The Role of Frizzled-7 in Gastric Cancer

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Abstract

Gastric cancer (GC) has an extremely low 5-year survival rate of only 30% and is the third-leading cause of cancer-related deaths worldwide. This is predominantly due to the highly metastatic nature of GC and the lack of available treatment strategies, highlighting the urgent and unmet need to identify novel therapeutic targets. The Wnt receptor Frizzled-7 (FZD7) regulates cell proliferation, epithelial-mesenchymal-transition (EMT), and invasiveness in many cancers. GC patients have mutations in genes that participate in or regulate Wnt signalling at the level of the Wnt receptor binding. Moreover, FZD7 is reported to be overexpressed in human gastric tumours suggesting that aberrant FZD7-mediated Wnt signalling drives GC growth and highlights FZD7 as a potential therapeutic target. However, the precise involvement of FZD7 in GC remains unclear and the specific Wnt receptor transmitting oncogenic Wnt signalling is unknown. Additionally, loss of function mutations to the negative regulator of the Wnt pathway, RNF43, has been implicated in the poor prognosis of GC. However, its functional significance in GC remains unknown.

We have implicated FZD7 as the predominant Wnt receptor involved in the growth, EMT, migration and invasion of GC cells irrespective of APC mutation. Here we used inhibitors of Wnt/FZD (OMP-18R5/LGK-974) and shFZD7 to test the therapeutic potential of targeting Wnt signalling in GC. Pharmacological targeting of FZD inhibited the growth of GC *in vitro* and *in vivo*. Furthermore, we have confirmed the functional significance of RNF43 in GC. Conditional deletion of *RNF43/ZNRF3* led to gastric tumours supporting the hypothesis of stratifying GC patients based on RNF43 mutations. OMP-18R5 and LGK-974 are currently in phase Ib clinical trials for multiple cancers. Our data expands the scope of patients that may benefit from these therapeutic approaches as we have demonstrated that these drugs are effective in treating GC patients regardless of APC mutation status.

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Abbreviations

SYMBOLS

°C = Degrees Celsius

Δ = Delta

µg = Mircograms

µL = Microlitre

µm = Micrometre

µM = Micromolar

fl/fl = Homozygously floxed allele

3D = Three dimensional

A

APC = Adenomatous Polyposis Coli

Arm = Armadillo

B

bp = Base pair

BMP = Bone Morphogenic Protein

C

CagA = Cytotoxin-associated gene product

CaMK = Calcium/calmodulin-dependent protein kinase

CK1-α = Casein kinase 1 alpha

cDNA = Complementary DNA

COSMIC = The Catalogue of Somatic Mutations in Cancer

CRC = Colorectal cancer

CRD = Cytosine eich domain

Cre = Cre recombinase

CRISPR = clustered regularly interspaced short palindromic repeats

CSC = Cancer stem cell

CT = Cycle threshold

D

DAB= 3,3'-Diaminobenzidine

DAG = 1,2 diacylglycerol

dH_2O = Distilled H_2O

Dkk = Dickkopf

DMSO = Dimethyl sulfoxide

DSB = Double strand break

DVL = Disheveled

E

E1-4 = ß-propeller/epidermal growth factor repeats

EC = Enterochromaffin

ECL = enterochromaffin-like

EDTA = Ethylenediaminetetraacetic acid

EEC = Enteroendocrine cell

EMT = Epithelial-mesenchymal-transition

ER = endoplasmic reticulum

EtOH = Ethanol

F

FAP = familial adenomatous polyposis

fl/fl = Homozygously floxed allele

FU = Furin-like

FZD = Frizzled

FZD7 = Frizzled-7

G

GC = Gastric cancer

GEMM = Genetically engineered mouse model

gDNA = Genomic Deoxyribonucleic Acid

GIT = Gastrointestinal tract

GSK-3ß = glycogen synthase kinase-3ß

H

HB-EGF = Heparin-binding EGF-like growth factor

HCC = Hepatocellular carcinoma

HCl = Hydrochloric acid

$HCO3^-$ = Bicarbonate

HDAC = Histone deacetylase

HDR = Homology directed repair

H&E = Hematoxylin & Eosin

HER2 = Human epidermal growth receptor 2

hMSC = Human mesenchymal stem cells

HSPG = Membrane-bound heparin sulfate proteoglycan

I

IHC = Immunohistochemistry

Indel = Insertion/deletion

IP3 = Inositol 1,4,5-triphosphate

L

LDLR = LDL repeats

LEF = Lymphoid enhancing factor

Lgr5 = Leucine-rich repeat-containing G-protein coupled receptor 5

LOF = Loss of function

LoxP = Locus of crossover of

Bacteriophage P1

M

MCR = Mutational cluster region

MET = Mesenchymal-epithelial-transition

MiRNA = MicroRNA

MMTV = mouse mammary tumour virus

MUC5AC = Mucin 5AC

MUC6 = Mucin 6

N

NFAT = nuclear factor associated with T cells

NHEJ = Non-homologous end joining

NLS = Nuclear localization signal

P

PBS = Phosphate buffered saline

PCP = Planar Cell Polarity

PCR = Polymerase chain reaction

PD = Peritoneal dissemination

PKC = Protein kinase C

PORCN = Porcupine

Q

qRT-PCR = Quantitative reverse transcription polymerase chain reaction

R

RNA = Ribonucleic acid

Ror1/2 = Receptor-tyrosine kinase-like orphan receptor 1/2

RPMI = Roswell Park Memorial Institute

R-Spo = R-Spondin

RTK = Receptor tyrosine kinase

Runx3 = Runt related transcription factor 3

S

SAMP = serine alanine methionine proline

sFRP = Secreted frizzled-related proteins

SHH = Sonic hedgehog

shRNA = Short-hairpin RNA

SPEM = Spasmolytic Polypeptide Expressing Metaplasia

T

TALENS = transcription activator-like effector nucleases

TCF = T-cell factor

TCGA = The Cancer Genome Atlas

Tff1 = Trefoil Factor-1

Tff2 = Trefoil Factor-2

TGF-α = Transforming growth factor alpha

TME = Tumour microenvironment

Troy = Tumour necrosis factor superfamily, member 19

W

Wls = Wntless

Wg = Wingless

Z

ZFNS = zinc-finger nucleases

Publications arising from this thesis:

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Other publications during PhD Candidature:

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Chapter 1: Literature Review

1. Introduction

1.1 Gastric Cancer

Gastric cancer (GC) is the fifth most common cancer in the world (1,313,000 cases) and the third leading cause of cancer death globally (819,000 deaths) (Bray et al., 2018). The incidence shows wide geographical variation, with approximately 50% of all cases emerging from Eastern Asia, where China has the highest incidence rate, and where novel cases of GC and mortalities account for ~ 45% of all cases globally (Ferlay et al., 2015). The majority of cases are usually not diagnosed until an advanced stage due to being asymptomatic until this point and the lack of screening programs in the majority of countries. Therefore, in European countries the outcome is often poor, with very low survival rates ranging from ~ 10% to 30%, including patients who have undergone surgery (Katai et al., 2018). Interestingly, the five-year survival rate is relatively good in Japan, where it reaches 90% (Stock and Otto, 2005), likely due to early diagnosis by endoscopic examinations and consecutive early tumour resection. Overall, GC remains a main contributor to the global burden of disability-adjusted life-years from cancer (Van Cutsem et al., 2016).

1.1.1 Risk Factors

GC results from a combination of environmental factors and accumulation of specific genetic alterations. The most common risk factors for GC include a diet high in salty and smoked foods and low in fruits and vegetables, smoking, obesity, chronic gastritis and infections (Buckland et al., 2015; Massarrat and Stolte, 2014). GC has been found to be inversely related to socioeconomic status: high socioeconomic position is associated with a reduced risk of GC (Nagel et al., 2007).

Family clustering of GC has been reported for centuries with the most world-famous example being the family of Napoleon Bonaparte; five first degree relatives were affected by GC, affecting three consecutive generations (Setia et al., 2015) (Sokoloff, 1938). In 1998, truncating mutations of *CDH1* were

described in the germline of three New Zealand Maori families predisposed to diffuse GC (Guilford et al., 1998b). In general, the risk of developing GC is 1.5-3 fold increased in individuals with a family history of GC (La Vecchia et al., 1992).

In developed countries one of the most common infections predisposing people to GC is *H.pylori*. In the general population, *H.pylori* infection reaches ~60%, but in GC patients it is markedly more common, present in ~84% (González et al., 2012). *H.pylori* is a class I carcinogen (Ferlay et al., 2015) and is one of the major causative agent in the cascade leading to GC. *H.pylori* is a gram-negative flagellated bacterium which has evolved to survive the hostile environment of the stomach by colonizing the gastric mucosa. Following successful colonization, *H.pylori* migrates to the stomach epithelium where it triggers a variety of adaptive cellular mechanism, including ER stress, autophagy, oxidative stress and inflammation, all participating in the development and progression of precancerous gastric lesions. The responses in host gastric epithelial cells are located in the gastric pits and attributed to the action of the bacterial virulence factors (Díaz et al., 2018). These include, but are not limited to, urease, vacuolating cytotoxin A, cag pathogenicity island, cytotoxin-associated gene A, peptidoglycan outer membrane proteins and γ-glutamyl transpeptidase (Polk and Peek, 2010; Valenzuela et al., 2013). A recent study identified that treatment of *H.pylori* infection decreased the risk of GC only if eradication was 100% successful (Kumar et al., 2020). Therefore, finding novel targets for the treatment of GC is still key as the mechanisms of GC prevention are still unclear.

1.1.2 Gastric Cancer Treatment Options

As mentioned above, GC is largely asymptomatic with the majority of patients presenting to their general practitioner in the late stages of the disease with generic symptoms such as abdominal pain, unexplained weight loss, a sense of fullness after eating a small meal and acid reflux. Before any systemic treatment is initiated, the status of the human epidermal growth factor

receptor 2 (HER2) is determined. Approximately 20% will present with HER2-positive GC (Discussed in detail later). The treatment options for the majority of patients with HER2-negative GC is dependent on the stage. Although rarely the case, if a patient presents with an early stage GC (Stage 0 and IA) they are best treated by minimally invasive endoscopic surgery. Stage IB, II and III GC is treated with total or partial gastrectomy and lymphadenectomy. Unfortunately, this is life-changing surgery with often serious and sometimes fatal side effects, such as blood clots, malnutrition and anastomotic leakage (Ikeguchi et al., 2012). Chemotherapy may be given before or after surgery to either reduce the tumour prior to surgery or to remove remaining cancer cells if they are identified in removed lymph nodes. Patients not able to undergo surgery, are treated with chemotherapy, radiation, or chemoradiation. For advanced stage cases (Stage IV), chemotherapy is the current standard of care for first-line treatment for patients due to the tumour often being too invasive for surgery (Wagner et al., 2006). However, this fails in >95% of non-operable gastric tumours due to the various mechanisms of chemoresistance (J.J.G. Marin, 2016). Therefore, patients with locally advanced stage GC and/or metastatic disease often only receive palliative treatments to improve their quality of life.

As mentioned, approximately 20% of GC are characterized by overexpression and/or amplification of the *HER2* gene. However, the prognostic value of HER2 in GC is controversial (Kim et al., 2014). Despite this, there is one approved drug targeted against HER2, Trastuzumab. This is the only molecular target with an approved drug for first-line treatment of GC. Trastuzumab is a recombinant humanized IgG1 monoclonal antibody directed against HER2. It is approved by both the FDA and the EMA for the treatment of HER2-postive metastatic adenocarcinoma of the stomach in combination with cisplatin and capecitabine or 5-fluoroouracil. Patients must not have received prior treatment for their metastatic disease and have tumours expressing high levels of HER2 as defined by a positive immunohistochemistry (IHC) score of 3

(Spackman et al., 2013). HER2 protein is a tyrosine kinase receptor, which upon activation triggers a broad spectrum of downstream cascades to promote numerous effects, including cell proliferation, apoptosis, adhesion, migration, and differentiation. Trastuzumab blocks the activation of HER2 by binding to its extracellular domain leading to the inhibition of cancer cell proliferation (Gunturu et al., 2013).

Immuno-oncology is a rapidly growing research area and has great success in the treatment of many cancers, especially with PD1/PD-L1 inhibitors. However, there are currently no approved first-line immunotherapies for GC. There are some promising clinical trials currently place which could potentially lead advancements in the treatment strategy of GC patients, particularly those with locally advanced or metastatic disease, and lead to a much improved quality of life. Excitingly, some recent clinical data has shown significant reduction in tumour burden in patients with untreated metastatic HER2-positive GC when treated with a combination of Trastuzumab and PD1 inhibitor (Pembrolizumab). A response rate of 52% was observed even without chemotherapy (Janjigian et al., 2019).

Clearly there is an urgent and unmet need to develop better, more effective and more targeted treatments for GC. This will require further work at a basic research level to identify novel therapeutic targets and drugs to ultimately lead to a longer and improved quality of life for GC patients.

1.2 Normal Gastric Architecture & Dynamics

Before deciphering the complicated and complex process of GC formation it is important to first have an understanding of the normal gastric morphology. An ongoing challenge that the tissue of the gastrointestinal tract (GIT) face, especially the stomach and small intestine, is allowing the efficient digestion, exchange, and absorption of nutrients, and water, whilst simultaneously preventing passage of harmful molecules and organisms. To overcome this challenge, all tissues of the GIT are lined by a specialized single layer of cells, the gastrointestinal epithelium. This functions as a protective barrier, as well as secreting a cocktail of factors (hormones, proteases, gastric acids, and mucus) to aide in food breakdown and nutrient uptake. This epithelium is continually renewed to preserve tissue homeostasis, due to the unrelenting exposure to chemical, biological and mechanical stresses. This constant replenishment of epithelial cells also serves as a protective mechanism to rid the epithelium of cells that may have undergone somatic mutations or cellular transformation.

The gross anatomy of the mammalian stomach also addresses this challenge. The stomach is divided into three anatomically distinct regions; the corpus the antrum, and the non-glandular fundus (Figure 1.1A)). The corpus is the main body of the stomach, primarily responsible for its digestive action. This is achieved through the release of a cocktail of hormones, enzymes and acids (O'Connor and O'Moráin, 2014; Willet and Mills, 2016). In contrast, the antrum secretes large amounts of mucous, as well as gastric hormones. This reflects its protective function, shielding the gastric epithelium from the hydrochloric acid secreted in the corpus region (Barker et al., 2010a; Karam, 1993). It is worth noting that the gross anatomy of the stomach differs between species. Of note, the stomach of mice includes a large non-glandular forestomach (Kararli, 1995) not present in humans.

Unsurprisingly, due to their close proximity and shared role in digestion, the stomach shares a number of features with the intestine. They have a common endodermal origin and an epithelial lining that is continually renewed by distinct populations of adult stem cells (Hoffmann, 2008). Much like the intestinal crypts, the gastric epithelium is comprised of invaginations, termed gastric units. Structurally, these units are made up of a pit, which is continuous with the surface epithelium and a flask-shaped gland which is organized into the isthmus, neck, and base regions. There are four major differentiated cell types that populate the gastric units; parietal cells, chief cells, gastric mucous cells and a variety of endocrine cells.

The distribution of these cell types and the turnover rate varies between the corpus and the antrum, mirroring their difference in function. The corpus gastric units are composed of several long glands that feed into short pits. Their epithelium is heterogeneous, containing vast quantities of parietal cells, a small number of base and neck mucus cells, endocrine cells (or G cells) and unique to the corpus, chief cells (Figure 1.1C). In contrast, the antrum is comprised of several short glands that feed into a single extended pit. They have a much simpler cellular composition characterized by abundant gastric mucus-secreting cells, endocrine cells and very low numbers of parietal cells (Figure 1.1B).

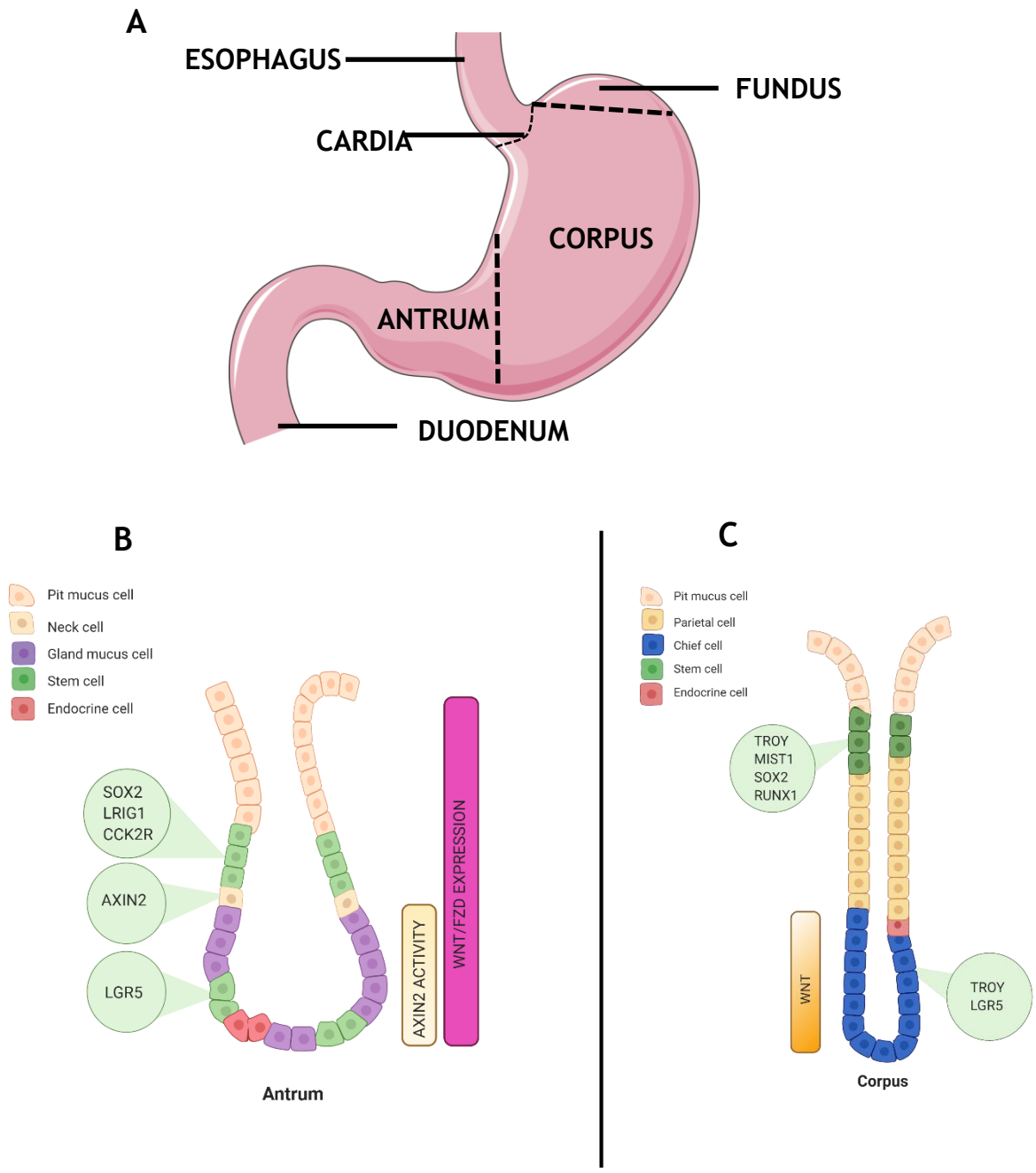


Figure 1.1. Structural organization of the stomach.

