cell contact via downregulation/mutation to *CDH1* (E-cadherin) (Guilford et al., 1998; Guilford et al., 1999).

While surgical intervention is the common practice for GC treatment, most patients are often at an advanced stage of disease progression at the time of diagnosis, which limits the value of surgery. As such, chemotherapy is the next appropriate countermeasure for these patients. However, only 20-40% of patients respond to first-line chemotherapies that target DNA replication and repair mechanisms (FOLFOX, FOLFIRI), with a median overall survival of 6-11 months (Jemal, Bray, Center, Ferlay, Ward & Forman, 2011). The past decades of fervent research into gastric

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tumour biology has significantly illuminated our understanding of tumour genomics, heterogeneity, immunoediting and drug resistance, which have culminated in the development of molecular targeted therapies. Targeted therapy not only confers higher anti-cancer specificity and selectivity than chemotherapy, but also reduces unwanted non-selective toxicity. While targeted therapies such as Trastuzamab/Pertuzamab (HER2), Certuximab (EGFR) and Ramucirumab (VEGFR-2) have been utilised in the clinic, often in combination with chemotherapy, they typically yield only a partial response and are prone to resistance (Blackham et al., 2016). Of note, the proportion of Epstein Barr-Virus positive gastric cancers that display increased PD-L1 expression (Cancer Genome Atlas Research, 2014) has prompted several clinical trials testing 'breakthrough' immune checkpoint inhibitors (Tremelimumab and Nivolumab) in patients at various stages of disease (Kang, 2017) Despite the impressive anti-tumour effects of immune checkpoint inhibitors observed in other solid tumours, gastric cancer patients only show a modest increase in survival - 5.32 (nivolumab) vs 4.41 (placebo) months, highlighting the need for fully randomised trials to properly identify gastric cancer patients who will benefit from immunotherapies. The limited response and frequent resistance to first-line chemotherapy and adjuvant molecular therapies is partially attributed to an incomplete understanding of the biology of gastric cancer (Zhang & Fan, 2010). As such, the continual discovery of additional signalling pathways that drive gastric tumourigenesis and progression provide novel therapeutic avenues that are desperately needed in the clinic.

To date, there is a tome of data demonstrating that Wnt signalling plays a critical role in driving gastric tumourigenesis, invasion and metastasis (Radulescu et al., 2012; Zhao, Ma, Bu, Wang & Zhang, 2013). Here we review the relevant studies implicating aberrant Wnt signalling in gastric cancer and how Wnt-targeted therapies may offer therapeutic benefit to gastric cancer patients.

A brief overview of Wnt signalling

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The Wnt signalling pathway is an ancient instructive genetic program, which is conserved from humans through to *Hydra* (Phesse, Flanagan & Vincan, 2016). It plays a vital role for orchestrating complex cellular behaviours during development, tissue homeostasis and regeneration where it coordinates cell proliferation, cell fate decisions, cell motility and tissue polarity. Due to its biological pervasiveness, Wnt signalling is implicated in many human cancers and degenerative diseases following pathway deregulation (Clevers & Nusse, 2012). Historically, Wnt signalling is considered to operate in two distinct modalities based on downstream involvement – or lack thereof – of the cytoplasmic protein β-catenin. These are referred to from hereon in as β-catenin-dependent or β-catenin-independent Wnt signalling. Of the two Wnt pathway branches, the β-catenin-dependent pathway has received the most research interest and is thus better characterised, and as such will be the primary focus of this review.

Wnts are secreted lipid-modified glycoproteins that act as both short (Farin et al., 2016) and long-range (Mulligan, Fuerer, Ching, Fish, Willert & Nusse, 2012) ligands to engage with cell surface receptors that can establish complex morphogen gradients, to promote subtle yet sophisticated biological outcomes, depending on cell and tissue context (Clevers, 2006; Clevers, Loh & Nusse, 2014; Clevers & Nusse, 2012). The hallmark of β-catenin-dependent Wnt signalling is the cytoplasmic accumulation of β-catenin. In the absence of Wnt activation, cytosolic levels of free β-catenin are kept to a minimum, despite the gene being continuously transcribed. This pool of free β-catenin is sequestered in a multi-protein "destruction complex" that consists of adenomatous polyposis coli (APC) tumour suppressor protein, AXIN, glycogen synthase kinase-3β (GSK-3β) and casein kinase 1 (CK1). Formation of the β-catenin destruction complex induces phosphorylation of β-catenin by CK1 at Ser45, which in turn primes GSK-3β phosphorylation of β-catenin on Thr41, Ser37 and Ser33 residues (Liu et al., 2002). Phosphorylated β-catenin is ubiquitylated by the F-box-containing protein β-TrCP E3 ligase tagging it for proteasomal degradation (Aberle, Bauer,

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Stappert, Kispert & Kemler, 1997; Kitagawa et al., 1999; Li et al., 2012). In the presence of Wnt, a heterodimeric receptor complex is formed, consisting of the seven-pass transmembrane protein Frizzled (FZD) and Low-density lipoprotein receptor-related protein 5/6 (LRP5/6). Through an unresolved mechanism involving the adaptor protein Dishevelled (DVL), both receptor components participate in separate intracellular interactions that trigger both the initiation and amplification of Wnt signalling via the inhibition of the β-catenin destruction complex (MacDonald, Tamai & He, 2009; Mao et al., 2001; Zeng et al., 2008; Zeng et al., 2005). This allows newly synthesised unphosphorylated β-catenin to accumulate, stabilise and translocate from the cytoplasm to the nucleus. β-catenin can then generate a transcriptionally active complex with T-cell factor/lymphoid enhancing factor (TCF/LEF) family of transcription factors to induce Wnt target gene transcription (for detailed reviews on Wnt signal transduction see (Acebron & Niehrs, 2016; Clevers & Nusse, 2012; Niehrs, 2012).

Recent experimental findings have revealed inherent pathway crosstalk and complexity that cannot be accounted for by current linear signal transduction models, with components at virtually every level of Wnt signal transduction been shown to affect both β-catenin-dependent and β-catenin-independent outputs (Mikels & Nusse, 2006; Niehrs, 2012; Topol, Jiang, Choi, Garrett-Beal, Carolan & Yang, 2003). As such, Wnt signalling is beginning to be viewed as a signalling network (van Amerongen & Nusse, 2009). An example of Wnt pathway complexity is the dazzling number of possible ligand-receptor interactions from the vast repertoire of mammalian Wnts (19), Fzds (10) and co-receptors (>6). These combinations influence just one facet of signal output following Wnt pathway activation and will yield different biological outcomes depending on cell/tissue context. This illustrates that despite major breakthroughs over the last several decades, there are still gaps in our understanding of how this pathway operates including precisely how Wnt-Fzd selectively is achieved.

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Duplicitous Wnt signalling – regulator of both normal and cancer stem cell biology

Multiple adult tissues such as the skin and gut undergo constant renewal, meaning, a balance between cell extrusion and replacement by newly born cells. It is now understood that cellular attrition, either through natural exhaustion or injury, within diverse tissues is fuelled by stem cells. Stem cell activity is often controlled by the microenvironment (niche) so that stem cell output is matched to the homeostatic needs or regenerative demands of the tissue (Clevers, Loh & Nusse, 2014). Wnt signalling controls stem cell activity in a variety of tissues such as the intestines, stomach, skin, bone and hematopoietic system (Clevers & Nusse, 2012; Visvader & Clevers, 2016). For example, intestinal stem cells sustain the constant turnover of the intestinal epithelium and express the cell surface receptor Lgr5 (Barker et al., 2007). *Lgr5* is a Wnt target gene (Barker et al., 2007), and *Lgr5*$^{+}$ intestinal stem cells require the Wnt receptor *Fzd7* to maintain homeostasis in the intestinal epithelium (Flanagan et al., 2015). Indeed, Lgr5 is a co-receptor for Wnts, which are expressed by neighbouring Paneth cells in the small intestine (Sato et al., 2011) and c-kit^{+} goblet cells and Reg4^{+} deep secretory cells in the colon (Rothenberg et al., 2012; Sasaki et al., 2016). *Lgr5*$^{+}$ stem cells are also located in the gastric epithelium suggesting Wnt signalling also regulates gastric homeostasis. This is supported by the observation that deletion of *Fzd7* in the gastric epithelium is deleterious and triggers rapid repopulation (Flanagan, Barker, Clevers, Ernst, Phesse, Vincan, 2017, in press). The TNF-family receptor *Troy* was recently identified as a marker of a subset of chief cells which act as 'reserve' stem cells in the stomach following injury, which are characterised by elevated expression of Wnt target genes (Stange et al., 2013). Together these data suggest Wnt signalling regulate gastric stem cell activity, however, the full extent of Wnt regulated homeostasis in the gastric epithelium is relatively poorly understood in comparison to the intestine.