(A) Anatomic schematic of the mammalian stomach
(B) Gastric unit of a human antral epithelium depicting the various cell types, stem cell populations (highlighted in green bubbles) and signalling gradients (Adapted from (Flanagan et al., 2018).
(C) Gastric unit of a human corpal epithelium depicting the various cell types, stem cell populations and signalling gradients (Adapted from (Flanagan et al., 2018).

1.2.1 Chief cells

The chief (or zymogenic) cells are only present in the glandular corpus region of the stomach and secrete digestive-enzymes. These cells occupy the majority of the base of a gastric gland, with the upper ones organized as simple cuboidal-columnar cells, and the lower ones outpouching into an acinar configuration at the very base (Karam, 1993). The primary function of chief cells is the secretion of enzymes involved in the digestion of protein. The main enzyme, secreted in its inactive, form is pepsinogen. Upon exposure to stomach acid, inactive pepsinogen undergoes a conformational change, exposing its catalytically active site and allowing the generation of active pepsin by proteolysis (Raufman, 1992).

Normal chief cells have a unique lineage, and do not derive directly from gastric epithelial progenitor cell lineages or involve cell division. Instead they arise by transdifferentiation. Pre-neck cells differentiate into mucous neck cells as they migrate toward the base of the glands and then re-differentiate at the bottoms of glands into chief cells, with a distinct pattern of gene expression; *MIST1* in mature chief cells and *TFF2* and *MUC6* in the more proliferative progenitor mucous neck cells (Hanby et al., 1999; Ramsey et al., 2007). While this dramatic and malleable phenotypic transition exhibited by chief cell lineages during normal homeostasis is remarkable, it can leave them vulnerable. In a pathological setting it can lead into a mucus cell metaplasia of the gastric glands, known as Spasmolytic Polypeptide Expressing Metaplasia (SPEM) (Lennerz et al., 2010).

1.2.2 Parietal cells

Parietal cells occur throughout the gastric unit of the corpus, with the majority located in the neck of the gland (Bredemeyer et al., 2009; Karam, 1993). They have multiple roles in gastric secretion, protection, and coordination of physiological repair. Their primary purpose is the production of hydrochloric acid (HCl) which aides in the digestion of food, absorption of minerals, control of harmful bacteria, and the maintenance of a strong acidic environment (pH<2) (Quigley and Turnberg, 1987). In addition to their unique

ability to produce HCl, parietal cells play a role in gastric homeostasis through the secretion of multiple growth factor molecules such as Heparin-binding EGF-like growth factor (HB-EGF) (Murayama et al.), transforming growth factor alpha (TGF-α) (Beauchamp et al., 1989) and sonic hedgehog (SHH) (Zavros et al., 2008).

The production of HCl by partial cells requires the gastric H^+/K^+ ATPase enzyme, a P_2-type ATPase. This is expressed only by the parietal cells, and is therefore a robust molecular marker of parietal cells (Spicer et al., 2000), and regulates the exchange of cytoplasmic H^+ for extracellular K^+. When parietal cells are stimulated by gastrin, their proton pumps secrete H^+ in the gastric lumen, which combines with luminal Cl^- to form HCl, creating the acidic environment of the stomach (Engevik et al., 2019). To ensure the required digestion of food while preventing damage to the gastric and duodenal mucosa, parietal cell-medicated acid secretion is highly regulated. This is achieved through a fine balance of activators and inhibitors including gastrin, histamine, the vagus nerve, somatostatin and glucagon-like peptide (Powley et al., 2011).

Parietal cells role in general mucosal homeostasis is highlighted by the observation that loss of parietal cells (termed oxyntic atrophy) is linked to the development of metaplasia in the corpus mucosa (El-Zimaity et al., 2002), which is a common precursor to gastric cancer (GC). The most common cause of parietal cell loss is chronic infection of the stomach with *Helicobacter pylori*. Although the mechanism of this still remains unclear, it is thought to require the action of T cells (Roth et al., 1999). More recent studies suggest that cytokines may lead to parietal cell death (Howlett et al., 2012) (Buzzelli et al., 2015). Loss of parietal cells is also linked to SPEM, which as described above, develops from the transdifferentiation of chief cells into mucous cell metaplasia (Huh et al., 2012) (Nam et al., 2010). However, the exact signals that coordinate these lineage changes remain unclear, with a recent publication indicating that parietal cell loss alone is not sufficient to induce SPEM (Burclaff et al., 2017).

1.2.3 Mucous-secreting cells

The entire GIT is covered by mucus, which has different properties in the stomach, small intestine, and colon. Within the stomach there is a two-layered system with an inner and an outer mucus layer, a system which is also observed in the colon (Atuma et al., 2001). The inner mucus layer of the stomach is very firmly attached to the epithelial cells. The outer layer is less so, although is still more attached than that of the colon (Ermund et al., 2013). This is to ensure robust protection of the gastric epithelium from the harsh HCl present in the gastric lumen. Additionally, this firmly attached mucus layer is important for the maintenance of a pH gradient across the mucus layer (Phillipson et al., 2002). A pH gradient exists across the gastric mucus barrier, with a near-neutral pH at the mucus surface (Schreiber and Scheid, 1997). The buffering of the H^+ secreted from the parietal cells occurs in the mucus via HCO_3^-, which is released from gastric surface mucus cells via an apical Cl^-/HCO_3^- exchanger (Allen and Flemström, 2005).

There are two types of gastric mucus cells: surface mucus cells and mucus neck cells. Surface mucus cells secrete Mucin 5AC (MUC5AC) and Trefoil Factor-1 (Tff1), whilst mucus neck cells secrete Mucin 6 (MUC6) and Trefoil Factor-2 (Tff2) (Pelaseyed et al., 2014). Tff1 and Tff2 are small mucin-associated secreted peptides of gastric mucus cells and have a role in gastric mucosal protection (Newton et al., 2000).

As well as protecting the gastric epithelium from the acidic environment of the stomach, the gastric mucus regulates the colonization of *H.pylori* (Skoog et al., 2012). This function is especially important as approximately half of the world's population are infected with *H.pylori*, with 1-3% of infected individuals going on to develop GC (Suerbaum and Michetti, 2002). The mucus layer prevents the majority of the colonizing *H.pylori* from attaching directly to gastric epithelial cells (Hessey et al., 1990), with them instead living in the mucus layer of the superficial gastric mucosa where they bind to highly glycosylated mucins (Lindén et al., 2002). Tff2 secreted by the mucus neck cells has been observed

to have a protective function against the progression of premalignant lesions in *H.pylori* infected mice (Fox et al., 2007) and epigenetic silencing of tff2 by *H.pylori* infection leads to gastric tumour development (Peterson et al., 2010). *H.pylori* is also able to alter gastric mucins with MUC6 becoming aberrantly expressed in the surface mucus cells (expressed mucous neck cells in a non-pathological setting) in *H.pylori* patients (Byrd et al., 1997).

1.2.4 Enteroendocrine cells

There are over thirty different hormones that have been identified as being produced in the GIT, and it is the enteroendocrine cells (EECs) that produce them. Hormones are released by the EECs in response to meal-related stimuli, and exert actions ranging from the local control of gut motility to the regulation of insulin and food intake (Gribble and Reimann, 2016). EEC populations in the stomach include enterochromaffin cells (EC cells), enterochromaffin-like cells (ECL cells), D-cells, G-cells, A-cells, and P/D1 (in humans) or X/A (in rats) cells. Table 1.1 highlights their individual secretions and functions (Håkanson et al., 1986; Ku et al., 2003; Lamberts et al., 1991; Sjölund et al., 1983; Stengel and Taché, 2009). Within the GIT, the EECs are usually located in the epithelial layer and make direct contact with the luminal constituents. However, in the stomach, especially the corpus glands, a large population of EECs are closed-type cells that do not make contact with the gastric lumen (Gribble and Reimann, 2016).

Table 1.1 Summary of gastric Enteroendocrine cells.

EEC Cell	Location	Secreted hormone	Function
EC cells	Antrum	Serotonin	Motility and mucosal growth & maintenance
ECL cells	Corpus proximal to parietal cells	Histamine	Aides in production of gastric acid
D-cell	Corpus & Antrum	Somatostatin	Inhibits gastric acid secretion
G-cells	Antrum	Gastrin	Controls gastric acid secretion by inhibiting ECL cells
A-cells	Corpus	Ghrelin	Regulates appetite
P/D1 cells	Gastric chief cells	Leptin	Regulates appetite

1.2.5 Gastric epithelial renewal

Due to the dynamic function and harsh environment of the stomach, the surface epithelium needs to be continually renewed. This is fueled by a small population of stem cells housed within the isthmus region of the gastric glands, both corpus and antrum. There are also populations of reserve stem cells, activated upon injury, that are located in the base of the glands. The population of reserve stem cells differ between the corpus and the antrum. A summary of gastric stem cells can be found in Figure 1.1. However, the mechanisms and factors that regulate gastric homeostasis have only been partially characterized.

The first studies looking into the dynamics of gastric epithelial renewal used radioactive labels to identify cellular migration, position, turnover rate, and kinetics (Karam, 1993). In the corpus, mucus-secreting cells migrate from the neck of the gland to the surface epithelium, where they live for 2-3 days. Parietal cells are located within the corpal gland neck, live for approximately 2 months and migrate bi-directionally towards the surface and the base of the gland. Chief cells positioned at the lower third of the corpus gland migrate to the base of the gland, where they reside for approximately 6 months (Karam, 1993). As the antral glands lack these long-lived chief and parietal cells, their turnover rate is much more rapid at 1-60 days.

Moving on from the early studies, the generation of transgenic mice to mark the progeny of specific cell types via lineage tracing (the gold-standard to assess stem cell function *in vivo*) has permitted the identification of several gastric stem cell markers, marking both active and reserve populations, with common genes often marking stem cell pools in both the corpus and antrum. (Flanagan et al., 2018). Corpal homeostasis is maintained by stem cells in the highly proliferative isthmus region, marked by *Mist1*, *Runx1*, *Sox2* and *Troy*. Lineage tracing has also been observed from Troy+ cells located in the base (Hayakawa et al., 2015a). Within the antrum four stem cell markers gave been identified: *Lgr5*, *Sox2*, *Lrig1* and *CCKBR* (Arnold et al., 2011; Barker et al.,

2010a; Hayakawa et al., 2015b; Powell et al., 2012), which all contribute to the homeostasis of the antral tissue.

Of the stem cell markers mentioned above, *Lgr5* is the best characterized. It resides at the base of both gastric glands and all gastric epithelial cell lineages can be derived from it as part of normal homeostasis or in response to injury (Barker et al., 2010b; Stange et al., 2013). It was initially identified as a Wnt target gene in the intestinal epithelium (Barker et al., 2007). It encodes a 7-transmembrance protein that participates in the Wnt receptor complex where it binds R-Spondin proteins (de Lau et al., 2011). Like the intestine, Lgr5+ cells populate the base of the gland, however the average number of four Lgr5+ cells per gastric gland is lower than that seen in the intestinal crypt (14). Also similar to the intestine is the requirement of gastric cultures for Wnt effectors (R-Spondin & Wnt3a) for effective culture growth. This is strongly suggestive of a Wnt active stem cell niche *in vivo* (Barker et al., 2010a). However, the exact source of Wnt ligands in the gastric epithelium remains to be established. Lgr5+ cells have a probable role in homeostatic maintenance of the gastric epithelium, due to them being mitotically active. Another difference between Lgr5+ cells of the intestine and stomach is that intestinal Lgr5+ cells at the crypt base act as active stem cells, whereas within the stomach they behave as reserve stem cells (Hata et al., 2018). Lgr5+ can act as an active stem cell at the base of the antrum (despite being more quiescent than those of the intestine) and generate transit-amplifying cells, yielding differentiated cells that constitute the bulk of the glandular epithelium (Barker et al., 2010b). However, within the corpus their role is solely as a reserve stem cell. This was observed through investigation with a non-variegated mouse model, *Lgr5-2A-CreERT2,* which detected *Lgr5* expression in a subpopulation of chief cells at the base of the corpal gland. Lineage tracing showed that although this population of cells do not give rise to fully traced glands during homeostasis, they were activated in response to damage, after which they could give rise to whole corpus glands (Leushacke, 2017).

The gastric epithelium also has other populations of reserve stem cells that are activated in response to injury, and are able to generate all gastric lineages. In particular, the stem cell marker *Troy* (Tumour necrosis factor superfamily, member 19) is expressed at the corpal gland base by a small population of fully differentiated chief cells. Lineage tracing with *Troy-eGFP-IRES-CreERT2* mice demonstrated that single marked chief cells are able to generate fully labeled gastric units over periods of approximately 3 months after recombination. This phenomenon accelerates upon tissue damage. Additionally, despite *Troy*+ chief cells rarely being proliferative, cultured *Troy*+ chief cells can generate long-lived gastric organoids that can be differentiated towards the mucus-producing cell lineages of the neck and pit *in vitro* (Stange et al., 2013). Furthermore, selective killing of the highly proliferative isthmus cells results in the activation of *Troy*+ chief cells (Li and Clevers, 2010). This demonstrates that *Troy* marks a specific subset of chief cells with the capability to replenish entire gastric units thus serving as quiescent reserve stem cells. Moreover, a rare population of *Troy*+ cells in the isthmus of corpus has been identified (Hayakawa et al., 2015a). This suggests there may be two populations of *Troy*+ cells in the corpus: one residing in the isthmus which is able to lineage trace during normal homeostasis, and the other in the base of the gland which functions as a reserve cell during healing.

Together these findings present a model that gastric epithelial renewal involves several unique populations of stem cells co-operating together to maintain homeostasis and yield and efficient and effective response to damage and injury. Wnt signalling plays an important role in the regulation of these stem cell populations which will be discussed in detail next.

1.3 Gastric Cancer Initiation

The acquisition of oncogenic mutations and ultimately the development of GC is influenced by a variety of environmental and pre-disposed genetic factors that can alter the changes of tumour formation.

GC initiation is a multi-step process upon which various genetic and epigenetic alterations accumulate (Oue et al., 2015). However, its precise carcinogenesis is less well characterized than colorectal cancer with many questions and missing links remaining. *H.pylori* colonization and/or other environmental factors drive superficial gastritis, which progresses to chronic inflammation which develops into intestinal metaplasia (or SPEM), evolves into dysplasia and finally adenocarcinoma which can progress into invasive cancer (Figure 1.2) (Correa, 1988).

A key histological characteristic of GC is the loss of parietal cells (also termed oxyntic atrophy) with this event being a prerequisite for the triggering of metaplasia and linked to the initiation of both dysplasia and neoplasia (Goldenring and Nam, 2010). Loss of parietal cells results in the transdifferentiation of the chief cell lineage into a mucus cell metaplasia identified as Spasmolytic Polypeptide Expressing Metaplasia (SPEM) (Nam et al., 2010) which is a neoplastic precursor in gastric carcinogenesis and an alternative type of metaplasia to intestinal metaplasia. SPEM cells express high levels of Tff2 and MUC6 (Weis and Goldenring, 2009) in contrast to intestinal metaplasia which is characterized by the expression of Tff3 and MUC2 (Nam et al., 2009).

The precise mutations and alternations that trigger each step of GC development are still being investigated and identified due a complex etiology by interactions between bacteria, host and environmental factors. Despite this, the development of GC converges on several signaling pathways that become disrupted through activation of oncogenes, *K-ras* and *c-met*, and inhibition of tumour suppressors *Apc* and *p53* (Hara et al., 1998; Horii et al., 1992; Yokozaki et al., 1992). Despite a number of genes having been identified as potential

driver genes in GC, the association between somatic mutations and clinical features has not been thoroughly elucidated to date. Research groups have been attempting to screen gastric tumours to identify driver mutations and determine if there is any genotype-phenotype correlation, however, results have been conflicting. This suggests that GC is not enriched with known driver mutations, highlighting why many targeted drugs useful in the treatment of other tumours are not effective in GC (Nemtsova et al., 2020). Additionally, *TP53* and *CDH1* (both common validated mutations in GC) mutation status does not alter GC treatment (Katona and Rustgi, 2017). Therefore, there needs to be a focus on epigenetics and other molecular characteristics to stratify patients and develop better treatments for patients with GC.

Germline mutations in some driver genes determine predisposition to the development of hereditary GC. Mutations in *CDH1*(E-cadherin) are responsible for the development of early hereditary diffuse-type GC (Luo et al., 2018), as are mutations in *STK11* (Peutz-jeghers syndrome), *TP53* (Li-Fraumeni syndrome), *BMPR1A* or *SMAD4* (gastrointestinal polyposis) and *PTEN* (Cowden syndrome) (Colvin et al., 2015).