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The cancer stem cell model suggests that tumour growth is driven by a small sub-population of cells (cancer stem cells, CSCs) rather than the bulk of the tumour cells (Visvader, 2011). Over several years this model has been refined as researchers have discovered significant plasticity between CSCs and non-CSCs within a tumour depending on the exposure of cells to growth factors and cytokines expressed by tumour cells or surrounding cells. Importantly, despite the progresses that have led to an improved standard of care, resistance to chemotherapy, whether intrinsic or acquired, is a complex and multifactorial phenomenon and remains the main cause of treatment failure and death in gastric cancer patients (Brungs, Aghmesheh, Vine, Becker, Carolan & Ranson, 2016). Gastric CSCs (gCSCs) have shown to be resistant to gastric cancer therapy and subsequently responsible for tumour recurrence and metastasis (Brungs, Aghmesheh, Vine, Becker, Carolan & Ranson, 2016; Mayer et al., 1993). Consequently, identifying the mechanisms of CSC regulation and maintenance is crucial to understand how these mechanisms influence the development of chemoresistant tumour cells in gastric cancer patients. Indeed, the similarities between normal adult somatic stem cells and CSCs suggest that the same signalling pathways that are involved in regulating somatic stem cell maintenance are also involved in the regulation of CSCs. As such, deregulated Wnt signalling has shown to increase 'stemness', trigger transformation and influence the development of chemoresistant CSCs (Barker et al., 2009; Melo et al., 2017; Sansom et al., 2007). For instance, elevated expression of the transcription factor and Wnt target gene *SOX9* (Blache et al., 2004) is observed in human gastric cancer patients and correlates with decreased patient survival (Santos et al., 2016). Targeted knockdown of SOX9 is sufficient to reduce CSCs viability/formation with concomitant suppression of Wnt signalling and target gene expression, which was phenocopied following β-catenin knockdown (Santos et al., 2016). Importantly, the increase in $Sox9^{+}$ resistant cells following exposure to cisplatin were largely rescued in Sox9 knockdown gastric cancer cells, demonstrating that agents targeting Sox9-β-catenin signalling can overcome chemoresistant cells (Santos et al., 2016). Similarly, Wnt6 expression is positively associated with tumour stage and inversely correlates with response to the

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anthracycline chemotherapeutics epirubicin (Epi) and doxorubicin (Dox) (Yuan et al., 2013). Treatment with Epi or Dox increases Wnt6 expression by enhancing the binding of cavolin-1 to β-catenin at the Wnt6 promoter, which in turn boosts cell survival. As such, targeted knockdown of Wnt6 reduces gastric cancer cell survival via increased caspase-3 induction (Yuan et al., 2013). While it is likely that $Wnt6^{+}$ resistant cells represent gCSCs, this was not formally demonstrated. More recently, investigations to identify cell-surface proteins that mark chemoresistant and self-renewing gastric cancer cells following chemotherapy (cisplatin) reveal enrichment for the cell-surface glycoprotein PMP22 (Cai et al., 2017). PMP22, a Wnt target gene, is expressed in patients that had undergone perioperative chemotherapy, highlighting a strong correlation between PMP22 expression and tumour recurrence. Gastric cancer cells and tumour xenograft sensitivity to cisplatin was significantly increased when combined with pharmacological inhibition of PMP22 (Cai et al., 2017). The cell-surface water channel protein Aquaporin-3 (AQP3) is overexpressed in gastric cancer tissues and promotes invasion and metastasis via EMT (Chen et al., 2014), however, its role in chemoresistance has only recently been investigated. Gastric cancer cells expressing high levels of AQP3 are refractory to treatment with cisplatin, but when combined with targeted AQP3 knockdown, gastric cancer cells show increased sensitivity to chemotherapy (Dong, Wang, Zhou, Wen, Wang & Shen, 2016). Of note, an independent study revealed modulation of β-catenin-dependent signalling, via Gsk-3 inhibition or Axin stabilisation, is sufficient to regulate the abundance and behaviour of $AQP3^{+}$ gastric cancer cells (Zhou et al., 2016), which implies targeted modulation of Wnt signalling may represent an avenue to combat therapy resistant gastric cancer cells. This observation of Wnt regulated chemo-resistance has also been observed in other cancer types including medulloblastoma and colon cancer, in which Wnt regulation of the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT), restored chemo-sensitivity *in vitro* and in tumour xenografts (Wickstrom et al., 2015). Collectively, these data demonstrate that Wnt signalling could be an attractive target to inhibit gastric CSC activity, which will impact on tumour growth and recurrence.

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Evidence for deregulated Wnt signalling in gastric cancer

The role of β-catenin-dependent Wnt signalling in gastric cancer is now well established, with approximately 10-30% of human gastric tumours displaying deregulated Wnt signalling (Cristescu et al., 2015; Wang et al., 2014), with the latest TCGA study confirming significant Wnt pathway mutations in gastric cancers (Cancer Genome Atlas Research, 2014). Functional evidence also demonstrates that deregulated Wnt signalling can trigger tumourigenesis in the stomach, as deletion of *Gsk3-β*, a component of the β-catenin degradation complex, resulted in gastric tumour formation (Radulescu et al., 2012), as does deletion of *Apc* in Mist1^{+} cells of the gastric epithelium (Hayakawa et al., 2017). Several WNTs are upregulated in GC including *WNT1* (Mao et al., 2014), *WNT5A* (Boussioutas et al., 2003; Kurayoshi et al., 2006) and *WNT6* (Yuan et al., 2013). These recent discoveries provide a timely backdrop to review the potential of targeting the Wnt pathway for the treatment of gastric cancer, as resistance to current chemotherapy is common, and the exact role of each component of the pathway and its potential as a therapeutic target is described in the sections below.

Genetic lesions of the β-catenin destruction complex

Somatic mutation is a common mechanism to facilitate Wnt pathway deregulation in many solid cancers, including gastric cancer. Loss-of-function (LOF) mutations of multiple downstream Wnt pathway components such as *APC*, *AXIN* or activating mutations of *CTNNB1* (gene encoding β-catenin) feature in the initiation and progression in both intestinal-type and diffuse-type gastric cancers (Cancer Genome Atlas Research, 2014; Wang et al., 2014). This section will review strategies targeting these three components of the destruction complex in gastric cancer.

Axin

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Gastric cancers positive for microsatellite instability (MSI) comprise one of several molecular subgroups identified by large-scale molecular characterisation studies (Cancer Genome Atlas Research, 2014). Frameshift mutations of cancer associated genes with mono- or dinucleotide repeats in the coding sequences are a feature of gastric tumours positive for MSI (Simpson, Caballero & Pena, 2001). The scaffold protein AXIN serves as a critical rate-limiting protein in the assembly of the β-catenin destruction complex (Lee, Salic, Kruger, Heinrich & Kirschner, 2003), highlighting its role as a negative regulator of Wnt signalling and tumour suppressor protein (Li et al., 2012). As such, approximately 30% of MSI-high human gastric cancers harbour frameshift mutations in *AXIN2* (Kim, Kim, Ahn, Yoo & Lee, 2009). The *AXIN2* frameshift mutation identified by Kim et al. is predicted to introduce a premature stop of amino acid synthesis in the C-terminus of AXIN2 protein and hence resemble a typical LOF mutation (Kim, Kim, Ahn, Yoo & Lee, 2009). This frameshift mutation (p.Gly665Alafs24) would eliminate a C-terminal half of PP2Ac-binding domain and the entire AXIN-binding domain. In addition, missense mutations in Axin have been reported in gastric adenocarcinomas (http://www.cbioportal.org/), however their functional significance has not been properly investigated. Recent work in *Drosophila* and human cells has identified missense mutations in *AXIN1* that disrupt the conserved core of the N-terminal Axin RGS domain, which is necessary for binding *APC* (Anvarian et al., 2016). Cells with a non-functioning Axin scaffold gain proneoplastic properties by forming protruding nanoscale aggregates, which engage with atypical signal transducers to confer cell-growth advantages (Anvarian et al., 2016). Indeed, *Axin1* null mice develop liver tumours, confirming its role as a tumour suppressor *in vivo* (Feng et al., 2012). It remains to be addressed if this same mutation-aggregate mechanism occurs in gastric cancers harbouring missense mutations to *AXIN*.

Adenomatous Polyposis Coli

Since its discovery as a major tumour suppressor in colorectal cancer (Kinzler et al., 1991; Korinek et al., 1997; Su et al., 1992), LOF mutations to *APC* have been

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commonly reported in many other epithelial cancers, including gastric cancer (Sano et al., 1991). Sequencing of human gastric tumours showed several different mutations to *APC*, however the small sample size of this study makes it difficult to conclude any correlation between *APC* mutation status and tumour sub-type (Nakatsuru et al., 1992). Recent large-scale genomic characterisation of gastric tumours reveal frequent somatic mutations to *APC* in non-hypermutated chromosomal instable (CIN) gastric cancers (Cancer Genome Atlas Research, 2014), which is further supported from an independent patient dataset reporting an even higher incidence of somatic mutations to *APC* (Cristescu et al., 2015). Likewise, in gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) patients, mutation analysis and Sanger sequencing successfully mapped point mutations in *APC* promoter 1B, which was shown to reduce binding of the YY1 transcription factor and impaired the activity of *APC* 1B promoter (Li et al., 2016). Importantly, allelic imbalance of *APC* is detectable in patient blood and saliva samples, serving as an excellent biomarker for prospective GAPPS patients (Li et al., 2016). In support, gastric cancer cell lines reveal mutations at codon 1450 of *APC* that encodes a truncated form of *APC* that fails to negatively regulate β-catenin, which causes constitutive activation of Wnt signalling (Sasaki et al., 2001). Importantly, several reports have demonstrated that conditional deletion or loss-of-heterozygosity (LOH) of wild type *Apc* is sufficient to drive gastric hyperplasia and subsequent adenoma formation (Powell et al., 2014; Radulescu et al., 2012; Sarkar et al., 2016; Tomita et al., 2007). Collectively, patient genomics and pre-clinical mouse models highlight the relevance of *APC* mutations in driving gastric tumourigenesis, which offer an attractive therapeutic target to treat gastric cancer patients. As such, the application of tankyrase inhibitors (discussed in detail below) has been shown to be efficacious in CRC cell lines and mouse models with *APC* mutations, which suggests a similar therapeutic benefit could be observed in pre-clinical models of gastric cancer harbouring *APC* mutations (Chen et al., 2009; Huang et al., 2009; Waaler et al., 2011).

CTNNB1 (β-catenin)

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Nuclear β-catenin, a hallmark of active Wnt signalling, is detected in approximately 30% of gastric cancer tumours, identifying β-catenin as a suitable target for therapeutic intervention (Clements et al., 2002). Gastric tumours displaying nuclear β-catenin frequently harbour mutations at exon3 of *CTNNB1* (Clements et al., 2002; Woo, Kim, Lee, Kang, Yang & Kim, 2001). Activating mutations at exon3 alter targeted serine-threonine phosphorylation sites by Gsk-3, which confer resistance to phosphorylation and lead to the accumulation of cytoplasmic and nuclear β-catenin and subsequent changes in expression of genes that regulate proliferation (*Cyclin D1*, *D2* and *E*) (Akama et al., 1995; Arici, Tuncer, Ozer, Simek & Koyuncu, 2009; Liang, Wang, Yang, Ye, Yu & Cui, 2003; Takano, Kato, van Diest, Masuda, Mitomi & Okayasu, 2000). Genetic association analysis investigating the correlation between tagged single nucleotide polymorphisms (SNPs) spanning *CTNNB1* and gastric cancer incidence and survival showed that SNPs rs1880481, rs4135385, rs11564475 and rs2293303 were significantly associated with gastric cancer susceptibility (Wang et al., 2012b). In addition, the rs4135385 AG/AA genotypes were associated with a 0.74-fold reduced adjusted hazard ratio for favourable overall 5-year survival of non-cardia gastric cancer (Chiurillo, 2015; Wang et al., 2012b). A recent proof-of-principle study demonstrated that conditional mutation of exon3 in *CTNNB1* is sufficient to induce intestinal-type gastric adenomas in the antral stomach of adult mice and increased activation of Wnt signalling (Radulescu et al., 2012). As such, targeted siRNA knockdown of β-catenin in human gastric cancer cells leads to inhibition Wnt target gene transcription, decreased cell proliferation and an increase in apoptosis of gastric cancer cells (Jiang, Shen, Ye, Yang & Wang, 2010). The expression of *Survivin (BIRC5)*, a Wnt target gene, was deregulated following β-catenin knockdown, suggesting that elevated Wnt signalling might inhibit apoptosis by regulating *Survivin* during gastric cancer (Jiang, Shen, Ye, Yang & Wang, 2010). A complementary gene-targeted approach demonstrated that recombinant adenovirus carrying p53 up-regulated modulator of apoptosis (PUMA) under the control of β-catenin/TCF-responsive promoter (AdTOP-PUMA) selectively targeted and killed AGS gastric cancer cells with active Wnt signalling (Dvory-Sobol, Sagiv, Liberman,

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Kazanov & Arber, 2007). Synergistic cell killing was observed when AdTOP-PUMA was used in combination with standard chemotherapeutic agents (5-florouracil, doxorubicin, paclitaxel) highlighting the potential of adjuvant Wnt targeted therapies in gastric cancer (Dvory-Sobol, Sagiv, Liberman, Kazanov & Arber, 2007). Of note, even in gastric cancers with no detectable mutations to *CTNNB1* or *APC*, the abundance of β-catenin mRNA is greatly enhanced (Ebert et al., 2002), and although post-translational rather than transcriptional regulation of β-catenin is at the core of active Wnt signalling, increased β-catenin protein production may suggest that upstream components of the Wnt pathway are deregulated, thereby activating Wnt signalling in gastric cancer.

Genetic lesions of the Wnt receptor complex

Aberrant activation of the Wnt pathway can also occur at the level of Wnts and/or Fzds. Genetic and/or epigenetic events that alter the function of Wnt regulators, thereby de-regulating Wnt and/or Fzd expression resulting in pathway activation, have been identified in several cancers, including gastric cancer, and also represent a target for cancer therapy which is reviewed in this section.

Loss-of-function mutations to RNF43

Mammalian tissues that undergo constant renewal, such as the skin, blood, and the gut, rely on tightly controlled Wnt signalling to maintain stem cell populations that fuel the replenishment of exhausted cells. For example, $Lgr5^{+}$ intestinal stem cells, which are exquisitely sensitive to Wnt, sustain the constant turnover of the intestinal epithelium (Barker et al., 2007). More recently, the potent Wnt agonist R-Spondin (R-Spo) was shown to be the receptor for Lgr5 and sufficient to potentiate β-catenin-dependent signalling (Carmon, Lin, Gong, Thomas & Liu, 2012; de Lau et al., 2011; Kazanskaya, Glinka, del Barco Barrantes, Stannek, Niehrs & Wu, 2004; Kim et al., 2008). Following the discovery of R-Spo as the ligand for Lgr5, independent investigations identified two highly related transmembrane E3 ubiquitin ligases, ZNRF3 and RNF43, as negative regulators of Wnt signalling that are integral to R-

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Spo/Lgr5 Wnt potentiation (Hao et al., 2012; Koo et al., 2012). Extensive biochemical experiments reveal that ZNRF3 and RNF43 regulate Wnt signalling by promoting the ubiquitylation, internalisation and degradation of Fzd-Lrp5/6 complexes following Wnt activation, thereby limiting the duration and intensity of Wnt signalling (Hao et al., 2012). These findings support the following model: in the absence of R-Spo, ZNRF3/RNF43 ubiquitylates Fzd via the DEP domain of Dvl and promotes the degradation of Fzd-Lrp5/6 complex (Jiang, Charlat, Zamponi, Yang & Cong, 2015), thus keeping Wnt signalling to low levels. However, in the presence of R-Spo, an interaction between Lgr5 and ZNRF3/RNF43, via R-Spo Furin domains (1&2), leads to the clearance of ZNRF3/RNF43, allowing Fzd/Lrp5/6 to accumulate at the membrane to enhance β-catenin-dependent Wnt signalling (Hao et al., 2012; Peng et al., 2013).