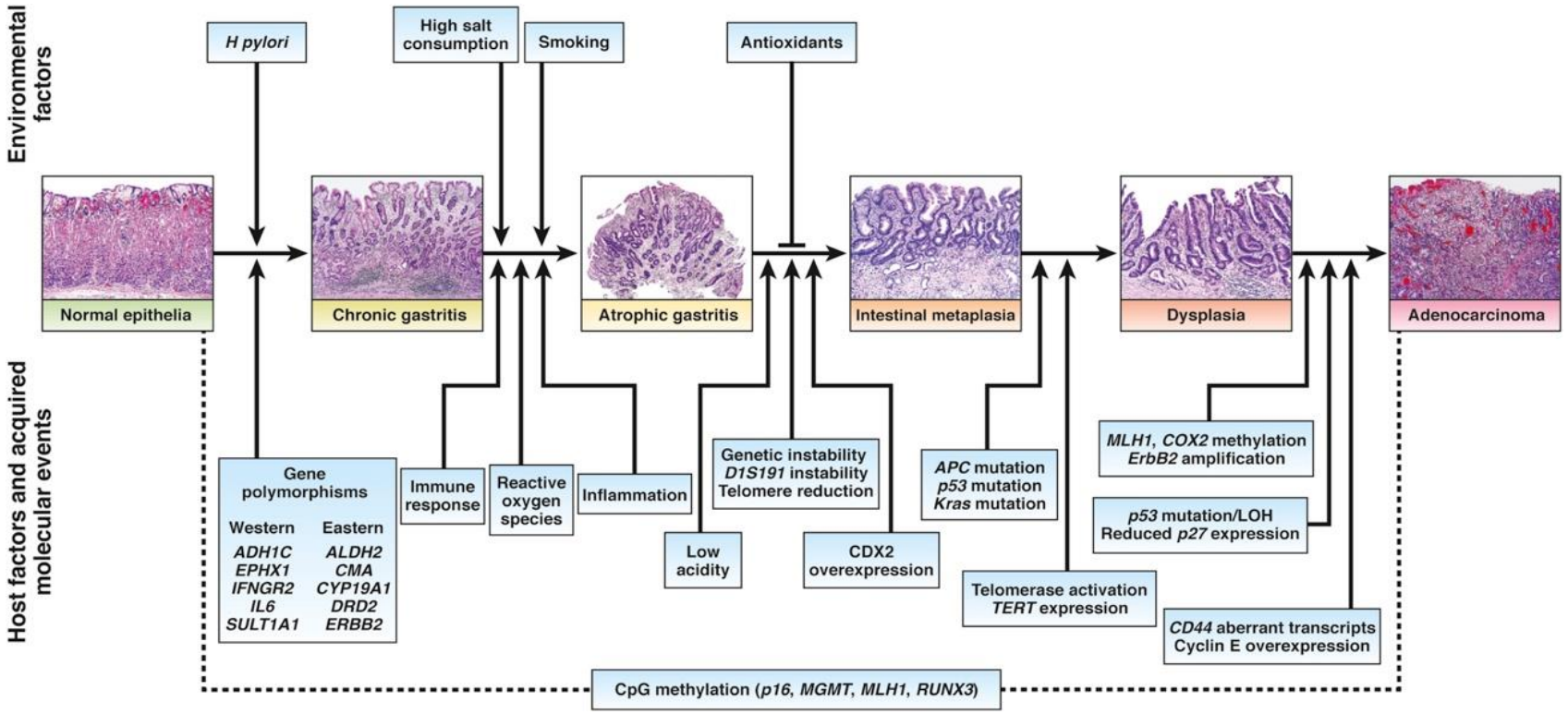


Figure 1.2 Pathogenesis of gastric cancer (Adapted from (Tan and Yeoh, 2015). A summary of current knowledge of the cause and pathogenesis of gastric cancer, including host and environmental factors as well as acquired molecular events. Many gaps in knowledge remain.

2

1.3.1 GC Classification

The majority (>90%) of GCs are adenocarcinomas. Non-Hodgkin's lymphomas and gastrointestinal stromal tumours make up the remaining 10% (Kelley and Duggan, 2003). GC adenocarcinomas are highly heterogeneous in regard to architecture, growth, cell differentiation and molecular pathogenesis. Therefore, there is an array of diversity of histopathological classification schemes. The most commonly used is the Lauren classification (Lauren, 1965). This classification broadly divides GC into two main pathological groups, intestinal-type and diffuse-type, in addition to mixed and indeterminate types. Diffuse-type carcinomas are poorly differentiated and are composed of solitary or poorly cohesive tumour cells in the absence of gland formation. By contrast, intestinal carcinomas are mostly well to moderately differentiated and form glandular structures that are reminiscent of colorectal adenocarcinomas (Van Cutsem et al., 2016).

The cancer genome atlas (TCGA) classification, made possible thanks to the advancements in next generation sequencing, was a milestone for the molecular characterization of GC. They performed full genomic profiling of 295 primary gastric adenocarcinomas (Cancer Genome Atlas Research, 2014), which through complex statistical analyses lead to the identification of four tumour subtypes: tumours positive for Epstein-Barr Virus (9%), microsatellite unstable tumours (22%), genomically stable tumours (20%) and chromosomally unstable tumours (50%). Table 1.2 highlights key features of each subtype. Identification of these subtypes has provided the start of a roadmap for patient stratification and trials of targeted therapies. TCGA network found a correlation with histological characteristics showed enrichment of the diffuse subtype in the genomically stable group (73%). The frequency of chromosomally unstable tumours was increased in gastro-esophageal-junction adenocarcinomas, and most tumours positive for Epstein-Barr virus were located in the fundus or corpus regions of the stomach. They also identified that tumors positive for Epstein-Barr virus were mostly found in males (81%) but predominance of microsatellite unstable tumors marginally favored females (56%) (TCGA, 2014).

Clinical translation of these molecular findings is vital to provide novel strategies for early GC detection and promote precision therapies for GC patients. For example, Epstein-Bar Virus-positive and microsatellite subtypes of GC have shown to have increased expression of PD-L1 and therefore may be good candidates for treatment with immune checkpoint therapy with PD-1/PD-L1 inhibitors (Derks et al., 2016) However, despite these clear views of the genetic diversity across human GCs, the mechanistic connection between their genotypes and phenotypes has largely remained unclear. Several research groups have performed studies to start making this connection clearer. Traditionally genetic mouse models have been used to phenotypically interpret genetic mutations, however most knockout mouse models for GC imply clinically irrelevant mutations. This renders it difficult to translate the phenotype to clinic. To address this, organoid technology has been utilized to culture gastric tumours and their corresponding normal tissues. After complex optimization of culture conditions, three independent studies by Nanki *et al.*, Yan *et al.*, and Seidlitz *et al.,* generated biobanks of patient-derived GC and normal gastric organoids which succeeded to captures all the GC molecular subtypes. Within their own studies they identified various patterns within subtypes however overall, the results between studies gave conflicting data, and no robust genetic distinction amongst GC subtypes has been elicited as of yet.

While genomic analysis has identified 46% of gastric tumour exhibit deregulation of the Wnt/ß-catenin pathway (Ooi et al., 2009), with several Wnt ligands upregulated, including WNT1, WNT2b, WNT5a, WNT6, and WNT10a (J Mao, 2014). Research is still ongoing to link deregulated Wnt to the specific histological and molecular GC subtypes. There are no results published as yet and this is a significant gap in the field.

Table 1.2. Key features of gastric cancer molecular subtypes.

EBV	MSI	CIN	GS
PIK3CA mutation	Hypermutation	Intestinal histology	Diffuse histology
PD-L1/2 overexpression	Gastric-CIMP	TP53 mutation	CDH1, RHOA mutations
Extreme DNA hypermethylation	MLH1 silencing	RTK-RAS activation	CLDN18-ARHGAP fusion
CDKN2A silencing	Mitotic pathways		Cell adhesion
Immune cell signalling			

1.4 Gastric Cancer Metastasis

Cancer metastasis is the process of cancer cells spreading from the primary site to other organs, which contributes to the major cause of death in cancer patients. Approximately 50% of patients with advanced GC die from recurrence and metastasis, even after curative surgery and chemotherapy (Zhou et al., 2017), with the medium survival of only 4 months (Zhao et al., 2019). However, the underlying mechanisms driving metastasis are even more complicated than those resulting in carcinogenesis (Gómez-Cuadrado et al., 2017). Metastatic tumours largely rely on the same driver mutations found in primary tumours (Zehir et al., 2017), suggesting the hallmark functions for tumour maintenance and progression remain critical in metastases.

1.4.1 Stages of Metastasis

For successful metastasis, in all cancer types, to take place, a compatibility between circulating tumour cells and a premetastatic niche is required. This is referred to as the seed and soil hypothesis (Paget, 1989). Improved sequencing technology built upon this and helped elucidate the model of the metastatic cascade, which can be broadly divided into four main processes: invasion, intravasation, extravasion and colonization (Shimizu et al., 2018). The metastatic cascade of GC consists of lympathic metastasis, hematogenous metastasis and peritoneal dissemination (PD). These processes require a number of important intra and extracellular changes which have the potential to be targeted for therapeutic gain.

GC metastasis follows a non-random distribution among distant organs. This phenomenon is termed “organotropism” or “organ-specific metastasis”. Common sites of spread for GC other than peritoneum in order of incidence are liver, lung and bone (Riihimäki et al., 2016). Ever-increasing evidence suggests that organotropism is regulated by a multitude of factors, including tumour-intrinsic factors, organ-specific niches, circulation patterns, and the interaction between tumour cells and the host microenvironment (Gao et al., 2019).

Typically, lymphatic and hematogenous metastasis are the major dissemination processes in solid cancers, with many metastasis models based on them. However, PD is the most frequent metastatic type in GC patients, occurring in more than 60% of patients with metastatic GC (Hu et al., 2018). Unlike lymphatic and hematogenous metastases, PD is driven by direct invasion from the gastric wall to the peritoneal cavity. This multistep process involves the cancer cells detaching from the primary tumours, adapting to the microenvironment of the peritoneal cavity and developing disseminated nodules (Figure 1.3). E-cadherin is a major contributor to a cells ability to undergo PD. It is a calcium-dependent cell-cell adhesion molecular that has a key role in establishing epithelial architecture and maintaining cellular polarity. Therefore, dysregulation of E-cadherin contributes to tumour invasion by promoting cell motility (Guilford et al., 1998a; Liu and Chu, 2014) and resulting in PD. Furthermore, E-cadherin and the E-cadherin-catenin complex may promote invasion and migration by modulating various signalling pathways, including Wnt signalling (Kuphal and Behrens, 2006), as well as EMT (Kourtidis et al., 2017).

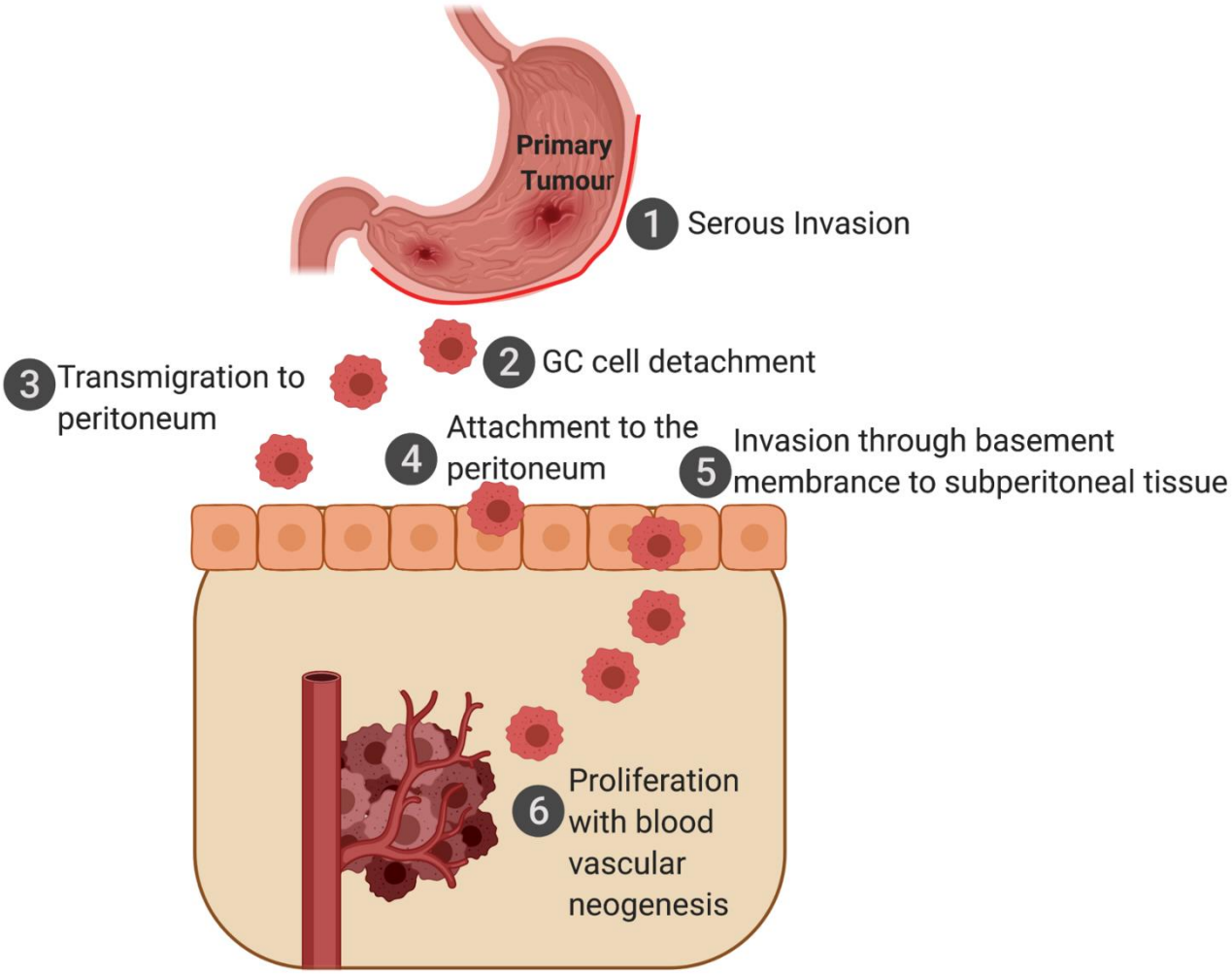


Figure 1.3 Peritoneal dissemination of gastric cancer. (**1**) Penetration of tumour tissue through serous layer. (**2**) Detachment from the primary site (down-regulation of E-cadherin). (**3**) Transmigration to the distant peritoneum, EMT involvement. (**4**) GC cell attachment to the peritoneum. (**5**) Invasion into subperitoneal tissue. (**6**) Proliferation with infiltration of stromal cells and vascular neogenesis, colonization of secondary site.

1.4.2 EMT

The EMT process is thought to play a central role in the departure of GC cells from primary tumour in all metastatic cascades (Lambert et al., 2017). EMT is a phenotypic conversion mechanism that refers to the loss of epithelial features and acquisition of mesenchymal properties, which is vital for preparing cancer cells to invade the surrounding parenchyma and intravasate into the bloodstream, lymph nodes or invade into the peritoneal cavity. EMT has been implicated in the initiation of metastasis and tumour progression in many cancer types, including GC (Huang et al., 2014). During EMT, epithelial cells exhibit enhanced motility and invasiveness (Peng et al., 2014), low expression of E-cadherin, high expression of vimentin and N-cadherin, a spindle-like shape and reduced adhesion. The key ligands involved in EMT are TGFß, Wnt and Notch. The major transcription factors that induce EMT via downregulation of E-cadherin (Kourtidis et al., 2017) are Twist, Snail, Slug and ZEB1 (Prieto-García et al., 2017). In the context of PD it has been found that it is the discoidin domain-containing receptor 2 that promotes PD in GC via induction of EMT (Kurashige et al., 2016)

1.4.3 Tumour Microenvironment

The microenvironment of the free abdominal space is hypoxic, contains immune cells, and is deficient in glucose (Gilkes et al., 2014) making it challenging, but not impossible, for the cancer cells seeded in the peritoneal cavity to survive, proliferate and migrate in this environment. Cell adhesion to appropriate extracellular matrix components with integrin and cadherin is essential for the cells survival. Loss of this adhesion leads to anoikis. Therefore, anoikis resistance is required for cells surviving in the peritoneal cavity through anchorage-independent growth (Simpson et al., 2008). Cancer cells develop this resistance via several mechanisms such as changes in integrin repertoire expression, induction of EM, oncogene activation and adaption of their metabolism (Buchheit et al., 2014; Douma et al., 2004; Paoli et al., 2013).

Cancer cells that are successful in adapting to the harsh environment of the peritoneal cavity attach directly to the peritoneal surface. Due to the

mesothelium, a membrane composed of simple squamous epithelium that forms the lining of the peritoneum, the cancer cells are prevented from penetrating into the sub-mesothelial space (Hu et al., 2018). The connective tissue under the subendothelial layer contributes to the formation of a microenvironment niche for the seeding of cancer nodules during PD (Psaila and Lyden, 2009; van der Wal and Jeekel, 2007). It has also been recently reported that mesothelial cells are able to create a novel tissue niche that facilitates GC invasion, resulting in PD (Tanaka et al., 2017). Interestingly, this study also demonstrated that targeting the Wnt3a pathway, through Dkk1 effectively suppressed peritoneal mesothelial cells infiltration. This is due to GC cancer cells releasing Wnt3a, which in association with extracellular vesicles act as a chemoattractant for invading peritoneal mesothelial cells (Tanaka et al., 2017). This highlights the importance of the tumour microenvironment and Wnt/β-catenin signaling in GC progression and the need to investigate this further.

1.5 Wnt Signaling Pathways

Wnt signalling is essential for embryonic development and adult tissue homeostasis. The Wnt pathways have been extensively studied and are divided into the canonical Wnt/ß-catenin pathway, non-canonical planar cell polarity (PCP) pathway, and the non-canonical Wnt/calcium pathway. The PCP pathway is responsible for regulating cytoskeletal changes (Butler and Wallingford, 2017) while the Wnt/calcium pathway controls calcium levels within the cell (Thrasivoulou et al., 2013). These pathways are indispensable for planar cell polarity and extension movements during gastrulation and epithelial cell migration, however they have distinctive roles compared to the canonical pathway. This pathway is fundamental in development and maintenance of GIT homeostasis through its regulation of numerous biological processes including: regulation of the stem cell pool, proliferation, differentiation, EMT, and apoptosis. The canonical Wnt pathway is implicated in many GCs and other cancers and diseases due to its role in regulating stem cell function and EMT. For this reason, this thesis will focus on the canonical Wnt pathway.