Recent large-scale genomic data has identified frequent *RNF43* LOF mutations, predominantly truncating or missense alterations, in >50% and 4.8% of microsatellite instable (MSI) and microsatellite stable (MSS) gastric tumours respectively (Cancer Genome Atlas Research, 2014; Wang et al., 2014). A recent examination of the progressive genomic and transcriptomic alterations from early-stage gastric adenomas through to later-stage disease validate the recurrent mutations to *RNF43* previously described (Min et al., 2016). Furthermore, the frequency of *RNF43* mutations in early-mid stage gastric tumours, identify deregulated Wnt signalling as a critical driver and potential biomarker of early gastric tumourigenesis. Thus, determining if a gastric tumour has *RNF43* mutations will help stratify which patients are more likely to benefit from therapies targeted to the Wnt receptor complex or the production of Wnts (Min et al., 2016). To date, the efficacy of Fzd-blocking antibodies and/or inhibitors of Wnt secretion have not been thoroughly tested in pre-clinical models of gastric cancer. However, one study has shown significant cell growth arrest and Wnt pathway inhibition using an inhibitor of Wnt secretion, IWP-2 (Chen et al., 2009), on human intestinal-type gastric adenocarcinoma cells (MKN28), however, there have been no

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reports of *RNF43* mutations in this cell line (Mo, Li, Chen, Liu, Sheng & Zhou, 2013).

Encouragingly, other pre-clinical models of solid cancers harbouring *RNF43* mutations such as pancreatic, colon and breast cancer treated with inhibitors directed to the receptor complex (inhibitors of Wnt secretion or Fzd-blocking antibodies) have yielded potent anti-tumourigenic effects, especially when used in combination with standard chemotherapeutics (Gurney et al., 2012; Jiang et al., 2013; Steinhart et al., 2017).

Epigenetic silencing of Wnt antagonists

Until relatively recently, it was considered that constitutive Wnt activation triggered by mutation to *APC*, *CTNNB1* or *AXIN* was impervious to further regulation from upstream Wnt components ie. ligands and receptors. However, it has been demonstrated that the secreted frizzled-related protein (sFRP) family of Wnt negative-regulators are frequently silenced via promoter hypermethylation in a variety of cancers, including gastric cancer (Caldwell et al., 2004; Cheng et al., 2007; Suzuki et al., 2004). The family of sFRP glycoproteins is comprised of 5 family members, which can bind directly to Wnt via its cysteine-rich domain (CRD), the Wnt-Fzd binding interface (Janda, Waghray, Levin, Thomas & Garcia, 2012), thereby competing with Fzd for Wnt binding. Given sFRPs can bind directly to Wnt, they are able to inhibit both β-catenin-dependent and β-catenin-independent signalling. In normal gastric mucosa, the expression of sFRP1, sFRP2 and sFRP5 is readily detected. However, in primary gastric tumours and gastric cancer cell lines, the expression of sFRP is absent, which is attributed to significant DNA methylation within the promoter-associated CpG islands (Cheng et al., 2007; Nojima et al., 2007; Zhao, Bu, Zhang & Wang, 2009). The silencing of *sFRP* via methylation is detected in pre-neoplastic gastric tissue, suggesting this is a mechanism of tumour initiation in the stomach, and thus could be used as a biomarker to screen for patients with an enhanced risk of developing gastric cancer (Cheng et al., 2007). Much like the studies

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performed in CRC cell lines (Caldwell et al., 2004; Caldwell et al., 2006; Suzuki et al., 2004), transfection of *sFRP-1,-2 or -5* successfully suppressed Wnt signalling, which is sufficient to block proliferation and induce apoptosis in gastric cancer cell lines harbouring *APC* or *CTNNB1* mutations (Nojima et al., 2007). Xenografts have also been used to demonstrate that transfecting mice with *sFRP2* could block tumour growth, thus illustrating the potential for sFRP2 to act as a functional tumour suppressor (Cheng et al., 2007). The ability of sFRP to attenuate Wnt signalling is dependent on a functional Wnt-binding CRD as sFRP constructs lacking a functional CRD failed to inhibit proliferation and induce apoptosis (Nojima et al., 2007).

Similar to the sFRP family of Wnt negative-modulators, the Dickkopf (Dkk) family of glycoproteins (Dkk1 - 4) are potent inhibitors of Wnt signalling (Niehrs, 2006). However, in contrast to sFRPs, Dkk selectively inhibits β-catenin-dependent Wnt signalling through interacting with Lrp via the epidermal growth factor (EGF) repeat domains within Lrp6 (Mao et al., 2001), thus preventing Wnt and Fzd from forming a ternary complex (MacDonald, Adamska & Meisler, 2004). Dkk can also modulate Wnt by associating with Kremen1 and 2 to form a complex that regulates the internalisation of Lrp (Niehrs, 2006). Much like sFRPs, the expression of Dkk-3 is commonly silenced (70%) in gastric cancer tissues via promoter methylation and is associated with poor patient survival, as shown by multivariate analysis (Yu et al., 2009). Reversal of DNA methylation with demethylating agents or ectopic expression of Dkk-3 is sufficient to restore Dkk-3 expression and subvert gastric cancer cell growth and Wnt signalling (Yu et al., 2009). More recently, conventional adenoviral gene therapy has been utilised to deliver functional DKK-1 to inhibit Wnt signalling and attenuate gastric tumourigenesis (Wang et al., 2012a). Following infection with the chimeric Ad5/35-DKK-1 adenovirus, the number of $CD44^+$ gastric cancer stem cells and volume of tumour xenografts was significantly reduced (Wang et al., 2012a). While the authors report successful infection of gastric cancer cells *in vitro*, issues surrounding delivery, tissue penetration and immunological response currently

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limit the feasibility of gene therapy technologies in gastric cancer patients (Sutter & Fechner, 2006).

Collectively, the silenced expression of sFRP and/or Dkk inhibitors in gastric cancer undoubtedly contributes to unrestrained Wnt pathway activation at the level of the receptor complex. As such, patients with DNA-promoter modifications to sFRP and/or Dkk negative-modulators, which can be detected in pre-neoplastic tissue, might benefit from Wnt-targeted therapies at the level of Wnt receptors and/or Wnt secretion.

Overexpression of Wnt ligands and Fzd receptors

While the incidence of genetic lesions to genes that encode for Wnts and Fzds is low, de-regulated expression of Wnts and Fzds is a common feature of gastric cancers, which can be attributed to the Wnt pathway mutations described previously. This suggests that gastric cancers with abundant Wnts and Fzds can be targeted therapeutically. For instance, Wnt5a, a prototypic β-catenin-independent Wnt known to inhibit β-catenin-dependent Wnt signalling and promote cell migration and invasion (Moon, 2002), is overexpressed and correlated with aggressive gastric cancer phenotypes (Kurayoshi et al., 2006). Infiltrating macrophages secrete Wnt5a following *H pylori* infection, which induces the migration and invasion of gastric cancer cells via CXCR4 chemokine receptors (Zhao, Ma, Bu, Wang & Zhang, 2013). In support, a recent pre-clinical investigation has identified that Wnt5a produced by innate lymphoid cells supports diffuse gastric cancer progression by inhibiting anoikis, which enables anchorage-independent growth of gastric cancer cells (Hayakawa et al., 2015). Indeed, chimeric mice transplanted with bone marrow from *Wnt5a*$^{flox/flox}$ mice (Wnt5a deficient) exhibit significantly fewer signet-ring foci than wild-type bone marrow recipient chimeras (Hayakawa et al., 2015). Furthermore, therapeutic targeting of Wnt5a using a novel anti-Wnt5a polyclonal antibody (pAb5a-5) has shown to reduce the migration and invasion of gastric cancer cells *in vitro* and *in vivo* by blocking receptor complex internalisation, which is necessary to activate

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target gene expression required for cell motility (Hanaki et al., 2012). Originally identified as a gene that preferentially integrates into mouse mammary tumour virus (MMTV) proviral DNA (Nusse & Varmus, 1982), *WNT-1* is overexpressed in gastric cancer tissues and has been shown to induce the expression of cancer stem cell (CSC) genes (Oct4, Cd44), which is associated with disease progression and poor outcome (Mao et al., 2014). Modified AGS gastric cancer cells that overexpress WNT-1 confer increased gastric cancer cell proliferation and Wnt target gene expression *in vitro* and in xenograft tumours (Mao et al., 2014). Treatment of AGS-WNT-1 overexpressing cells with the antibacterial potassium ionophore Salinomycin, which was shown to inhibit β-catenin-dependent signalling by inducing the degradation of LRP6 (Lu, Lin, Roberts, Waud, Piazza & Li, 2011), effectively reduces tumour growth and the associated elevated expression of CSC and Wnt target genes (Mao et al., 2014).