1.5.1 Canonical Wnt/ß-catenin Pathway

The Wnt/ß-catenin pathway is an ancient and conserved signalling cascade that involves the transcriptional co-activator, ß-catenin (Logan and Nusse, 2004). In the absence of a Wnt signal, unstimulated cells regulate ß-catenin levels via a multi-protein complex, termed the destruction complex. The destruction complex is made up of the scaffold protein Axin, APC, glycogen synthase kinase-3ß (GSK-3ß) and casein kinase 1 alpha (CK1-α). This complex phosphorylates ß-catenin, marking it for subsequent ubiquitination and degradation by the proteasome, preventing its localization to the nucleus. The absence of nuclear ß-catenin initiates a complex of TCF/LEF and Groucho to recruit histone deacetylases (HDACs), repressing Wnt target genes. In the presence of a Wnt ligand, a heterodimeric receptor complex is formed, consisting of Frizzled and its co-receptor, an LRP5/6 protein. The LRP5/6 receptors are phosphorylated by CK1-α and GSK-3ß (Janda et al., 2012) which leads to the recruitment of disheveled (DVL) and axin proteins to the plasma

membrane, where they become polymerized and activated (Li VS, 2012). DVL inactivates the destruction complex, resulting in the stabilization and accumulation of ß-catenin and its translocation to the nucleus. In the nucleus, ß-catenin forms an active complex with TCF/LEF, inhibiting Groucho repression, and recruits histone-modifying co-activators to initiate Wnt target gene transcription (Figure 1.4) (Flanagan et al., 2018).

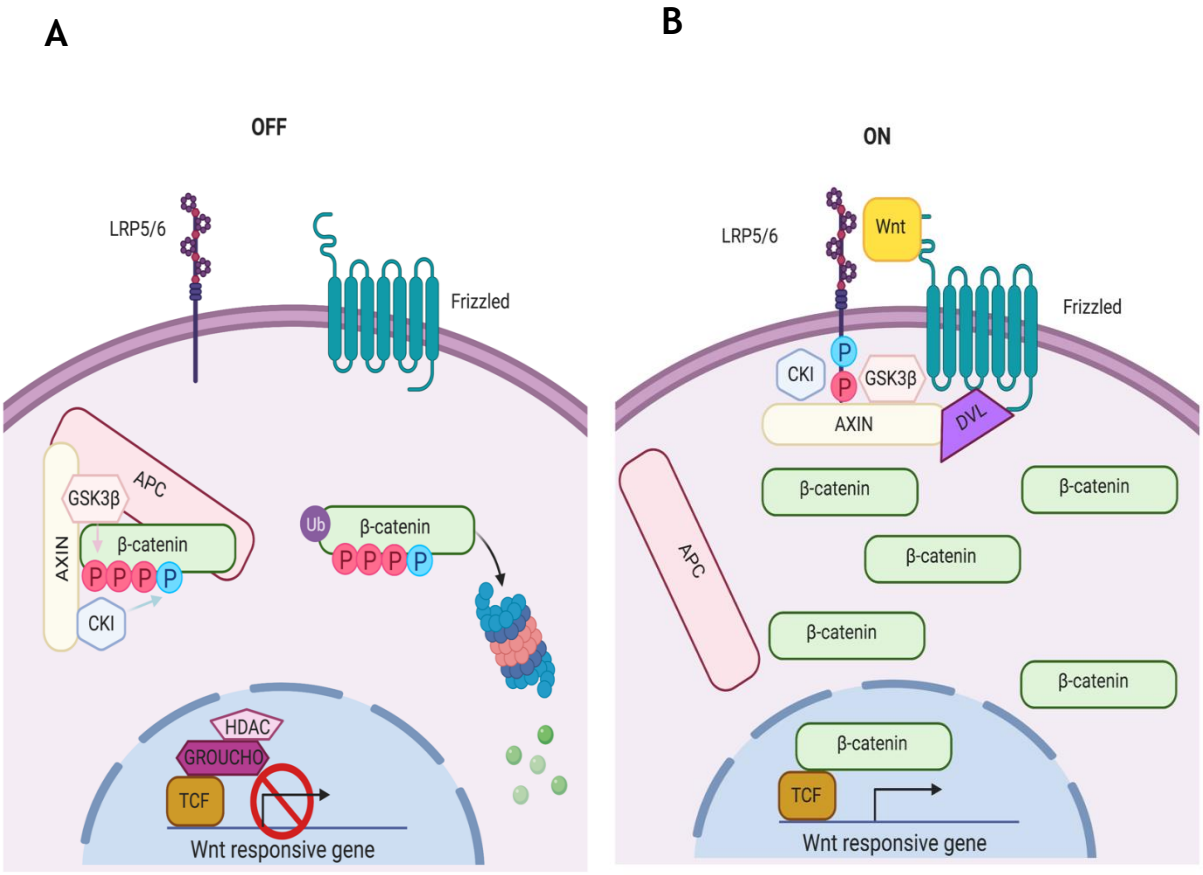


Figure 1.4. Overview of canonical Wnt/ß-catenin signalling (Adapted from (MacDonald et al., 2009). Wnt signalling in target cells. (**A**) In the absence of Wnt, a destruction complex consisting of Axin, Apc and GSK-3 ß resides in the cytoplasm, where it binds to and phosphorylates ß-catenin, which is then degraded by the proteasome. T-cell factor (TCF) is in an inactive state, preventing transcription of Wnt target genes, as a consequence of binding to the repressor GROUCHO. (**B**) Binding of Wnt to its receptors, Frizzled and Lrp5/6, induces the association of Axin with phosphorylated Lrp5/6. The destruction complex is inactivated by disheveled (DVL), and ß-catenin is stabilized, translocates to the nucleus and subsequently binds TCF to up-regulate Wnt target genes.

1.5.2 Wnt ligand synthesis, secretion and signalling

Wnt ligands are secreted glycoproteins, conserved in all metazoan animals. There are 19 Wnt ligands in the mammalian genome, which allow for complex and specific interactions with an equally broad array of receptors able to transduce the Wnt signal (Clevers and Nusse, 2012b). Wnt proteins are cysteine rich, approximately 350-400 amino acids long, and contain an N-terminal signal peptide required for proper secretion. Wnt ligand secretion is a tightly regulated and complex process, with all Wnt proteins undergoing two types of lipid modifications on the conserved residues: cysteine 77 and serine 209 (Harterink and Korswagen, 2012). The first is the addition of palmitate to cysteine 77. This renders the Wnt protein hydrophobic and tethers it to the cell membrane of cognate receptors (Willert et al., 2002). The second modification is the attachment of a palmitoleoyl to serine 209. This is required for the release of Wnt from the endoplasmic reticulum (ER). Furthermore, Porcupine (PORCN), an ER protein only active in Wnt-producing cells, is likely responsible for the serine 209 modification, as PORCN deletion causes Wnt retention in the ER (Takada et al., 2006) and a defect in Wg secretion in the Drosophila embryo (Kadowaki et al., 1996).

Additionally, the seven-transmembrane protein Wntless (Wls) provides an essential, though less understood, function in Wnt secretion (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). It is thought to principally act as a cargo receptor that ferries Wnt along the exocytic path (Herr et al., 2012). Wls localizes to the golgi network, endosomes and the plasma membrane, and binds Wnt proteins (Clevers and Nusse, 2012b). Therefore, Wls is critical for Wnt secretion and trafficking, as in Wls mutant cells, Wnt accumulates and is retained in the Golgi (Port et al., 2008).

Wnt signals were historically considered to be morphogens, molecules that exert their action across a long-distance in tissue, thus forming a gradient that determines cell fate in a concentration-dependent manner. However, there is limited evidence that this is actually the case and Wnts may signal over a

shorter distance. For example, originally, the Wg protein produced by a thin line of cells was thought to have long-range characteristics. However, it has been demonstrated that a non-diffusible, membrane-tethered form of Wg can largely compensate for the function of wild-type Wg (Alexandre et al., 2014). Therefore, Wnt signalling is now considered to occur mostly over short distances, such as between neighboring cells in a stem cell niche or neuromuscular junction (Korkut et al., 2009; Sato et al., 2011b). It is still not fully understood how Wnt ligands exert signalling activity with both secreted- and membrane-associated mechanism observed. Membrane-associated mechanisms include the transport of Wnt in exosomes (Gross et al., 2012) and cytonemes (dynamic actin-based membrane structures) (Stanganello and Scholpp, 2016). Cytoneme formation is regulated by non-canonical Wnt/PCP signalling, whereas in neighboring cells, cytoneme-associated Wnt8a activates the canonical ß-catenin signalling pathway (Mattes et al., 2018).

1.5.3 Frizzled receptors

Genetic screens in Drosophila looking for mutations that disrupt cell polarity led to the discovery of the Frizzled (FZD) genes. Since then, they have been found throughout the animal kingdom, including the most primitive metazoan, but they are not present in plants or simple single cell eukaryotes (Schenkelaars et al., 2015). FZD genes encode the principal receptors for Wnt family of signalling molecules (Bhanot et al., 1996; Yang-Snyder et al., 1996). The number of FZD genes varies between the class of organism: vertebrates have 10 known FZDs, *Drosophila* have 4 and *C.elegans* have 3.

Sequence analysis shows that vertebrate FZD genes can be divided into discrete classes based on structural homology. *FZD1*, *FZD2* and *FZD7* share approximately 97% identity, *FZD5* and *FZD8* share 70% identity, *FZD4*, *FZD9* and *FZD10* share 65% identity and *FZD3* and *FZD6* share 50% amino acid identity (Figure 1.5, Table 1.3) (Fredriksson et al., 2003; Sagara et al., 1998). The FZD genes encode proteins that share a common architecture: An N-terminal signal peptide, a conserved extracellular cysteine-rich domain (CRD), followed by a

7-pass transmembrane domain and an intracellular C-terminal PDZ domain. The structure of the extracellular domains is well reported however only the structure of the transmembrane domain of FZD4 was been recently described (Yang et al., 2018c). This revealed a unique compact organization and vacant ligand pocket which may provide a number of avenues for selective chemical probes and drug design against FZD4 and other FZDs (Zhang et al., 2018).

The conserved CRD on the FZD protein provides the binding site for Wnt ligands (Dann et al., 2001; Hsieh et al., 1999). The determined structure of the Wnt-Frizzled CRD complex displayed an unusual ligand-receptor interaction, characterized by a relatively small protein-protein interface and a relatively large interface between the CRD and a palmitoleic acid lipid group covalently attached to the Wnt (Janda et al., 2012).

On the cytoplasmic side of the plasma membrane, the C-terminal and the intracellular loops of the FZD receptors facilitate interactions with G-proteins and other regulatory signalling components. The short C-termini of the FZDs has a conserved motif which binds the PDZ domain of the scaffolding protein Dvl (Punchihewa et al., 2009; Tauriello et al., 2012). This interaction contributes to the disassembly of the Axin-based ß-catenin destruction complex (Cliffe et al., 2003). In Planar Cell Polarity (PCP) non-canonical Wnt signalling, Dvl accumulates with FZD in endosomes. These are actively transported in the posterior direction and released back to the plasma membrane at the apical posterior tip of the epithelia (Shimada et al., 2006). This is required for the amplification of the initial FZD-activating signal and establishment of the uniform planar polarization of the tissue.

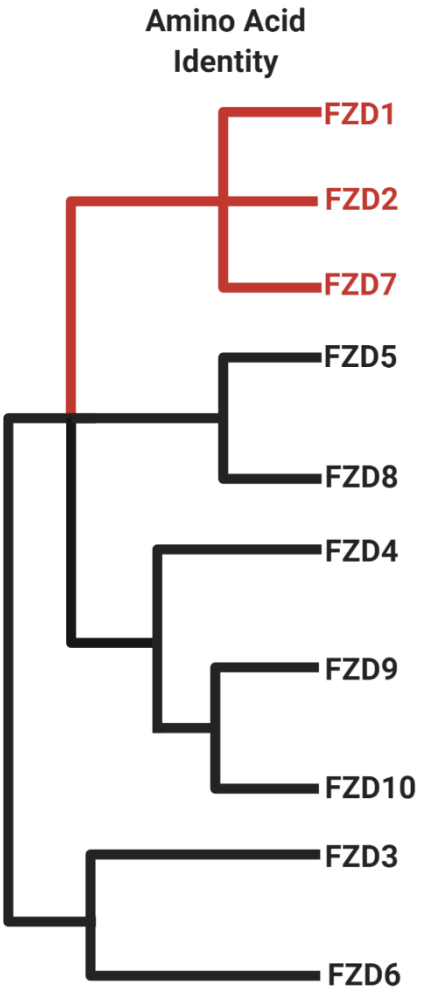


Table 1.3. Homology of Frizzled receptors to FZD7

Full length protein	Percent Identity (to fzd7)	Percent Similarity (homology) (to fzd7)	Percent Gaps
FZD1_HUMAN	79	86	5
FZD2_HUMAN	79	85	3
FZD3_HUMAN	46	62	6
FZD4_HUMAN	44	58	13
FZD5_HUMAN	49	63	12
FZD6_HUMAN	44	59	7
FZD8_HUMAN	46	59	19
FZD9_HUMAN	46	61	9
FZD10_HUMAN	47	62	7

Figure 1.5. Frizzled receptor identity. Dendrogram showing amino acid sequence identity among Frizzled proteins. FZD1, FZD2 and FZD7 constitute a distinct branch within the FZD family.

FZDs can function in all three distinct Wnt signaling pathways: the PCP pathway, the canonical Wnt/β-catenin pathway and the Wnt Ca^{2+} pathway. The Wnt-Fzd interaction can take many forms: a single Wnt can bind multiple FZD proteins and vice versa to activate canonical and/or non-canonical Wnt signalling. The structure of the Wnt-CRD complex allowing many binding combinations (Janda et al., 2012). This allows Wnt signalling to regulate a vast array of cellular functions through functional selectivity in different downstream pathways. In canonical signaling, FZD responds to Wnt proteins in the presence of co-receptor LRP5/6 to activate and propagate the Wnt/β-catenin pathway. Alternatively, FZD receptors can respond to Wnt proteins in the presence of the Wnt co-receptor Ror2 to activate the non-canonical pathway (Mikels and Nusse, 2006). The decision for a cell to activate canonical or non-canonical Wnt signalling largely depends on the receptor/co-receptor/ligand combination and availability (Figure 1.6).

The majority of the FZD receptor genes display developmental phenotypes when deleted from specific tissues. The close sequence homology between FZD members can make it difficult to interpret phenotypes, as in many cases, partial redundancy is observed. Partially redundant FZD pairs include FZD1 and FZD2, FZD3 and FZD6, FZD4 and FZD8, and FZD5 and FZD8 (Yu et al., 2012a). For example, defects in CNS axon guidance and hair follicle orientations, linked to PCP disruption, are observed in mice carrying single mutations in either FZD3 or FZD6, with additional phenotypes observed in double homozygous $Fzd3^{-/-}$; $Fzd6^{-/-}$ mutant mice (Dong et al., 2018; Stuebner et al., 2010; Wang et al., 2006), suggesting that Fzd3 and FZD6 play a redundant role in controlling polarity, but through non-identical mechanisms. Likewise, it has been shown that FZD2 and FZD7, that share ~95% structural homology, function in a redundant fashion during convergent extension and closure of the ventricular septum and palate (Yu et al., 2012a). However, it is not only structurally similar FZDs that can compensate for one another. It was demonstrated that in human mesenchymal stem cells (hMSC) that many FZD receptors were capable of

activating canonical Wnt signalling to maintain the proliferative and undifferentiated status of hMSCs in culture. However, only FZD7 and FZD5 were able to compensate for one another to sustain active signalling, thus demonstrating that two non-sequence related FZDs can act in a redundant fashion (Kolben et al., 2012)

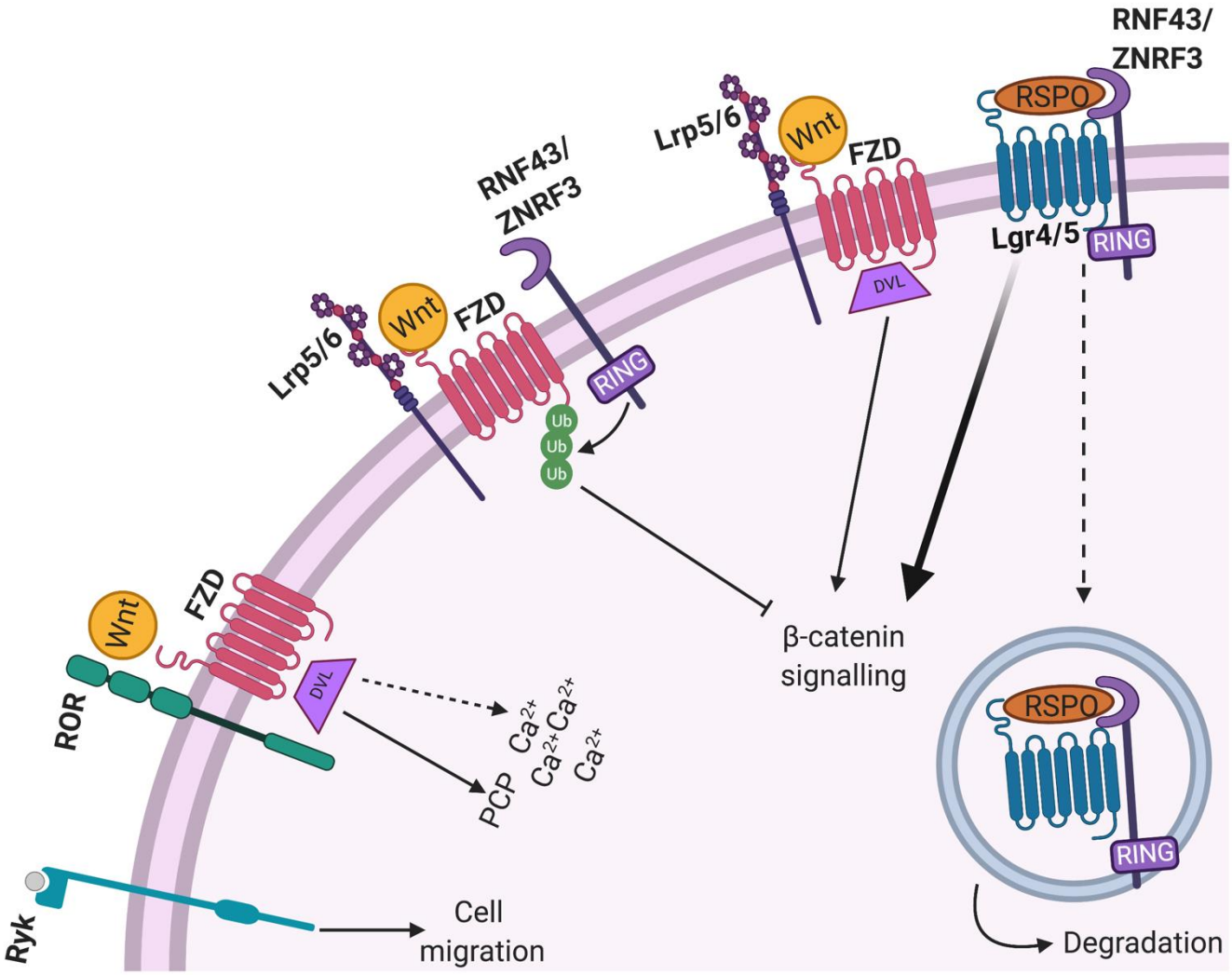


Figure 1.6. Schematic arrangement of Wnt membrane receptors (Adapted from (Phesse et al., 2016). Frizzled proteins are considered the main Wnt receptors. The outcome of the Wnt-FZD interaction is determined by co-receptors. Wnt-FZD-Lrp5/6 activates the canonical ß-catenin signalling pathway, which itself can be modulated by other cell surface proteins. For example, RNF43/ZNRF3 ubiquitylates FZD receptors and targets them for internalization and proteasomal degradation, thus resulting in turn-over of the receptor complex and inhibited ß-catenin signalling. In the presence of Lgr4/5 and RSPO it is RNF43/ZNFR3 which is internalized and degraded and FZD-LRP5/6 remains on the cell surface to enhance ß-catenin signalling. FZD receptors can associate with Ror2 to transmit non-canonical Wnt signals from ligands including Wnt5 or Wnt11, either via PCP or Ca2+. Some receptors such as Ryk are thought to function independently of Frizzled

1.5.4 LRP5/6

LRP5/6 forms one half of the canonical Wnt/ß-catenin receptor complex along with FZD. Shortly after the discovery of canonical Wnt1 gene, LRP5/6 was identified to be key for Wnt1-dependnet tumour development in Wnt1 transgenic mouse models (Badders et al., 2009; Goel et al., 2012; Lindvall et al., 2006) and LRP6 if often overexpressed in human breast tumours (Liu et al., 2010). The ectodomain of LRP5/6 is composed of four ß-propeller/epidermal growth factor repeats (E1-4) and three LDL repeats (LDLR). E1-4 but not LDLR is the binding domain of canonical Wnt ligands, as well as the canonical pathway inhibitor Dkk1 (Angers and Moon, 2009; Bhanot et al., 1996; Chen et al., 2011; Mao et al., 2001). Currently, the LDLR-binding proteins remain unexplored. The current view is that the close proximity of LRP5/6 and FZD coupled by canonical Wnt ligand binding to E1-4 OF lrp5/6 and the CRD of FZD is required for canonical pathway activation (Angers and Moon, 2009; MacDonald et al., 2009). In contrast, Dkk1 promotes the internalization of LRP5/6 via binding with its receptor, Kremen, making LRP5/6 unavailable for Wnt binding, thus, inhibiting the canonical pathway (Mao et al., 2002).

Intracellularly, LRP5/6 participates in key molecular interactions with the scaffolding protein Axin and the kinases CK1-α and GSK-3ß via the evolutionarily conserved cytoplasmic PPPSPxS binding motif (P = Proline, S = Serine/Threonine, x = variable residue). Mutational analysis of LRP6 revealed that when cytoplasmic PPPSPxS binding motif is altered, LRP6 acts as a dominant negative mutant and conversely, LRP6 mutants that lack the extracellular domain behave as constitutively activated Wnt receptors (Tamai et al., 2004; Zeng et al., 2005).

The current model indicates that in response to Wnt, Dvl binding and recruitment of Axin to the FZD-LRP5/6 complex triggers the phosphorylation of LRP5/6 on the PPPSPxS motif via a dual-kinase mechanism, first by GSK-3ß then by CK1-α (Davidson et al., 2005). This duel-phosphorylation promotes the engagement of LRP5/6 with Axin. It is surprising that GSK-3ß mediates LRP5/6

phosphorylation and activation, due to its known inhibitory role in Wnt signalling via promoting ß-catenin phosphorylation and degradation (Liu et al., 2002). However, it has been shown that the membrane-associated form of GSK-3ß, in contrast to cytosolic GSK-3ß, stimulates Wnt signalling as well as Xenopus axis duplication (Zeng et al., 2005). Therefore, GSK-3ß can act as a switch dictating both the on and off states of the Wnt signalling pathway.

1.5.5 Ror and Ryk

Two other classes of receptor that forms a part of the Wnt-pathway components are Ror and Ryk. These are both tyrosine kinase evolutionarily conserved transmembrane receptors capable of participating in canonical and non-canonical Wnt signalling. However, the exact mechanisms by which they transduce Wnt signals are still be refined and understood.

Receptor-tyrosine kinase-like orphan receptor 1/2 (Ror1 & Ror2) are able to bind directly to Wnt ligands and therefore function as Wnt receptors (Mikels et al., 2009).This is thanks to their distinguishing feature in the extracellular domain of the presence of a CRD domain that shares close homology with the Wnt-binding domain of the FZD receptors (Mikels and Nusse, 2006; Oishi et al., 2003; Saldanha et al., 1998). The primary ligand for Ror2 is Wnt5a (Green et al., 2008). Wnt5a induces the formation of a complex between Ror2 and FZD, resulting in the phosphorylation of Ror2 and the recruitment of Dvl, Axin and GSK-3ß, the same machinery that mediates Wnt3A-induced phosphorylation of Lrp5/6 (Grumolato et al., 2010; Yamamoto et al., 2007). This results in Wnt3a and Wnt5a competing for binding to the FZD receptor; the identity of the Wnt ligand will determine which co-receptor will be activated. In contract to functioning as a co-receptor with FZD, the Wnt5a/Ror2 complex can function to inhibit the Wnt/ß-catenin pathway (Mikels et al., 2009), thus Ror has both kinase-independent and kinase-dependent functions (van Amerongen et al., 2012)

Ror2 has also been implemented in understanding how Wnt proteins travel between cells. Cytonemes, finger-like structures, grow out of the cell

membrane and carry Wnt proteins to their destination. Ror2 is activated by Wnt8a which triggers the assembly of the cytonemes and induces Lrp6-FZD clustering into the LRP6 signalosome to activate ß-catenin signalling. The amount of Ror2 activation is directly proportion to the amount of cytonemes produced by the cell and therefore the levels of Wnt signals and ß-catenin signaling. This mechanism has been observed in zebrafish embryos, the mouse intestine, and most excitingly, human stomach tumours (Mattes et al., 2018)

Ryk receptors have a critical role in axon guidance and neurite outgrowth in response to multiple Wnt ligands and therefore have mostly been studied in neurobiological contexts (Keeble et al., 2006). Ryk receptors are considered catalytically inactive and have been shown to bind to FZD suggesting a role as a co-receptor in Wnt signalling, however this function is cell-context dependent (Berndt et al., 2011; Kim et al., 2019). Therefore, it is likely that many independent mechanisms exist downstream from ligand-receptor binding for both FZD and non-FZD Wnt receptors.

1.5.6 The ß-catenin destruction complex

In the absence of Wnt/FZD interaction, the scaffolding proteins Apc and Axin sequester ß-catenin, which allows CK1-α to phosphorylate the N terminus of ß-catenin at Ser 45, a residue often mutated in cancers. Subsequently, GSK-3ß is recruited to phosphorylate Serine 33, 37 and threonine 41 residues (Lybrand et al., 2019). Phosphorylated ß-catenin is then recognized by the F-box-containing protein ß-TrCP, which mediates ubiquitylation and proteasomal degradation (Shi et al., 2015). Together, these proteins make up the ß-catenin destruction complex (as depicted in Figure 1.4). When Wnt ligands are present, they bind and activate heteromeric receptor complexes of FZD and LRP5/6 which initiate a signal via disheveled that inhibits the destruction complex.

Although this model of Wnt signalling is generally accepted, key aspects of how the destruction complex is regulated remain controversial. There are dozens of models that hypothesize how ß-catenin is regulated, including destruction complex inactivation by kinase inhibition, complex dissociation, separation of

the destruction complex from the ubiquitylation machinery and aggregation of the complex at the site of activated receptors (Lybrand et al., 2019). This uncertainty derives from the fact that most components of the destruction complex are multifunctional, serving other roles in addition to Wnt signaling. This results in only a small fraction of the total cytoplasmic pool of each protein actually participating in Wnt signalling (Papadopoulou et al., 2004). This has resulted in most investigations of the destruction complex using protein overexpression assays in highly artificial *in vitro* systems, thus, rendering their physiological significance uncertain.

A study by Li *et al.,* examined all the destruction complex components and interactions at endogenous levels Firstly, Li *et al.*, proposed that the composition of the Axin complex does not change in colorectal cancer cell lines, in which the Wnt pathway is locked to the "on" state, demonstrated by the presence of GSK-3β and β-catenin (Li VS, 2012). However, other studies have demonstrated that Axin1 complex can be degraded or sequestered to the plasma membrane by LRP5/6 following Wnt activation (Huang et al., 2009; Zeng et al., 2005). Secondly, Li *et al.*, demonstrated that the activities of GSK-3β and CK1-α are not inhibited upon Wnt signalling, and are free to phosphorylate β-catenin. Whereas other studies have suggested that GSK-3β activity is inhibited directly by LRP5/6 PPPSPxS motif or via its sequestration into multi-vesicular bodies (Taelman et al., 2010; Wu et al., 2009). This difference is settled by proposing that the phosphorylated form of β-catenin accumulates within an intact destruction complex following Wnt stimulation (Li VS, 2012).

The role for APC in the inhibition of the destruction complex following Wnt ligand presentation have recently been proposed. The results from Parker and Neufeld expand on the current model of Wnt signalling such that in response to Wnt, the destruction complex (Logan and Nusse, 2004) maintains composition and binding to β-catenin (Clevers, 2006), moves toward the plasma membrane (MacDonald et al., 2009) and requires full-length APC for this re-localization (Parker and Neufeld, 2020). They demonstrated that APC deletion

or truncation in non-transformed human colon epithelial cells completely abolished Wnt-induced destruction complex localization (Parker and Neufeld, 2020). This highlights APCs duel function as a scaffold protein and as a key player to enable full trafficking of the destruction complex towards a Wnt cue. It has also been recently shown that the central region of APC has a role in preventing clathirin-mediated endocytosis in the Wnt-off state (Saito-Diaz et al., 2018). Therefore, APC may also act as a molecular “gatekeeper” to block receptor activation via the clathrin pathway (Figure 1.7).

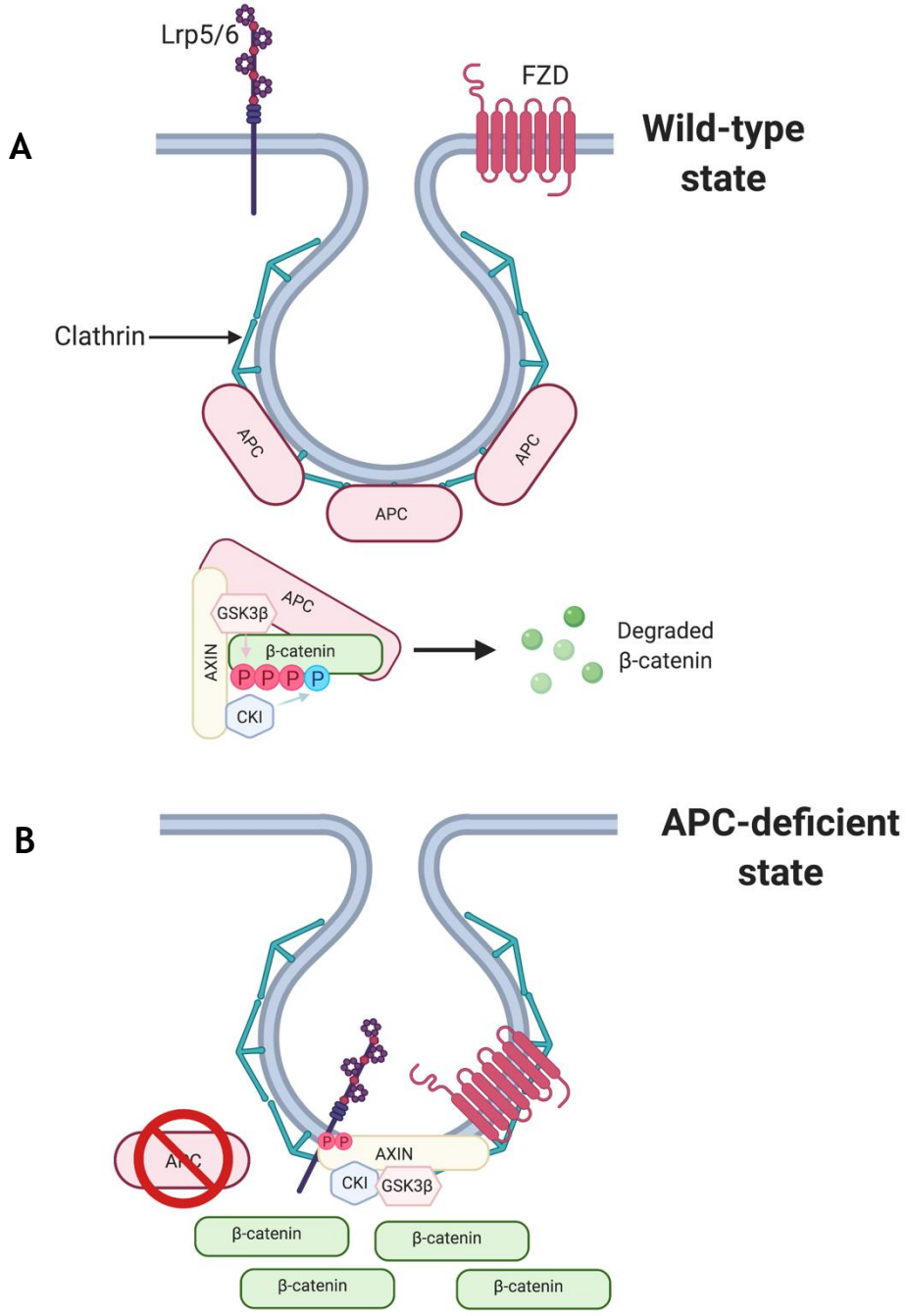


Figure 1.7 APC may act as a negative regulator of Wnt receptor activation via the clathrin endocytic pathway. (Adapted from (Saito-Diaz et al., 2018). In the absence of Wnt ligand, APC maintains low cytoplastimc ß-catenin as part of the destruction complex. A novel role for APC is its role in inhibition of Wnt receptor activation in the unstimulated state. (**A**) In the absence of a Wnt ligand (Basal state), APC localizes to clathrin-coated pits to block spontaneous Wnt receptor activation. (**B**) Upon loss of APC (APC-depleted state), ligand-independent LRP6 oligomerization is no longer inhibited, the Wnt signalosome forms, and the pathway is activated.

1.5.7 Nuclear Events

Nuclear ß-catenin is a hallmark of active Wnt signalling (Nusse and Clevers, 2017) but how ß-catenin is transported into and out of the nucleus is not well understood. Early studies suggested that ß-catenin enters the nucleus by interacting with nuclear pore proteins and is independent of the nuclear-localization signal (NLS) (Henderson and Fagotto, 2002). It has been reported that ß-catenin can be actively exported from the nucleus as cargo of Axin (Cong et al., 2004) or that APC can shuttle between the cytoplasm and nucleus (Rosin-Arbesfeld et al., 2000). A *Drosophila* genetic screen identified two nuclear binding partners, Bcl9 and Pygopus which are closely involved in the nuclear import and retention of ß-catenin (Townsley et al., 2004). They may also aide in the transcriptional ability of ß-catenin (Hoffmann, 2005).

The TCF/LEF family of transcription factors is the main partner for ß-catenin in gene regulation (Hoppler and Kavanagh, 2007). TCF represses gene expression by interacting with the repressor Groucho, which promotes histone deacetylation and chromatin compaction. Wnt-induced ß-catenin stabilization and nuclear accumulation leads to a complex of TCF with ß-catenin, which displaces Groucho (Daniels and Weis, 2005) and recruit other co-activators for gene activation (Figure 1.8). The vertebrate genome harbors four TCF/LEF genes: *Tcf-1*, *Lef-1*, *Tcf-3* and *Tcf-4*.

Wnt signalling regulates gene transcription in a highly context-dependent manner. Transcribed target genes contribute to a variety of cellular functions from stem cell function to proliferation. Additionally, Wnt signalling can promote the expression of several Wnt pathway components, highlighting feedback control as a key feature of Wnt signalling regulation. These “Wnt pathway” genes are often common across all cell types, whereas many other Wnt target genes are cell-type specific and are less often found to be induced across different kinds of cells. Therefore, expression of Wnt pathway genes, such as Axin2 (Jho et al., 2002) and Lgr5 (van de Wetering et al., 2002) are good hallmarks of active Wnt signalling.