Similar to their cognate ligands, the Frizzled family of Wnt receptors is frequently de-regulated in gastric cancers, which is often associated with poor clinical outcome (Phesse, Flanagan & Vincan, 2016; Schmuck, Warneke, Behrens, Simon, Weichert & Rocken, 2011). Of the ten family members, multiple reports have identified *FZD_7* to be overexpressed in gastric cancer tissues, which are associated with various stages of disease progression (Kirikoshi, Sekihara & Katoh, 2001; Schmuck, Warneke, Behrens, Simon, Weichert & Rocken, 2011; Zhao et al., 2014). FZD_7 is unique among other Fzd members as it is one of the few Fzds shown to transduce all major signalling branches of the Wnt pathway and is associated with maintaining proliferation in adult stem cell populations (Fernandez et al., 2014; Flanagan et al., 2015; Phesse, Flanagan & Vincan, 2016). This places FZD_7 in a unique position to mediate cell proliferation as well as tumour dissemination and metastasis (Ueno et al., 2009; Vincan, Darcy, Farrelly, Faux, Brabletz & Ramsay, 2007; Vincan et al., 2005). Side population (SP) of cells, which display cancer stem cell properties, isolated from human gastric cancer cell lines reveal increased expression of FZD_7 and other genes associated with CSCs, which supports a role for FZD_7 in promoting gastric cancer (Schmuck, Warneke, Behrens, Simon, Weichert & Rocken, 2011). Moreover, our

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unpublished data demonstrates that targeted molecular inhibition of Fzd_7 or conditional deletion of *Fzd_7* is sufficient to block the growth of human gastric cancer cells and in mouse models of intestinal-type gastric cancer (Flanagan, Phesse and Vincan 2017). Similarly, siRNA knockdown of FZD_2, which is structurally related to FZD_1 and FZD_7 (Fredriksson, Lagerstrom, Lundin & Schioth, 2003; Sagara, Toda, Hirai, Terada & Katoh, 1998), reduced cell proliferation and invasion in MKN45 and MKN74 gastric cancer cells (Tomizawa, Shinozaki, Motoyoshi, Sugiyama, Yamamoto & Ishige, 2015). In contrast, gastric cancer cell lines (AGS and SGC7901) reveal transcriptional silencing of *FZD_6* by miRNA-21 (Yan, Liu, Zhou, Dang, Yin & Zhang, 2016). Re-introduction of *FZD_6* expression represses gastric cancer cell proliferation by antagonizing β-catenin-dependent Wnt signalling, which can be blocked by siRNA-targeted *FZD_6* knockdown or pharmacological inhibition of miRNA-21 (Yan, Liu, Zhou, Dang, Yin & Zhang, 2016). Together, these data demonstrate that aberrant expression of Wnts and Fzds contribute to gastric tumourigenesis, and thus they represent a therapeutic target for gastric cancer patients with tumours showing elevated Wnt activity.

Infection and innervation influence Wnt activation in gastric cancer

The aetiology of gastric cancer is further complicated by complex interactions between bacteria, host and environmental factors (Gravaghi et al., 2008). Infection with the bacterial carcinogen *H. pylori* is the greatest risk factor for gastric cancer (Hardbower, Peek & Wilson, 2014), with ~75% of the global gastric cancer burden attributed to *H. pylori*-induced inflammation and associated tumourigenesis (Parkin, Bray, Ferlay & Pisani, 2005). Following successful colonization of the stomach epithelium, *H. pylori* drives superficial gastritis, which progresses to chronic inflammation (Correa, 1996). *H. pylori*-driven inflammation is sufficient to induce the expression of *CDX2* and *MUC2*, which enables the transdifferentiation of gastric cells to adopt an intestinal cell phenotype (intestinal metaplasia). This is characterised by the loss of parietal cells and presence of Paneth and goblet cells. The resulting intestinal metaplasia (predominantly in the antrum) or spasmolytic polypeptide-

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expressing metaplasia (SPEM; predominantly in corpus) progresses to dysplasia and ultimately cancer. *H. pylori* delivers bacterial virulence factors that modulate epithelial biology and inflammatory responses for its own benefit. Of the virulence factors produced by *H pylori* associated with gastric cancer development, cytotoxin-associated gene product (CagA) and its associated type IV secretion system (T4SS) have been shown to activate Wnt signalling and promote gastric tumourigenesis and progression (Amieva & Peek, 2016; Neal, Peterson, Kent & Guillemin, 2013).

Gerbils infected with a carcinogenic strain of *H. pylori* develop rapid and highly penetrant changes in the gastric mucosa of recipient animals that progress to gastric adenocarcinoma (Franco et al., 2005). The induction of gastric dysplasia following *H. pylori* infection was associated with increased expression of nuclear β-catenin, which was shown to be CagA-dependent (Franco et al., 2005). Research using transgenic zebrafish has extended previous findings, demonstrating that activation of Wnt and epithelial changes triggered by CagA are downstream or parallel to the β-catenin destruction complex, but upstream of Tcf-4 (Neal, Peterson, Kent & Guillemin, 2013). In addition, gastric cells co-cultured with *H. pylori* induce phosphorylation of Lrp6 within 30 minutes, which is sufficient to stabilise β-catenin where it can mediate β-catenin target gene transcription (Gnad, Feoktistova, Leverkus, Lendeckel & Naumann, 2010). The success of *H. pylori* to induce changes to the gastric epithelium hinge on its capacity to attach to and transform gastric stem cells. Adult gastric stem cells, marked by the Wnt target gene Lgr5, are not only reliant on Wnt signalling for their maintenance but are also the cell-of-origin in gastric tumourigenesis following $Lgr5^+$-targeted Wnt activation (Barker et al., 2010). Adult murine $Lgr5^+$ gastric stem cells infected with *H. pylori* increase proliferation and the expression of Wnt target genes, which is sufficient to transform $Lgr5^+$ gastric stem cells and their progeny (Sigal et al., 2015). Strains of *H. pylori* with defects in chemotaxis or that are CagA-deficient are unable to induce transformation of $Lgr5^+$ gastric stem cells and subsequent hyperplastic changes (Sigal et al., 2015). A complementary approach using *ex-vivo* human gastric organoids also demonstrated the transformation of human

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gastric stem cells following infection with *H. pylori* (Bartfeld et al., 2015). Taken together, these studies suggest that small molecule inhibitors targeted to the interaction between β-catenin and Tcf or other co-factors required for Wnt-driven transcription might abrogate the increased activation of Wnt signalling observed following infection and thus have therapeutic value in patients infected with carcinogenic strains of *H. pylori*.

Several recent studies from Timothy Wang's group have elegantly shown that functional nervous system innervation is required at all stages of gastric cancer in a Wnt dependent manner. The studies collectively reveal a Nerve Growth Factor (NGF)/Achetylcholine-muscarinic receptor-3 (ACh-M_3) signalling axis that activates Wnt signalling via promoting YAP/β-catenin complexes (Hayakawa et al., 2017) in gastric stem cells, which in turn fuels gastric tumour growth (Hayakawa et al., 2015; Zhao et al., 2014). Furthermore, pharmacological inhibition of cholinergic signalling with botulinum toxin (botox) was sufficient to block tumour growth in mouse models of gastric cancer, associated with down regulation of Wnt target genes (Zhao et al., 2014).

These data suggest that Wnt signalling is rate-limiting for cholinergic signalling-associated gastric cancer. Indeed, gastric tumours that developed in the antrum following *Apc* truncation in Mist1$^+$ cells of *Mist1CreERT2; Apc$^{flox/flox}$* mice were significantly reduced when the Ach-M3 receptor was co-deleted (Hayakawa et al., 2017). In addition to demonstrating that Wnt signalling is rate-limiting for ACh-M_3-driven gastric tumours, these data also highlight that the antrum is more sensitive to Wnt-driven tumourigenesis than the corpus, since Mist1$^+$ cells are found in both areas of the stomach (Hayakawa et al., 2017).

Suitable inhibitors of Wnt signalling for gastric cancers

Aberrant Wnt signalling in gastric cancer can be achieved through either mutational or non-mutational alterations. As such, molecular blockade of Wnt signalling is

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sufficient to inhibit tumour growth in several pre-clinical models of solid cancers, prompting the recent development of Wnt-targeted inhibitors, several of which are in early-stage clinical trials. However, it is clear that the advancement of anti-Wnt drug candidates is slow both in numbers of candidates entering the trials and in the stage of advancement through the phase 1-2-3 clinical trials. Pharmacological modulation of Wnt signalling can be divided into compounds that modulate the ligand/receptor interface, stabilise the degradation complex or interfere with β-catenin-dependent gene transcription (Figure 2A and B) (Tai et al., 2015). Note, the pharmacology and anti-tumour effects of Wnt inhibitors described below have not been validated in pre-clinical models of gastric cancer, although there is now sufficient biological evidence to suggest they may be of therapeutic benefit for the treatment of gastric cancer in the future.

There is also a strong rational for the repositioning of existing FDA approved drugs for the treatment of diseases with deregulated Wnt signaling such as cancer. For example, the anthelmintic agent Niclosamide was originally approved for clinical use in treating tapeworm infections, but has been shown to inhibit the growth of several cancer cell lines including colon, breast, pancreas, lung and prostate (Mook et al., 2015). Niclosamide is a multi-functional drug and has been shown to inhibit several oncogenic signalling pathways including Notch, mTOR, Stat-3, NF-κB and Wnt. Indeed, Niclosamide treatment significantly inhibited the growth of *APC* mutant colon cancer xenografts, independently of mTOR and NF-κB signalling, which was associated with reduced Wnt signalling. As the stomach is more easily accessible than other organs such as the pancreas and brain, the problem of delivery for anti-cancer treatments is less of an issue, and therefore drugs such as Niclosamide which have a poor bioavailability have a greater chance of success in gastric cancers.

Inhibitors of Wnt signalling at the receptor complex

The gaps in our understanding pertaining to intrinsic Wnt pathway complexity pose technical concerns when considering how best to inhibit β-catenin-dependent and β-

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catenin-independent Wnt signalling. One approach to block *both* β-catenin-dependent and β-catenin-independent Wnt signalling is to focus on inhibiting the interaction between Wnts and their cognate Wnt receptors, Fzd, which can be achieved using monoclonal antibodies and decoy receptors. Vantictumab (OMP-18R5, Oncomed Pharmaceuticals) is an anti-Fzd blocking antibody, originally identified to bind Fzd_7, which can functionally bind to 5 out of 10 mammalian Fzd receptors (1, 2, 5, 7 and 8) by binding a series of highly conserved residues spanning the Fzd extracellular cleft (Gurney et al., 2012). Vantictumab can successfully inhibit the ability of several Wnts to activate β-catenin-dependent Wnt signalling, which is accompanied by decreased Wnt transcription (Gurney et al., 2012). Furthermore, when tested on tumour xenografts, Vantictumab significantly inhibited the growth of several types of solid human tumours including breast, pancreatic and colon. Significant synergy was observed when Vantictumab was combined with standard-of-care chemotherapies, such as paclitaxel and taxol (Gurney et al., 2012). Vantictumab is currently in phase 1b clinical trials for HER2-negative breast cancer (NCT01973309) and advanced pancreatic cancers (NCT02005315) and was well tolerated up to the current dose of 15mg/kg every three weeks. Reported side effects include fatigue, abdominal pain, constipation and nausea. An interim efficacy assessment has demonstrated an overall response rate of 48 percent for patients given Vantictumab and chemotherapy. Patient free survival and overall survival data is not yet available for these clinical studies. Of note, significant bone turnover was observed in a subset of patients, which resulted in a temporary hold on three phase 1b clinical trials (Tai et al., 2015). The temporary hold has been lifted after reviewing substantial clinical safety and efficacy data and revised protocols submitted by Oncomed. Other strategies employ soluble decoy receptors that sequester Wnts, akin to sFRPs, thus preventing Wnt-Fzd interactions. Ipafricept (OMP-54F28, Oncomed Pharmaceuticals) is a soluble Fc fusion protein that consists of the CRD of FZD_8 fused to the Fc domain of human IgG1 (http://www.oncomed.com/). Ipafricept inhibits the growth of patient-derived xenografts, which is characterised by reduced CSC frequency and proliferation, as well as promoting tumour cell differentiation. As such, Ipafricept has progressed to

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phase 1b clinical trials and is tested in combination with chemotherapy for the treatment of various solid tumours such as hepatocellular carcinoma, ovarian and pancreatic cancer (clinicaltrials.gov). Interim safety data demonstrate that the combination of Ipafricept with chemotherapy was well tolerated and an overall response rate of 39 percent was observed (Tai et al., 2015).

An alternate approach to inhibit Wnt signalling is to block the biogenesis and secretion of Wnt proteins. Wnts undergo two types of known lipid modification. The first is a palmitate to cysteine 77, which is conserved among all Wnt family members (Willert et al., 2003). The second reported modification is the addition of a mono-unsaturated palmitoleate moiety to serine 209, which is required for release of Wnt from the endoplasmic reticulum (ER) and binding to Fzd (Takada et al., 2006). Porcupine (PORC) is an essential non-redundant enzyme that is responsible for the serine 209 modification and as such, PORC inhibition causes Wnt retention in the ER and thus blocks their secretion and subsequent pathway activation (Takada et al., 2006). However, there are some cells, including $CD8^+$ T-cells and human astrocytes, that have a PORC-independent mechanism of Wnt secretion, as treatment with the PORC inhibitor IWP-2 did not prevent Wnt secretion in these cells (Richards, Seaton, Wallace & Al-Harthi, 2014). Several small molecule PORC inhibitors have been developed in recent years and have shown promising anti-tumour effects in pre-clinical models with minimal off-target effects (Chen et al., 2009; Jiang et al., 2013; Madan et al., 2016), which have been extended to phase 1 clinical trials for Wnt ligand-dependent malignancies such as pancreatic adenocarcinoma and BRAF-mutant colorectal cancer which are currently ongoing (NCT01351103).

Stabilisers of the β-catenin destruction complex

The scaffolding protein AXIN is the rate-limiting component of the β-catenin destruction complex (Lee, Salic, Kruger, Heinrich & Kirschner, 2003) with the levels of AXIN1 and AXIN2 proteins being constantly surveyed and regulated by tankyrases, TNK1 and TNK2. Tankyrases regulate the stability of AXIN1 and AXIN2

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through poly(ADP-ribosyl)ation (PARsylation) (Huang et al., 2009), which directs AXIN ubiquitylation by RNF146 and proteasomal degradation (Callow et al., 2011; Zhang et al., 2011).