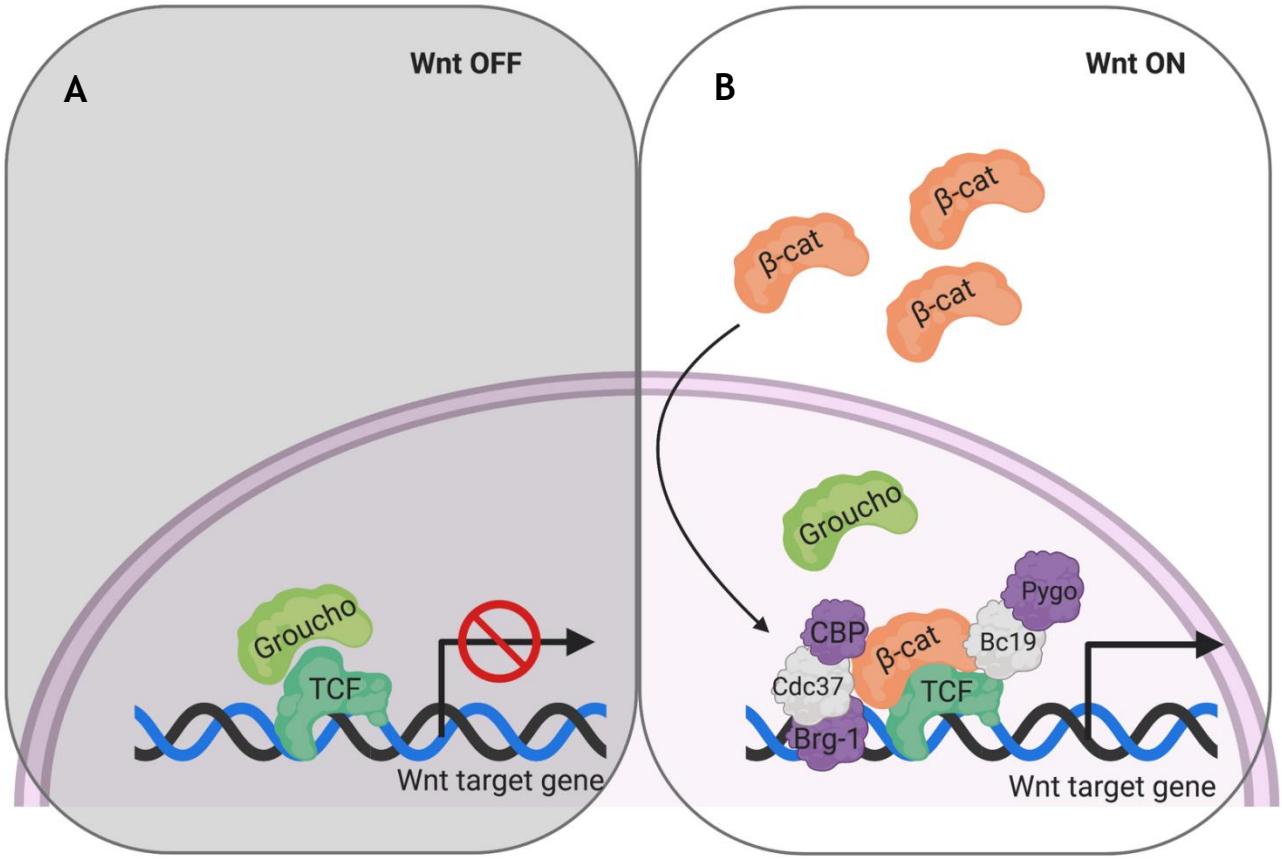


Figure 1.8 Wnt signalling in the nucleus (Adapted from (Clevers and Nusse, 2012a). (**A**) In the absence of Wnt signals, TCF occupies and represses its target genes, helped by transcriptional co-repressors such as Groucho. (**B**) Upon Wnt signalling, ß-catenin displaces Groucho from TCF and recruits' transcriptional co-activators and histone modifiers such as Brg1, CBP, Cdc47, Bcl9 and Plygopus (Pygo) to drive target gene expression.

1.5.8 Wnt Antagonists & Agonists

Wnt signalling is regulated at every stage of the pathway from the extracellular receptors and ligands to the intracellular components by a wide range of effectors. These effectors function as agonists or antagonists and are important in the control of the fine-tuning of Wnt signalling and in inhibiting or activating Wnt-regulated developmental processes. Critically, their role in Wnt pathway regulation continues through adulthood and is implicated in pathological events, including cancer (Cruciat and Niehrs, 2013).

Secreted frizzled-related proteins (sFRPs) are a family of five (sFRP1-5) secreted glycoproteins that have been identified as extracellular modulators of the Wnt signalling pathway (Jones and Jomary, 2002). They are the largest family of secreted Wnt inhibitors. All sFRP family members contain an N-terminal domain that is 30-50% homologous to the CRD of the FZD receptors, this is both necessary and sufficient for Wnt binding and inhibition (Lin et al., 1997; Rehn et al., 1998). In contrast to FZD family proteins, sFRPs lack a transmembrane region and the cytoplasmic domain required for signal transduction into the cells. Instead they have a C-terminal Netrin-related motif domain that is required for their ability to induce optimal Wnt inhibition (Bhat et al., 2007). sFRPs modulate the Wnt signalling pathway by directly binding with Wnt ligands to block the interaction between Wnt and FZD receptors. Additionally, sFRPs can bind with FZD receptors to form non-functional complexes that prevent Wnt signalling activation (Bafico et al., 1999).

A recent study found that abnormal nuclear localization of sFRPs bound to ß-catenin was able to modulate TCF4 recruitment. This exerted either promoting or suppressive effects on the Wnt/ß-catenin-elicited cancer stem cell (CSC) phenotype (Liang et al., 2019). This suggests an alternative mechanism in which sFRPs act as biphasic modulators of Wnt-signalling-elicited CSC properties beyond extracellular control. However, the exact mechanism in which sFRPs translocate into the nucleus remains unclear.

The Dickkopf (Dkk) genes represent a small family of evolutionarily conserved glycoproteins that potently antagonize Wnt/ß-catenin signalling. There are four Dkk genes (Dkk1-4) in the vertebrate genome. Dkk proteins selectively antagonize canonical Wnt signalling by binding to LRP5/6, thus, preventing FZD and Wnt from forming a ternary complex (Semënov et al., 2001). In addition to LRP5/6 Dkk1 can form a ternary complex with the cell surface receptor Kremen which promotes LRP6 internalization and degradation (Mao et al., 2002). A novel role for Dkk1 in the absence of Kremen proteins has been identified in stabilizing Lrp6 while inhibiting Wnt/Lrp6 signalling. Importantly, it was also demonstrated that Dkk1 blocks Wnt3A-induced LRP6 down-regulation (Li et al., 2010).

1.5.9 R-Spondin & E3 ligases (Rnf43 & Znrf3)

R-Spondin (R-Spo) proteins are potent agonists of canonical Wnt signalling, but only in the presence of Wnt ligands (Kazanskaya et al., 2004; Kim et al., 2008a). R-Spos are cysteine-rich secreted glycoproteins and through their role in activating Wnt signalling control a variety of cellular and tissue functions (Yoon and Lee, 2012). In mammals, there are four R-Spos which display high structural similarity and 60% sequence homology (Nam et al., 2006). All four contain four distinct domains: a putative signal peptide domain, a cysteine-rich furin-like (FU) domain, a thrombospondin type I repeat domain and a basic amino acid-rich domain (Kazanskaya et al., 2004). It is the FU domains that are essential to amplify the Wnt ligand-dependent activation of canonical Wnt signalling (Kim et al., 2008a; Li et al., 2009). Crystal structure analysis showed that one of the FU domains binds to Lgr receptors (Peng et al., 2013a; Wang et al., 2013b). The other FU domains to the cell-surface transmembrane E3 ubiquitin ligase Znrf3/Rnf43 (Chen et al., 2013; Zebisch et al., 2013), which antagonize Wnt signaling by ubiquitinating FZD receptors followed by endocytosis of the Wnt receptor complex (Hao et al., 2012; Koo et al., 2012). Here, the R-Spo-Lgr complex binds to ZNRF3/RNF43 to block the ubiquitination of FZD receptors which leads to the augmentation of the Wnt

signalling cascade (Figure 1.6). It has also recently been reported that R-Spo2 and R-Spo3 can also amplify canonical Wnt signalling independently of Lrg receptors, via membrane-bound heparin sulfate proteoglycans (HSPG) (Lebensohn and Rohatgi, 2018).

The negative regulators of Wnt signalling, ZNRF3 and RNF43 were identified from gene expression profiling to identify genes that positively correlated with known negative regulators of Wnt signalling, such as Axin2 or Dkk-1 (Hao et al., 2012). As mentioned, ZNRF3 and RNF43 regulate Wnt signalling via their ability to promote the degradation of the FZD-Lrp6 complex, however, it has been shown that R-Spo induces membrane clearance of ZNRF3 though ZNRF3-Lgr4 dimerization. This leads to the accumulation of Wnt receptors on the cell surface (Hao et al., 2012; Peng et al., 2013b). These findings support a model in which in the absence of R-Spo, the E3 ligases ubiquitylates FZD and promotes the degradation of FZD-LRP6 complex, thus keeping Wnt signalling to low levels. However, in the presence of R-Spo, lgr4 and ZNRF3 interact, via the FU domain on R-Spo. This leads to the clearance of the E3 ligases, thus, allowing the FZD-LRP6 complex to accumulate at the membrane to enhance canonical Wnt signalling.

1.5.10 Wnt signalling in the homeostasis of the Gastric Epithelium

The role of Wnt signalling in maintaining gastric homeostasis is less well defined than its close counterpart, the intestinal epithelium. However, the requirement of Wnt signalling in gastric homeostasis is widely accepted. Gastric organoid cultures require Wnt3a in the culture medium in addition to the Wnt agonist R-Spondin (Barker et al., 2010b; Flanagan et al., 2016) thus demonstrating that Wnt is required for the activity of gastric epithelial cells. It has been demonstrated that Wnt signalling is more active in the antrum than the corpus (Flanagan et al., 2017a). . Furthermore, over-expression of Wnt can lead to intestinal differentiation of the stomach (Khurana and Mills, 2010). Further supporting evidence for the role of Wnt in gastric homeostasis was provided by the genetic profiling of laser-capture micro-dissected granule-free

isthmal cells from both the antral and corpal epithelium (Giannakis et al., 2006). The expression profile of isolated isthmal cells revealed significant up-regulation of Wnt target genes in comparison to isolated parietal, chief, and mucous cells. This is consistent with the upregulation of Wnt target genes observed in other stem cell populations such as hematopoietic and embryonic stem cells (Assou et al., 2007; Fernandez et al., 2014; Vijayaragavan et al., 2009; Willert et al., 2002). Furthermore, deregulated Wnt signalling in the adult stomach, achieved by activating Wnt mutations in the gastric epithelium, results in a marked change in stomach homeostasis (Radulescu et al., 2013).

The discovery of Lgr5+ gastric stem cells gives weight to the role of Wnt signalling in gastric homeostasis. Lgr5+ gastric stem cells are responsive to Wnt ligands and (along with their immediate progeny) share a significant overlap of Wnt target genes with intestinal Lgr5+ stem cells, indicative of robust Wnt activity at the base of antral gastric glands (Barker et al., 2010b). It has been demonstrated that FZD7 is the predominant Wnt receptor in regulating homeostasis in the intestinal epithelium, in which deletion of FZD7 in either the whole epithelium or specifically in the *Lgr5*+ intestinal stem cells, triggers rapid repopulation (Flanagan et al., 2015a). More recent studies following on from this have demonstrated that FZD7 is also expressed in the antrum of the gastric epithelium, and is required for the growth of gastric organoid cultures. Deletion of FZD7 in the gastric epithelium *in vivo* was shown to be deleterious and triggered rapid repopulation of the epithelium (Flanagan et al., 2017a). This data identifies that FZD7 is crucial for transmitting Wnt signalling to regulate homeostasis in the gastric epithelium. However, unlike the intestinal stem cells, the function of Lgr5+ gastric stem cells do not require FZD7 *in vivo*. Deletion of FZD7 does not inhibit the capacity of Lgr5+ cells to lineage trace full gastric units in the antral epithelium (Flanagan et al., 2019b). This highlights key differences in the way Wnt regulates homeostasis and Lgr5+ stem cells in the stomach compared to the intestinal epithelium. Together, this

demonstrates that Fzd7 is required for at least one population of stem cells in the gastric antrum, but this population has yet to be identified.

The Wnt pathway is more active in the antrum than in the corpus, however, further data has demonstrated that the Wnt target gene, *Troy* marks mature chief cells in the corpal stomach, consistent with a role for Wnt signalling in gastric epithelium. Isolated Troy+ cells up-regulate a significant proportion of Wnt-ß-catenin target genes expressed by Lgr5+ gastric stem cells and contribute to the homeostasis of the corpal epithelium both under basal and injury conditions (Stange et al., 2013). The plasticity displayed by Troy cells may be regulated by Wnt signalling due to their expression of many Wnt target genes. However, the precise source of Wnt ligands that active this pathway remains to be identified.

Detailed analysis of Wnt signalling in the epithelium of the antrum using *in situ* hybridization showed that several Wnt ligands were expressed (Wnt2b, 3a, 4, 5a, 9a, 9b and 11), as well as all 10 FZD receptors (Sigal et al., 2017). The expression patterns of some FZD receptors was focused in specific areas of the gastric units, for example FZD10 was expressed predominantly in the pit region, FZD6 predominantly in the base, FZD5 expression was markedly decreased in the gland base, whilst FZD6 and FZD7 expression was mainly confined to the bottom two thirds of the gastric unit (Stange et al., 2013). These specific expression patterns suggest a distinct role for Wnt signalling in each area of the gastric unit, which has yet to be fully elucidated. Further studies are required to understand the various niche factors required by the various stem cell populations of the gastric epithelium. This would provide a greater and more complete understanding of the role of gastric stem cells in homeostasis, regeneration and disease.

1.6 Aberrant Wnt Signalling & Gastric Cancer

As mentioned one of the best characterized signalling pathways implicated in GC is the Wnt pathway. This pathway is involved in the

tumorigenesis of many cancers, but especially so in cancers of the gastrointestinal tract (Pai et al., 2016). In brief, the hallmarks of this pathway include increased ß-catenin (encoded by *CTNNB1*) signalling, which is often facilitated by inactivating mutations in APC. In the TCGA study, APC was mutated in 17% of GC tumours and 8% with mutations to *CTNNB1*(TCGA, 2014). Another regulator of Wnt signalling is the E3 ubiquitin ligase RNF43, which was found to be mutated in 18% of GC tumors in the TCGA study, as well as in other data sets (Wang et al., 2014b). This further supports the likely importance of the Wnt signaling pathway in GC, which can become dysregulated at any level of the pathway (Table 1.5).

1.6.1 H.pylori-mediated Wnt signalling

As previously mentioned, a major risk factor for GC is chronic infection with *H.pylori*. A virulence factor produced by *H.pylori* following colonization of the stomach lumen, is cytotoxin-associated gene product (CagA). This has been shown to activate Wnt signalling and promote gastric tumorigenesis and progression by its translocation to the cytoplasm of epithelial cells (Silva-García et al., 2019). Once inside the host cell, CagA interacts and phosphorylates the oncoprotein c-Met receptors that activates NF-κB and the expression of numerous pro-inflammatory cytokines and chemokines, enzymes and angiogenic factors (Tohidpour, 2016). This functional ternary complex, CagA-c-Met-CD44 also induces nuclear ß-catenin accumulation by activating the PI3K/Akt signalling pathway (Suzuki et al., 2009; Wroblewski et al., 2015). Additionally, CagA can directly interact with the gastric tumour suppressor transcription factor Runx3 (runt related transcription factor 3), labelling it for ubiquitination and proteasome degradation (Tsang et al., 2010). Runx3 normally interacts with Tcf4 which represses ß-catenin-dependent gene expression. The interaction of CagA with Runx3 exposes the TCF4 binding site for ß-catenin, which activates the upregulation of ß-catenin target genes and can induce GC carcinogenesis (Ito et al., 2011). Another tumour suppressor that is negatively affected by *H.pylori* is Tff1 . In a normal setting Tff1 increases the phosphorylating activity of GSK3ß on ß-catenin, which results in a reduction in

ß-catenin nuclear translocation and Tcf4 transcriptional activity (Soutto et al., 2015). In *H.pylori* infected epithelial cells, *H.pylori* promotes hypermethylation of the Tff1 gene which leads to an increase in ß-catenin-dependent gene expression (Tomita et al., 2011). Additionally, promoter methylation and mRNA downregulation of Wnt/ß-catenin antagonists (sFRP, DKK1 and WIF1) have also been demonstrated during gastric carcinogenesis and affected by *H.pylori* infection (Yang et al., 2018a). As a result of this genetic modification, ß-catenin nuclear translocation was increased in gastric epithelial cells, highlighting that epigenetic modification is an important factor for GC.

In addition to epigenetic modifications, *H.pylori* infection promotes cancer stem cell characteristics in GC cells by activating Wnt/ß-catenin signalling in a process dependent on CagA (Yong et al., 2016). It was also demonstrated that Nanog and Oct4, two transcription factors associated with EMT, had increased expression in GC samples from patients infected with CagA-positive *H.pylori* (Chiou et al., 2010). It has also been demonstrated that *H.pylori* is able to directly inject CagA into Lgr5+ gastric stem cells. This significantly increases the number of Lgr5+ gastric cells, which in turn leads to an increase in Wnt signalling activity (Sigal et al., 2015). A population of Wnt responsive Axin2+ gastric stem cells has also been shown to increase following *H.pylori* infection. This is linked to an increase in R-spondin2 signalling from sub-epithelial myofibroblasts (Sigal et al., 2017). This increase in expression of R-Spo and Lgr5 is positively correlated with poor patient survival and outcomes (Wilhelm et al., 2017; Xi, 2019). Gastric epithelial cells infected with *H.pylori* also induce the expression and activation of other Wnt receptor components such as FZD (Geng et al., 2016) and LRP (Gnad et al., 2010), propagating Wnt signalling and cell transformation. Specifically it was found that *H.pylori* colonization leads to the activation of the Wnt/ß-catenin pathway through upregulation of FZD7. Knockdown of *FZD7* in *H.pylori* infected GC cells reduced cell proliferation and colony forming. Furthermore, it was found that miR-27b harboured a putative binding site for FZD7 and that miR-27b was able to suppress *H.pylori* infection

and the Wnt pathway through inhibition of FZD7 (Geng et al., 2016) This suggests that designing therapeutics to target Wnt signalling at the level of the receptor complex in *H.pylori* infected GC cells could be beneficial.