Recent efforts using cell-based screens have discovered several small-molecule tankyrase inhibitors, which successfully stabilise AXIN1 and AXIN2 by preventing PARsylation, thus promoting β-catenin destruction complex stability and functionality (Huang et al., 2009). First generation tankyrase inhibitors, inhibitor of Wnt response (IWR) and XAV939, were independently discovered and both shown to inhibit β-catenin-dependent signalling and cell viability in *APC* or *CTNNB1* mutant cancer cell lines, highlighting their therapeutic promise for the inhibition of Wnt-dependent cancers (Chen et al., 2009; Huang et al., 2009). Second generation tankyrase inhibitors have been optimised for potency, selectivity and tested in pre-clinical mouse models of intestinal cancer, which demonstrate stabilisation of AXIN to reduce β-catenin-dependent signalling and subsequent tumour burden (Lau et al., 2013; Waaler et al., 2012). The translation of this class of drugs into the clinic has been frustrating as significant intestinal toxicity is observed in pre-clinical models (Kahn, 2014; Lau et al., 2013). Next generation tankyrase inhibitors are being developed to reduce off-target effects on genes such as PARP1 and are currently being tested in preclinical models (Nathubhai et al., 2016; Tai et al., 2015). As such, it will be of interest to see if these new compounds can be optimised to balance inhibiting Wnt activity whilst reducing toxicity, which may advance their progression into the clinic.

Inhibitors of β-catenin interaction partners and transcription

A key mechanism of β-catenin-dependent Wnt signalling is the dynamic regulation, and subsequent activation, of a β-catenin-centric complex where β-catenin interacts with and recruits a series of nuclear co-activator proteins such as TCF/LEF, cyclic AMP response element-binding protein (CBP), B-cell CLL/Lymphoma-9 (BCL-9), p300 and others, to activate target gene transcription (Valenta, Hausmann & Basler, 2012). Thus, drugging such protein-protein interfaces offers an attractive avenue for

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blocking Wnt signalling downstream of common pathway mutations such as *APC*, which would limit the risk of mutational circumvention leading to drug resistances. However, this therapeutic approach is not suitable for oncogenic mutations identified in genes responsible for β-catenin transcriptional activity, such as *TCF7L2* (Kim, Kim, Ahn, Yoo & Lee, 2009). To date, a number of small-molecule inhibitors have been identified via cell-based high throughput screens that successfully inhibit β-catenin interactions necessary for transcriptional activation, which significantly limit Wnt signalling and subsequent tumourigenesis in pre-clinical models.

An early approach to detect β-catenin-TCF interactions utilised a high-throughput enzyme-linked immunosorbent assay (ELISA) screen. Subsequent screening of extensive compound libraries (>45,000 compounds) identified eight compounds that dose-dependently inhibited β-catenin-TCF complex formation (Lepourcelet et al., 2004). Functional inhibition of β-catenin-TCF interaction was confirmed via decreased activation of Wnt-specific reporter assays (TOPflash), colon cancer cell proliferation/viability and β-catenin-induced axis duplication in *Xenopus laevis* embryos (Lepourcelet et al., 2004). Of the eight compounds, two structurally related compounds, PKF115-584 and CGP049090, proved the most potent and were found to also disrupt the β-catenin-APC interaction, implicating potential direct binding to β-catenin (Lepourcelet et al., 2004). However, the exact molecular mechanism by which these two compounds inhibit Wnt signalling remains unresolved (Lepourcelet et al., 2004, Kahn, 2014).

Using a similar approach, colon cancer cell lines challenged with a secondary structure-templated chemical library revealed a novel low-molecular weight inhibitor of β-catenin-dependent signalling, ICG-001. Characterisation of ICG-001 demonstrated potent suppression of TOPflash assays and Wnt target gene transcription. ICG-001 mediated Wnt pathway suppression by directly binding to CBP, thus disrupting the interaction between CBP and β-catenin (Emami et al., 2004). ICG-001 selectively induces apoptosis in transformed cells but not in normal colon

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cells as seen in the *Apc*$^{Min/+}$ mouse model and xenograft models of colon cancer (Emami et al., 2004). Second generation class of CBP-β-catenin inhibitors, PRI-724, have progressed into phase II and Ib clinical trials for metastatic CRC (NCI-2015-00436, NCT02413853) and advanced pancreatic adenocarcinoma (NCT01764477) respectively and are currently ongoing.

Implementing 'peptide stapling' technology, which involves the introduction of a synthetic hydrocarbon bridge into an α-helical peptide, both the β-catenin–BCL9 (Takada et al., 2012) and the β-catenin–TCF4 (Grossmann, Yeh, Bowman, Chu, Moellering & Verdine, 2012) interfaces have been targeted, yielding potent *in vitro* inhibitors (IC_{50} ~10–20nM), which displayed low micromolar cell-based inhibition. The clinical utility of the stapled peptide class of molecules has yet to be established (Takada et al., 2012; Grossmann, Yeh, Bowman, Chu, Moellering & Verdine, 2012; Kahn, 2014). As β-catenin-dependent Wnt signalling is deregulated in gastric cancer, with mutations in *APC* and *RNF43* observed (Cancer Genome Atlas Research, 2014; Wang et al., 2014), it will be interesting to see if this class of drugs is also effective in gastric tumours displaying high Wnt signalling.

Concerns surrounding drugging the Wnt pathway

As mechanisms of Wnt deregulation during cancer have been slowly exposed, this has been mirrored by considerable research effort into discovering Wnt-targeted therapeutics (Barker & Clevers, 2006; Kahn, 2014). However, drugging developmental pathways can have potentially devastating effects on embryonic development, adult stem cell populations and the regenerative response following injury (Kahn, 2014). A common concern relating to Wnt pathway therapeutics is the potential for acute toxicity in adult tissues that are maintained by adult stem cells (including intestine, haematopoietic system, skin, bone and hair) regulated by Wnt signalling (Clevers, Loh & Nusse, 2014). With respect to the intestinal epithelium, it is encouraging that targeted ablation of intestinal $Lgr5^{+}$ stem cells, which display

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exquisite sensitivity to Wnt, does not disrupt intestinal homeostasis and a full recovery of the stem cell compartment is driven by substantial cellular plasticity displayed by differentiated intestinal cell types (Buczacki et al., 2013; Tetteh et al., 2016; Tian et al., 2011; van Es et al., 2012). Additional side effects may include metabolic change due to the impact of Wnt on liver zonation, bone toxicity and potential neurological effects given the role of Wnt signalling in synapse formation and maintenance in the CNS and PNS (Burke, Reed, Phesse, Sansom, Clarke & Tosh, 2009; Harrison-Uy, Siegenthaler, Faedo, Rubenstein & Pleasure, 2013; Liu et al., 2011). Therefore, when considering the targeting of crucial developmental pathways that are utilised by both CSCs as well as normal somatic stem cells, the potential 'Jekyll and Hyde'-like behaviour of this class of therapeutic agents remains an ever-present issue (Kahn, 2014).

In addition, the appropriate dose of Wnt pathway inhibitor must be taken into account given the pleiotropic effects and varying thresholds of Wnt signalling across many tissues. For example, the level of β-catenin-dependent Wnt signalling in liver and melanoma oncogenesis is lower than intestinal tumourigenesis (Buchert et al., 2010). Therefore, therapeutic agents that alter Wnt signalling activity may reach an efficacious threshold in one tissue but fail to do so in another. Moreover, within the intestinal epithelium, different Wnt threshold levels distinguish stem and progenitor cells, which could be useful for exclusively targeting CSCs without damaging the renewal capability of the intestine (Tao et al., 2015).

Another question to consider is whether it is best to treat gastric malignancy through antagonising or agonising the Wnt pathway? As mentioned earlier, restoration of *FZD*$_6$ expression in gastric cancer cells is sufficient to inhibit β-catenin-dependent Wnt signalling and associated tumour-promoting phenotypes (Yan, Liu, Zhou, Dang, Yin & Zhang, 2016). Similarly, hyper activation of Wnt signalling in *Cited1* deficient mice reduced tumourigenesis in the intestine (Meniel et al., 2013). In contrast,

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inhibition of Wnt signalling at several different levels of the pathway has been demonstrated to provide anti-tumour effects in several cancers (Anastas & Moon, 2013; Polakis, 2007). Therefore, a greater understanding of how Wnt signalling is controlled in gastric cancer tissues is needed to guide which, if any, inhibitors of Wnt signalling should be applied. Another parameter to consider is the complex inter- and intratumour heterogeneity observed in gastric cancers (Zhong et al., 2016), which can confound drug responsiveness and lead to acquired resistance. It is therefore imperative to have a greater understanding of individual patient tumour biology and to identify predictive biomarkers of efficacy to aid therapeutic strategy.

Concluding remarks

When taken together, the increasing amount of proof-of-principle studies strongly identify that aberrant Wnt signalling contributes to gastric cancer and suggest that interfering with this signal could be an effective treatment for this disease. Although promising therapeutic leads have been advanced and tested in combination with other chemotherapeutics in several solid tumours, the same Wnt targeted therapy combinations have not been tested in pre-clinical models of gastric cancer. While the potential safety concerns associated with Wnt targeted therapies are legitimate, similar complications are also relevant with all drugs. If used and integrated correctly (Cancer Genome Atlas Research, 2017), the recent deluge of genomic (Cancer Genome Atlas Research, 2014), epigenomic (Ooi et al., 2016), and functional profiles from cancer patients (van de Wetering et al., 2015) will pave a clear path forward to identify the appropriate patients for a specific Wnt inhibitor.

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Table 1. Wnt mutations and modifications in gastric cancers

APC	Truncation, point mutation	Tankyrase inhibitors, inhibitors of β-catenin transcription	Wang *et al.* 2014, Li *et al.* 2016
AXIN2	Missense mutation	Inhibitors of β-catenin transcription	Kim *et al.* 2009
CTNNB1	Exon3 mutation, SNPs	Tankyrase inhibitors, inhibitors of β-catenin transcription	Radulescu *et al.* 2012 Wang *et al.* 2012
DKK1-3	Promoter methylation	Porcupine inhibitors, Fzd-blocking antibodies	Yu *et al.* 2009
FZD_1,FZD_2, FZD_7	Overexpression	Fzd-blocking antibodies	Zhao *et al.* 2014
RNF43	Truncation, missense	Porcupine inhibitors, Fzd-blocking antibodies	Wang *et al.* 2014
sFRP1-5	Promoter methylation	Porcupine inhibitors, Fzd-blocking antibodies	Nojima et al. 2007
TCF7	Missense mutation	Tankyrase inhibitors, inhibitors of β-catenin transcription	Kim et al. 2009
WNT-1, WNT-2B, WNT-3, WNT-5A, WNT6	Overexpression	Porcupine inhibitors, Fzd-blocking antibodies	Mao *et al.* 2014, Zhao *et al.* 2014 Yuan *et al.* 2013

Figure 1. Anatomy of the mammalian stomach and mucosa

(A) Gross anatomy of the stomach, illustrating the Gastroesophogeal junction (GEJ), Fundus, Corpus and Antrum. (**B and C)** Schematic of antral **(B)** and corpal **(C)** gastric units and their various epithelial cell types. Each unit is divided into a surface pit, isthmus, neck and base regions. Of note, the cellular composition and architecture varies between the antrum and corpus, which reflects functional specificity.

Figure 2. Inhibitors of Wnt signalling

(A) Following WNT/FZD binding (not shown), recruitment of the scaffolding protein AXIN to the receptor complex leads to inhibition of GSK3 and the β-catenin destruction complex, which allows newly synthesised β-catenin to accumulate and translocate to the nucleus (orange arrows), where it binds with co-factors to form a transcriptionally active complex. Indicated are some of the inhibitors that target various intracellular nodes of Wnt signal transduction that could have therapeutic

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benefit for gastric cancer patients with *APC*, *AXIN* or *CTNNB1* mutations. (**B)** Wnt signalling can be inhibited at the cell surface by various pathway inhibitors such as sFRPs and DKK, which bind to WNT and LRP5/6 respectively. Other approaches target WNT signalling using small molecules that inhibit Porcupine (PORCN), which prevent the secretion of WNT from the endoplasmic reticulum (yellow arrow). In addition, FZD-blocking antibodies (Vantictumab) and decoy FZD receptors (Ipafricept) inhibit Wnt signalling by blocking FZDs and sequestering WNTs respectively. Gastric cancer patients with *RNF43* mutations or WNT and/or FZD overexpression would be likely to benefit from these types of Wnt inhibitors.

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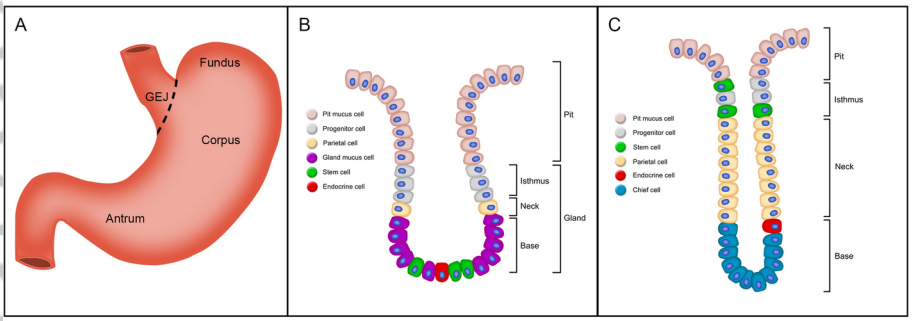
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Figure 1

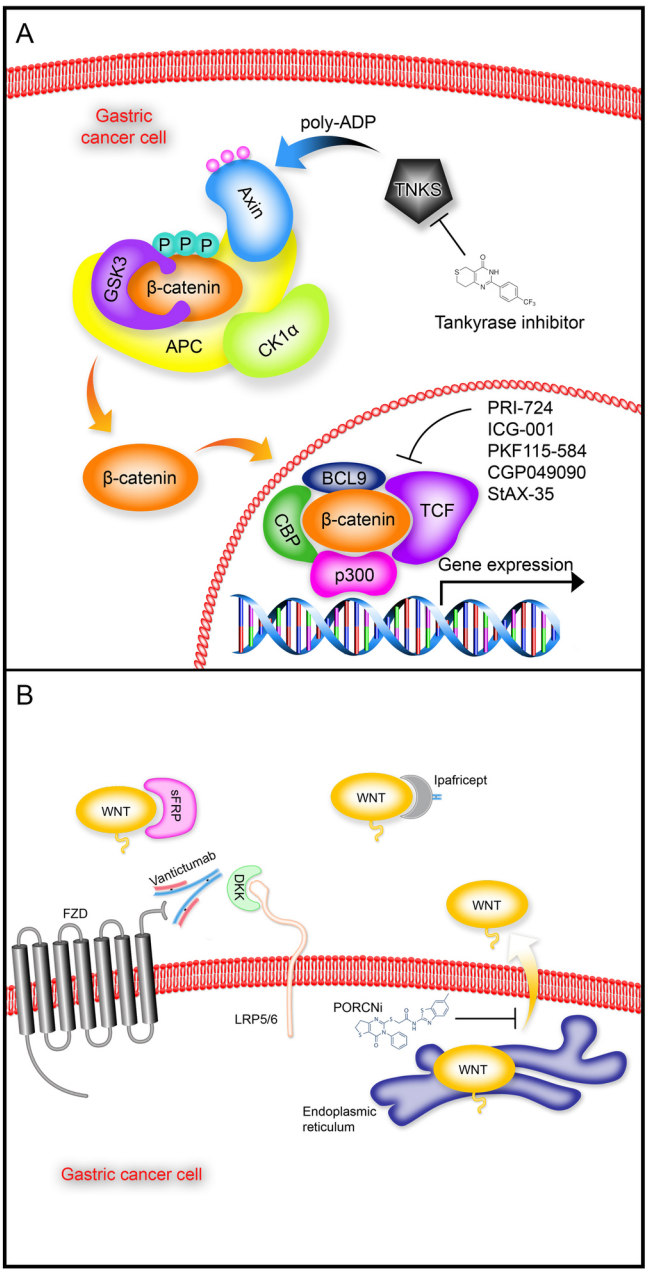


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Figure 2



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Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

List of hyperlinks to cross-reference:
FZD$_1$
FZD$_2$
FZD$_6$
FZD$_7$
WNT1
WNT2B
WNT5A
WNT6
sFRP1
sFRP2
sFRP5
DKK1
LGR5
R-SPO
GSK-3B
CTNNB1

TARGETS	
GPCRs	**Enzymes**
FZD$_1$	GSK-3B
FZD$_2$	
FZD$_6$	
FZD$_7$	
LGR5	

LIGANDS	
WNT1	sFRP5
WNT2B	DKK1
WNT5A	R-SPO
WNT6	CTNNB1
sFRP1	
sFRP2	

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