Despite the reported ability of *H.pylori* to induce and activate the Wnt pathway in GC, there is also distinct data demonstrating the involvement of multiple Wnt pathway components independent of *H.pylori* in the initiation and progression of both intestinal-type and diffuse-type GC.

1.6.2 Intracellular Component Mutations

The traditional view is that mutations to various intracellular components of the Wnt/ß-catenin signalling pathway lead to constitutive activation and the development of many cancers, including GC. In fact more than 70% of patients diagnosed with GC had deregulated Wnt/ß-catenin signaling (Ooi et al., 2009). The most common mutations occur as stabilized forms of ß-catenin (*CTNNB1*) or truncating mutations to APC (Morin et al., 1997).

First, work investigating Wnt signalling involvement in the pathogenesis of GC identified ß-catenin as a suitable target for therapeutic intervention. Nuclear ß-catenin, a surrogate marker of active Wnt signalling, is detected in approximately one-third of GC tumours. In normal gastric epithelium, ß-catenin is confined to the cell membrane. However, during aberrant activation of the pathway, ß-catenin is located predominantly in the nucleus. In gastric tumours displaying nuclear ß-catenin, one-third of them have mutations in exon-3 of the ß-catenin gene (*CTNNB1*) (Clements et al., 2002; Woo et al., 2001). The *CTNNB1* mutations are frequently missense mutations (Machin et al., 2002). Early studies reported that mutations in ß-catenin are confined to intestinal-type gastric tumours, suggesting that the tumorigenic mechanisms are different from diffuse-type gastric tumours (Ebert et al., 2002; Park et al., 1999). However, studies since have shown no significant difference in the number of tumours with ß-catenin mutation between diffuse and intestinal-type GC (Clements et al., 2002). The difference between these findings could be due to the

examination of a larger sample size of gastric tumours in research by Clements and colleagues. The functional significance of exon-3 of the ß-catenin gene is that it encodes serine-threonine phosphorylation sites for GSK3-ß which regulates degradation of ß-catenin by the ubiquitin-proteasome pathway.

Mutations in exon-3 of ß-catenin and alteration of these phosphorylation sites confer resistance to phosphorylation and lead to the accumulation of nuclear ß-catenin and subsequent changes in expression of genes that regulate proliferation, such as Cyclin D1, D2 and E (Akama et al., 1995; Arici et al., 2009) and Wnt target genes (Gao et al., 2017a). More recent work has shown that reduced ß-catenin protein expression by targeted silencing of ß-catenin in GC cells leads to inhibition of ß-catenin nuclear localization and Wnt transcriptional activity (Jiang et al., 2010). Additionally, it has been observed that the inactive form of GSK3-ß is up-regulated and down-regulated in gastric tumors and the surrounding non-neoplastic tissue, respectively (Zheng et al., 2010). Furthermore, high levels of ß-catenin and cytoplasmic GSK3-ß correlate with an invasive phenotype in gastric tumours (Miyazawa et al., 2000; Zheng et al., 2010).

It has been shown *in vivo* that constitutive Wnt activation, through conditional truncation of APC, deletion of GSK3-ß, or overexpression of ß-catenin in the gastric epithelium is sufficient to initiate gastric adenoma formation in both the antrum and corpus (Radulescu et al., 2013). Although loss of function of Apc or GSK3-ß is unable to drive the adenomas to malignancy, hinting that Wnt plays a role in the initiation of tumorigenesis. Initial studies examining mutations within gastric tumours revealed somatic mutations in a region of chromosome 5q (Sano et al., 1991), a region that is frequently mutated in colorectal carcinoma (Vogelstein et al., 1988), and that was subsequently found to harbor the coding region of APC, the gene responsible for familial adenomatous polyposis (FAP) (Kinzler et al., 1991; Korinek et al., 1997; Morin et al., 1997; Su et al., 1992). Loss of heterogeneity at 5q has been shown in approximately 40% of GCs regardless of histologic subtype (Rhyu et al., 1994).

Furthermore, spontaneous inactivation of Apc in the APC^{min} mouse model was found to promote the formation of gastric adenomas within the antral stomach (Gravaghi et al., 2008; Tomita et al., 2007).

Somatic mutations of APC were further characterized in gastric tumours, demonstrating that only very well differentiated adenocarcinomas, and not less differentiated intestinal gastric tumors, contained APC mutations (Nakatsuru et al., 1992). The location of somatic APC mutations varied between tumours, giving rise to the idea that different subsets of tumours have different mechanisms to their carcinogenesis. However, it was later discovered that both major types of GC harbor APC mutations (Kim et al., 2010). A possible explanation for this discrepancy is that early work did not screen the entire APC gene, thereby possibly missing mutations in other locations of the APC gene that are observed in other types of GC (Nakatsuru et al., 1992). Studies using GC cell lines have shown mutations at codon 1450 of APC, which leads to truncation of the protein causing constitutive activation of Wnt/ß-catenin signalling. This is characterized by nuclear localization of ß-catenin and upregulation of TCF/LEF regulated transcription (Sasaki et al., 2001).

There are over 1,000 known mutations in the APC gene (Béroud and Soussi, 1996; Laurent-Puig et al., 1998), which makes it extremely challenging to translate knowledge of the specific genomic mutation into clinically relevant information. Adding to this complexity, it has been shown that even patients with identical mutations can develop varying clinical manifestations (Giardiello et al., 1994), suggesting that there may be additional contributing genomic and environmental factors. Even though somatic mutation of the APC gene is most commonly associated to the colon, it has frequently been observed in many other tissues (Rubinstein et al., 2020) with The Catalogue of Somatic Mutations in Cancer (COSMIC) includes a total of 65,672 samples from diverse tissue types, of which 5,928 are noted to contain APC variants. Currently (At time of query), the catalogue contained 2,657 gastric carcinoma samples that had undergone APC testing, reporting 139 tumours with mutations (5.23%) (Forbes et al.,

2017). The recent TCGA study contained 293 gastric samples with 17% reporting genetic alteration to the APC gene (TCGA, 2014). The role of APC mutation in the pathogenesis of gastric adenocarcinoma is less well-studied than in the colon, however, there is evidence from recent molecular profiling studies to support bi-allelic loss of APC as a potential driver (Lim et al., 2016).

Due to the stomach and the colons proximity and shared role in digestion, one might expect similar APC mutational profiles, however, this is not the case. Within the TCGA dataset there are 43 APC mutations, 69% of all recorded mutations, that are unique to the stomach and not observed in colorectal adenocarcinoma genomic datasets. The remaining 31% of APC mutations are shared with the colon. Of note, the colon datasets record 309 different APC mutations, whereas there are only 63 recorded APC mutations for stomach. Of the 63 mutations recorded for the stomach, over 50% of them are truncating mutations (TCGA, 2014). Gastric and colonic APC variants also differ in genomic location across the gene. The frameshift and stop gain variants of the colon samples occurred almost exclusively in the 5'-portion of the gene, compared to a more uniform distribution in the gastric samples. In the colon samples, only 1.4% occur after codon 1650 compared to 26% of the frameshift and stop gain variants in the gastric samples (Rubinstein et al., 2020).

However, even in cancers with no detectable mutations to ß-catenin or APC, the mRNA level of ß-catenin is greatly enhanced (Ebert et al., 2002). This suggests that other more upstream components of Wnt signalling are being deregulated in GC, and understanding the mechanism by which they signal could lead to advances in therapy for GC.

1.6.3 Dysregulation of Wnt signalling at the Plasma Membrane

Until recently, it was believed that mutations in APC, Axin and/or ß-catenin which cause constitutive activation of Wnt signalling cannot be modulated further by upstream components of the Wnt pathway. However, it has been demonstrated that the sFRP family of Wnt antagonist are frequently silenced via promoter hypermethylation in a variety of cancer, including GC

(Caldwell et al., 2004; Cheng et al., 2007; Suzuki et al., 2004). The silencing of sFRP via methylation is detected in preneoplastic gastric tissue and thus could be used as a biomarker for GC (Cheng et al., 2007). Transfection of Sfrp-1, -2 or -5 successfully suppressed TCF/LEF activity, which is sufficient to block proliferation and induce apoptosis in GC cell lines even in those harboring Apc and/or ß-catenin mutations (Nojima et al., 2007). Building on this, one group has successfully reduced the size of gastric tumour xenografts by transfecting mice with sFRP2, thus demonstrating the potential for sFRP2 to act as a functional tumour suppressor (Cheng et al., 2007).

Similar to other Wnt antagonists, frequent promoter methylation of Dkk-1 and subsequent down-regulation has been observed in GC cell lines and primary gastric tumours (Kagey and He, 2017). GC cells transfected with functional Dkk1 lead to significant reductions in tumorigenicity (Yu et al., 2009). Similarly, GC cells enriched for CD44 expression, a gastric stem cell marker, were isolated and virally transfected with Dkk-1 to abrogate Wnt signalling. Successfully infected cells displayed large amounts of phosphorylated ß-catenin in addition to the inhibition of TCF/LEF target gene expression. This correlated with decreased survival and invasiveness of GC cells (Wang et al., 2012b). The functionality of Dkk-1 was also assessed *in vivo*, where Wang et al. demonstrated that the size of CD44+ gastric tumour xenografts significantly diminished following administration of Dkk-1. This showed that there is a potential therapeutic benefit to treating GC stem cells with Dkk-1 and that modulation of upstream Wnt components can attenuate gastric tumorigenesis irrespective of downstream mutations (Wang et al., 2012b).

Taking advantage of gastric stem cell markers allows the manipulation of signalling pathways exclusively in stem cell populations, thereby investigating the role stem cells play in disease etiology. The expression of gastric stem cell marker Lgr5 is observed at the base of gastric glands in both non-neoplastic and metaplastic gastric tissue (Barker et al., 2010b; Simon et al., 2012). Barker et al. used a well-characterized *Lgr5-EGFP-IRES-CreERT2* mouse model to

conditionally truncate Apc from antral Lgr5+ stem cells. Following Apc truncation in these stem cells, elevated expression of nuclear ß-catenin was observed at the base of the glands. This subsequently fueled the growth of highly proliferative gastric adenomas (Barker et al., 2010b). However, the development of the Lgr5+ gastric adenomas did not proceed, due to activating Wnt mutations in Lgr5+ intestinal stem cells leading to a high tumour burden in the intestine and the animals having to be sacrificed (Barker et al., 2010b). Interestingly, analysis of GC patients revealed that the spatial distribution of Lgr5+ cells within the tumour mass correlates with patient survival (Simon et al., 2012). Patients with Lgr5+ cancer cells at the luminal surface lived longer (compared with Lgr5-negative cases at the luminal surface), while those with Lgr5+ tumour cells in the tumour center and at the invasive front lived shorter when compared with Lgr5-negative cases at these sites (Simon et al., 2012).

Within the molecular classifications of gastric tumors, a significant proportion of chromosomal invasive tumors display mutations to Wnt pathway components; Apc, ß-catenin and Rnf43 (TCGA, 2014). These findings were further supported from an Asian GC patient dataset reporting even higher incidence of somatic mutations to Wnt pathway components (Cristescu et al., 2015). Of particular note, is the reported truncating mutation to the E3 ubiquitin ligase, *RNF43*, found in 54% of microsatellite instable gastric tumours (Maruvka et al., 2017; Wang et al., 2014a).

As previously mentioned, RNF43 together with ZNRF3 function to negatively regulate Wnt signalling via ubiquitinylation of Frizzled receptors, leading to their degradation (Hao et al., 2012; Koo et al., 2012). Loss of function (LOF) mutations to *Rnf43*/*Znrf3* are associated with driving proliferation in GC, presumably by allowing FZD receptors to stabilize, become overexpressed on the cell surface, thus rending cells hypersensitive to Wnt ligands and leading to sustained Wnt signaling (Figure 1.9). However, this has not been functionally demonstrated in GC yet. RNF43 mutations occur in high-grade dysplasia and GC or in carcinomas adjacent to adenomas, this indicates that RNF43 mutation

is one of the key events in the malignant transition of these tumours (Min et al., 2016; Spit et al., 2019). Diagnostically, RNF43 mutations leading to Wnt-dependent tumours occur in early-stage gastric lesions (Min et al., 2016), which could be used to stratify patients who may benefit from compounds that block the secretion of Wnt ligands, which have substantial efficacy in other 'Wnt-addicted' RNF43 mutant cancers (Jiang et al., 2013; Steinhart et al., 2016).Similarly, RNF43 mutations have been shown to be frequently present in colorectal cancers. In patients carrying the germline mutations E318fs, RNF43 was demonstrated to be inactivated by loss of heterozygosity or additional somatic mutations in polyps, giving rise to serrated polyps and tubular adenomas (Yan et al., 2017b).

RNF43 was also shown to be differentially expressed in GIT tumours compared with healthy tissue. A study conducted in gastric and colorectal cancers showed down regulation of RNF43 in tumors that was associated with distant metastasis and TNM staging leading to poorer survival (Gao et al., 2017b). More recently, RNF43 hotspot mutations were reported in colorectal polyps and colorectal tumours, recurrence was found to be higher in patients with colorectal cancer harboring mutated RNF43 (Eto et al., 2018). These findings were confirmed for GC where RNF43 expression was positively correlated with better survival, and poorly differentiated adenocarcinomas were shown to lack RNF43 expression (Niu et al., 2015). This suggests that RNF43 plays an important role in tissue homeostasis in the GIT and that its alteration can lead to malignant transformation. Therefore, there has been great interest to elucidate the functional role of RNF43.

In the context of the stomach, RNF43 overexpression has been shown to impair stem-like properties, and reduce proliferation and sphere formation capacity (Gao et al., 2017b) however until very recently there has been no formal investigation into the impact of RNF43 loss of function in the stomach. The recent study reported that the loss of endogenous RNF43 function enhanced proliferation and initiated tumour growth of GC cells (Neumeyer et al., 2019).

This was investigated both *in vitro* and *in vivo* by depleting the expression of endogenous RNF43 in gastric cells. GC stably transfected with shRNA targeting RNF43 showed increased proliferative and invasive capacity *in vitro*. In an *in vivo* xenograft model, tumours derived from shRNF43 cells were larger than tumors from control cells, confirming that loss of RNF43 enhances tumour growth. The authors generated mouse models to further investigate RNF43 but failed to induce a discernible phenotype using the Cre-Lox system (Discussed in further detail in chapter 4). They instead introduced a two-point mutation in the RING domain of RNF43. Organoids derived from the stomach of mice with mutations to RNF43 grew larger than organoids that originated from stomachs of WT mice, supporting the tumour suppressor function of RNF43 in the stomach (Neumeyer et al., 2019). However, this study did not utilize a robust genetic knockout mouse model or assess the changes due to mutant RNF43 in a long-term setting, making it less representative of tumors seen in a clinical setting. This study has confirmed that the gastric tissue homeostasis is altered in mice harboring point mutations disrupting the RING domain of RNF43. Importantly, it has still not been functionally demonstrated that the phenotypes associated with conditional deletion of RNF43 is due to deregulation of FZD and if phenotypes can be rescued with co-deletion of RNF43 and a FZD gene. This would allow further characterization of GC due to loss of RNF43 and help identify which other members of the Wnt signalosome may be working together to lead to GC.

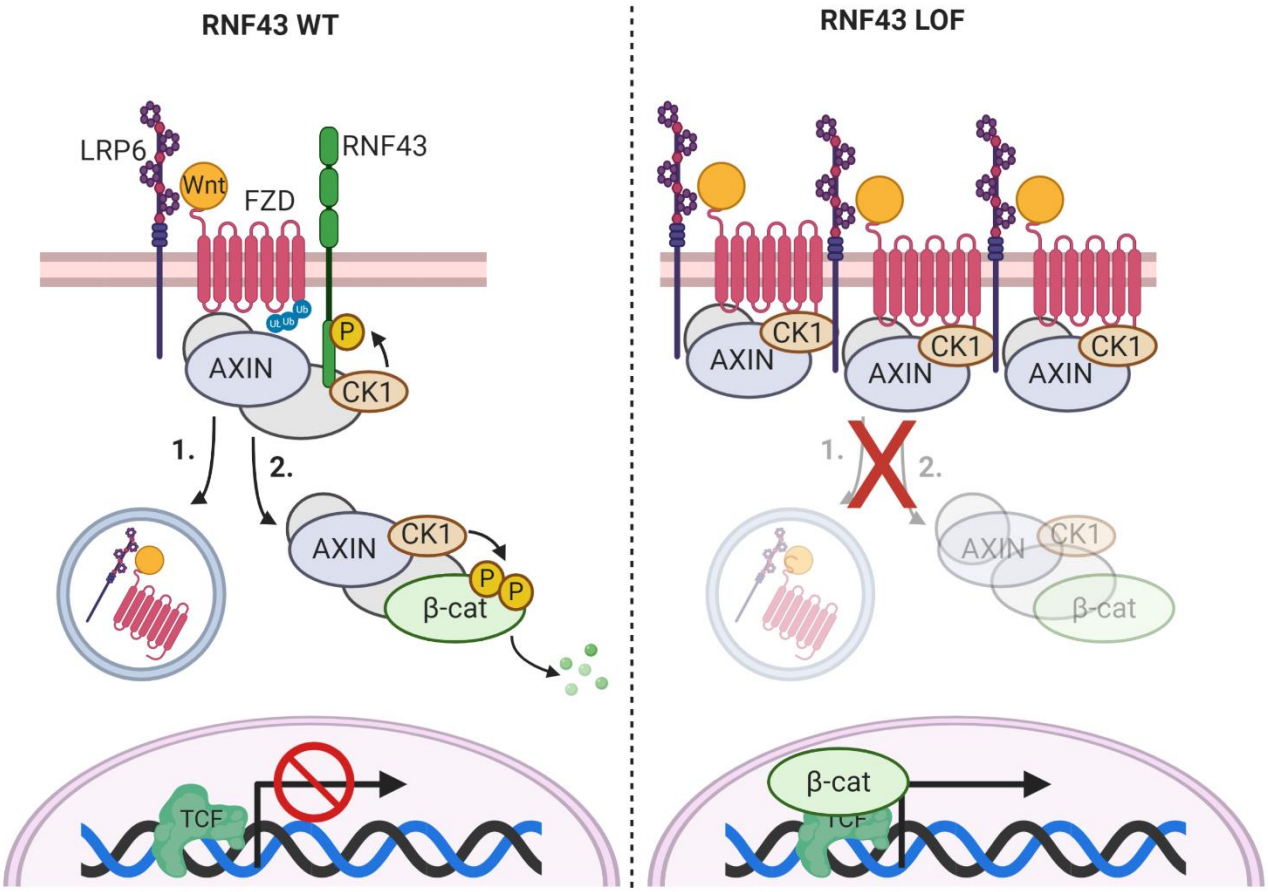


Figure 1.9 Model for mode of action for RNF43 LOF mutants. (**Left**) RNF43performs a bi-functional tumour suppressor role by 1. Targeting Wnt receptors for endocytosis and lysosomal degradation, and 2. By transiently interacting with the destruction complex to reconstitute its activity in the cytosol and re-establish Wnt pathway inhibition. (**Right**) LOF mutations prevent RNF43 function at the plasma membrane, leading to Wnt receptor overexpression and consequently hypersensitivity of cancer cells to Wnt.

A member of the signalosome that may also be involved is LRP6, the co-receptor for Wnt activation. LRP6 has been frequently overexpressed in colorectal, breast and liver adenocarcinoma in association with increased Wnt/ß-catenin signalling (de Voer et al., 2016; Liu et al., 2010; Tung et al., 2012). LRP5/6 is amplified in approximately 14% of GC (TCGA, 2014) and may play a part in oncogenic Wnt signalling and may be upregulated along with Frizzled receptors in GC, although investigations into LRP5/6 functional role in GC are scarce.. What has been elucidated is that infection of gastric epithelial cells with *H.pylori* induces rapid phosphorylation of LRP6 and downstream activation of Wnt/ß-catenin signalling (Gnad et al., 2010). Furthermore, curcumin and pantoprazole (proton pump inhibitors) have demonstrated inhibitory effects on the growth of GC (Shen et al., 2013; Zheng et al., 2017).

In the classical model, the role of APC is limited to ß-catenin proteolysis however, a recent study has reported that APC inactivation can induce ligand-independent LRP6 signalosome formation via clathrin-mediated endocytosis (Saito-Diaz et al., 2018). Endocytosis is required for sustained Wnt/ß-catenin signal activation and endocytosis of the LRP5/6-FZD receptor complex occurs quickly after Wnt binding (Gagliardi et al., 2014). This suggests that, without Wnt ligands, APC inhibits receptor activation via the clathrin pathway, thus, providing a new model for the mechanism by which the Wnt pathway is aberrantly activated upon APC loss with the assistance of LRP6. Consistent with this proposed new model, secreted inhibitors such as sFRP-1, which binds and sequesters Wnt ligands are not capable of inhibiting Wnt signalling in APC mutant cells. This is in contrast to DKK-1, which binds directly to LRP6 to inhibit signaling via disruption of the LRP6-FZD complex or promote the internalization of inactive LRP6 (Yamamoto et al., 2008). It has also been demonstrated that loss of APC could induce the formation of the Wnt signalosome (Saito-Diaz et al., 2018), suggesting that multiple signalosome components mediate the aberrantly increased signalling resulting from APC loss. These components,

including FZD receptors, need to be investigated further to understand the unique role they each play to induce Wnt signalling in GC.

Therefore, LRP5/6 could represent a promising actionable target for cancer therapy. For instance, LRP6 monoclonal antibodies targeting Wnt3 binding site potently reduced the proliferation and growth of APC-Mutant intestinal tumoroids (Saito-Diaz et al., 2018). Unfortunately, LRP6 extracellular domain is divided into two functional entities which bind either WNT1 or WNT3 glycoproteins. Therefore, the use of an antibody targeting the WNt3 binding site of LRP6 can sensitize cells to Wnt1 ligands most likely due to antibody-mediated LRP6 dimerization. Hence, it is necessary to develop specific domain antibodies to selectively inhibit LRP6 activation by certain classes of Wnts while leaving the binding of other Wnt ligands unchanged, limiting potential side effects. Excitingly a group has very recently identified a single-domain antibody fragments (VHH) that specifically bind the Wnt3-binding site (Fenderico et al., 2019). They have been able to show that by inhibiting cellular responses to Wnt3a but not those to Wnt1, their anti-LRP5/6 VHHs efficiently block Rnf43 mutant intestinal organoid growth and survival (Fenderico et al., 2019). Targeting specific regions of LRP6 may represent a potential strategy to reduce ß-catenin-dependent signaling in tumors, without altering other Wnt functions. Frizzled receptors, particularly FZD7, also play a key role in the aberrant Wnt signalling associated with GC, this will be discussed in detail in the next section.

Taken together current research suggests that Wnt signalling can be further modulated at the level of the ligand/receptor irrespective of downstream mutations that constitutively activate the pathway. Furthermore, a role for Wnt/FZD in gastric cancer as a therapeutic target is made more attractive by the observation of aberrant expression of Wnts and FZD receptors in gastric tumours (Flanagan et al., 2017b, Mao et al., 2014). Additionally, it has been recently demonstrated in gp130F/F mice, which develop Stat3-dependent gastric tumors, that they display high Wnt signalling despite there being no

mutations to the intracellular components of the Wnt pathway (Flanagan et al., 2019a).

1.6.4 The role of Frizzled Receptors in GC

There is an ever-growing body of evidence demonstrating that mutations at the level of Wnt receptors play a significant role in GIT tumorigenesis. Each of the Frizzled receptors is intimately linked to one or more cancer type, commonly through up-regulation of a specific FZD receptor (Table 1.4). With respect to the role of FZD receptors and GIT cancers, including GC, FZD7 has been shown to be commonly over-expressed. Targeted inhibition of FZD7 within colon cancers has greatly attenuated various cancer hallmarks (Gurney et al., 2012; Ueno et al., 2009; Vincan et al., 2007a; Vincan et al., 2005). FZD7 is located on human chromosome 2q33 and contains 3869 nucleotides that are translated into a 574 amino acid seven-transmembrane protein that contains a N-terminal extracellular CRD and a C-terminal cytoplasmic PDZ domain. FZD7 is expressed in a wide variety of tissues such as adult skeletal muscle, heart, brain, placenta, kidney and lung (Sagara et al., 1998; Ueno et al., 2013). However, of the 10 FZD family members, FZD7 is the only evolutionary conserved family member that regulates developing gastrointestinal systems (Minobe et al., 2000), where FZD7 has a critical role in controlling tissue separation during gastrulation (Winklbauer et al., 2001).

GC displays aberrant Wnt/ß-catenin pathway activation and although many of the genetic mutations contributing to hyperactive Wnt signalling originate from intracellular components, it is highly likely that additional modulation of the Wnt pathway can come from upstream signalling components. This hypothesis is strengthened by being demonstrated in CRC (Caldwell et al., 2004; Suzuki et al., 2004) and the fact that FZD7 has recently been implemented in regulating tumourigenesis and growth in the stomach (Flanagan et al., 2019a). FZD7 is abundantly expressed in human gastric tumours (Flanagan et al., 2017a; Kirikoshi et al., 2001b), which is also associated with poor patient outcome (Li et al., 2018). In spite of compelling evidence implicating FZD receptors in GC,

there has been no formal investigation, until now, of the therapeutic benefit of targeting FZD receptors in GC *in vivo*.

These type of investigations are especially important as FZD7 is capable of transducing both canonical and non-canonical Wnt signalling, which means that FZD7 can regulate cancer hallmarks associated with both signalling arms such as proliferation, evasion of apoptosis, angiogenesis and invasion (Asad et al., 2014; Vincan et al., 2007a). FZD7 has been shown to regulate many cancers, for example, in hepatocellular carcinoma (HCC) ~70% display elevated nuclear ß-catenin (Wong et al., 2001) showing a role for canonical Wnt signalling. Despite intracellular components of the Wnt pathway, such as Axin1 and ß-catenin, are mutated in some cases of HCC, they are not sufficient to account for the high frequency of Wnt pathway activation observed. Illustrating activation of the pathway at the level of the ligand/receptor. FZD7 is frequently overexpressed in HCC and has been shown to interact with Wnt3a to transmit canonical Wnt signalling in HCC (Kim et al., 2008b).

Wnt signalling is known to regulate the development and homeostasis of the breast and is deregulated in breast cancer (Zeng and Nusse, 2010). FZD7 is overexpressed in aggressive triple negative breast cancer (Yang et al., 2011) and knockdown of FZD7 via shRNA was able to inhibit migration, colony formation and xenograft growth of human breast cancer cells which was associated with reduced canonical Wnt signalling (Yang et al., 2011). Additionally, FZD7 expression is regulated by breast cancer stem cells with SIRT1, an NAD^+ -dependent deacetylate positively regulating FZD7 mRNA and protein levels, promoting cell migration and proliferation (Simmons et al., 2014). Inhibition of SIRT1 significantly reduced FZD7 levels and reversed the aggressive tumourigenic effect of FZD7 overexpression in breast cancer cell lines (Simmons et al., 2014).

Knockdown of FZD7 using siRNA has also been shown to inhibit proliferation of human ovarian adenocarcinoma cell lines (Asad et al., 2014). Interestingly, inhibition of FZD7 in ovarian cancer resulted in an increase in the level of

canonical signalling, whilst non-canonical signalling components were down-regulated (Asad et al., 2014). This demonstrates that FZD7 is likely regulating ovarian cancer cell growth via the Wnt/PCP pathway and illustrates FZD7s ability to transmit signals via both canonical and non-canonical pathways.

Nuclear ß-catenin is observed in ~70% of cervical tumours, however, mutations to *APC* and *ß-catenin* are rare in this cancer (Shinohara et al., 2001) suggesting Wnt signalling is activated at the level of the receptor. Inhibition of FZD7, via shRNA, reduced invasion and EMT in cervical cancer cell lines *in vitro* and the phenotypes observed were associated with changes in the regulation of EMT markers including E-Cadherin, Vimentin and Snail (Deng et al., 2015).

FZD7 has also been shown to regulate intestinal cancer even in those which harbor mutations to *APC*. Inhibition of FZD7 using a dominant negative extracellular domain is able to block the growth of human colon cancer cells *in vitro* and in xenograft experiments with stably transfected SK-CO-1 cells (Vincan et al., 2005). FZD7 also plays a predominant role in transmitting Wnt signalling in intestinal LGR5+ stem cells during homeostasis and regeneration (Flanagan et al., 2015b). Conditional deletion of *Fzd7* in intestinal organoids resulted in crypt atrophy and death, whist conditional deletion of *Fzd7* in specifically the Lgr5+ stem cells triggered rapid epithelial repopulation in transgenic mice (Flanagan et al., 2015b). This demonstrates that FZD7 is critical for intestinal stem cell function. FZD7 is also involved in the regulation of metastasis by regulating EMT in intestinal cancers (Vincan and Barker, 2008). Expression of dominant negative FZD7 in human SK-CO-1 cells blocked growth in xenograft experiments and induced morphological changes suggesting FZD7 promotes MET (Vincan et al., 2005).

Until now the role of FZD7 in GC has not been functionally investigated and the role of FZD *in vivo* was not known. These data suggest that therapeutic targeting of FZD7 could be advantageous in the treatment of GC as it could block all tumorigenic hallmarks associated with aberrant canonical and non-canonical Wnt signalling.

Table 1.4 Frizzled receptor up-regulation in cancer.

Frizzled	Wnt Signalling	Over-expressed in cancer	Reference
FZD1	Wnt/β-catenin	Pancreas and neuroblastoma	(Flahaut et al., 2009; Yang et al., 2018b)
FZD2	Wnt/β-catenin & Wnt/Ca2+	Wilms tumour, endometrial and lung	(Bian et al., 2016; Gujral et al., 2014)
FZD3	Wnt/β-catenin & Wnt/Ca2+ (PKA)	Colon, hepatocellular carcinoma and breast	(Bengochea et al., 2008; Mo et al., 2019; Wong et al., 2013)
FZD4	Wnt/β-catenin	Cervical, acute myeloid leukemia(Thiele et al., 2015) and prostate	(Gupta et al., 2010; Ma et al., 2017; Tickenbrock et al., 2008)
FZD5	Wnt/β-catenin	Kidney and prostate	(Peterson et al., 2017; Thiele et al., 2015)
FZD6	Wnt/Ca2+	Squamous cell carcinomas and breast	(Corda and Sala, 2017; Corda et al., 2017)
FZD7	Wnt/β-catenin & Wnt/Ca2+ (JNK)	Esophageal, gastric, hepatocellular, colon and Wilms' tumor	(Cao et al., 2017; Dekel et al., 2006; Merle et al., 2004; Ueno et al., 2009; Van Cutsem et al., 2016)
FZD8	Wnt/β-catenin	Renal cell carcinoma, prostate and lung	(Murillo-Garzón et al., 2018; Wang et al., 2012a; Yang et al., 2017)
FZD9	Wnt/Ca2+ (ERK)	Osteosarcoma and astrocytoma	(Wang et al., 2017c; Zhang et al., 2006)
FZD10	Wnt/β-catenin & Wnt/Ca2+ (JNK)	Colon and synovial sarcoma	(Nagayama et al., 2005; Nagayama et al., 2009)

1.6.5 Oncogenic Wnt signalling & GC metastasis

Approximately 50% of patients with advanced GC die from recurrence and metastasis, even after curative surgery and chemotherapy (Zhou et al., 2017). Therefore, it is important to understand the underlying mechanisms of GC metastasis and how these could be exploited for therapeutic gain. Wnt signalling has been implemented in multiple aspects of the process of metastasis in many cancers, including GC (Zhan et al., 2017). The process of EMT is a key step in the metastasis cascade, allowing tumour cells to adopt a more mesenchymal phenotype. The mechanism underlying the initiation of EMT in GC is unknown, however it has been investigated in other cancer settings. For example, it has been shown that the cytoplasmic concentration of SNAI2 (EMT transcription factor) is regulated by GSK-3β phosphorylation and subsequent ubiquitination by β-TrCP in breast cancer. Activation of Wnt/β-catenin signalling stabilizes SNAI2 by inhibiting GSK-3β kinase activity and initiates EMT (Wu et al., 2012). This highlights Wnt/β-catenin pathway as a key pathway involved in EMT and there, plays a critical role in metastasis. In the context of GC, an important player is Wnt-5a, which stimulates cell migration and invasion of GC cells through regulation of focal adhesion complexes by activating the small GTP-binding protein Rac (Kurayoshi et al., 2006). Wnt5a also contributes to GC progression by inducing expression of laminin γ2, which increases metastatic potential (Yamamoto et al., 2009).

It has previously been reported that loss of *CDH1* (E-cadherin), through loss of heterozygosity or promoter methylation, activates Wnt signalling by unleashing membrane-bound β-catenin, which in turn, activates Wnt/catenin signaling (Gottardi et al., 2001; Orsulic et al., 1999). With recent genomic data suggesting that while *CDH1* can act as an independent cancer driver in GC in some cases, it more commonly acts in concert with other driver mutations, such as *TP53* to gain a cancer phenotype. Additionally, diffuse-type GC requires *CDH1* mutations in addition to other molecular aberrations including RHOA and RNF43 mutations in order to gain diffuse-type GC characteristics (Nanki et al.,

2018). Therefore, understanding the other molecular components that are involved will be vital for identifying novel drug targets.

It has recently been demonstrated that LGR5 is a positive regulator of GC metastasis attributed to its indispensable role in regulating cytoskeletal reorganization and Wnt responses in GC cells (Wang et al., 2018). LGR5 overexpression is a signature mark of the stem cells derived from the stomach (Nakata et al., 2014) and therefore plays a role in the maintenance of stemness and, so its overexpression is likely linked to metastasis. LGR5 expression has been correlated with GC progression (Wu et al., 2013). This new data linking LGR5 to GC metastasis through activation of Wnt/β-catenin signaling helps explain the underlying molecular mechanism of LGR5 overexpression during GC development and metastasis. The study demonstrated that LGR5 upregulated the expression of β-catenin and affected the subcellular localization of β-catenin in GC cells (Wang et al., 2018). Therefore, this data indicates that LGR5-mediated Wnt signaling results in the accumulation and translocation of β-catenin to the nuclei, driving a positive feedback activation of the Wnt pathway and enhancing and maintaining GC progression. The exact molecular details of this regulation require further clarification and may involve other members of the Wnt pathway signalosome, such as FZD receptors.

FZD7 has been demonstrated to regulate gastric epithelium and be overexpressed in GC (Flanagan et al., 2017a; Kirikoshi et al., 2001b). Therefore, it is likely to be implicated in GC progression. FZD7 has been studied in more detail in the setting of the colon and it has been reported that down-regulation of FZD7 expression leads to a decrease in the metastatic capabilities of colon cells (Ueno et al., 2009). FZD7 is has also been reported to be required for metastatic growth of melanoma cells (Tiwary and Xu, 2016). A recently study in GC showed that over-expression of FZD7 was associated with GC metastasis, advanced clinical stages and poor patient prognosis (Li et al., 2018). Therefore, FZD7 is a likely candidate for facilitating GC progression. FZD receptors are known critical factors for EMT processes. For example, Wnt5a/FZD2-medicated

non-canonical signaling drives EMT in the lung, liver, breast and colon (Gujral et al., 2014). FZD4 ablation induces active ß1-integrin and E-cadherin expression highlighting its role in regulating EMT and cell adhesion in prostate cancer (Gupta et al., 2010). Down regulation of FZD7 expression significantly inhibited cell invasion and migration, accompanied with a decrease in vimentin and snail, and an increase in E-cadherin in cervical and ovarian cancers (Asad et al., 2014; Deng et al., 2015). Additionally, Wnt3a-FZD7 signaling is up-regulated in hepatocellular carcinoma (Kim et al., 2008b) and over-expression of FZD7 promoted cell mobility, metastasis and EMT in esophageal cancer (Cao et al., 2017). Fzd7 has also been shown to regulate MET in CRC cells (Vincan et al., 2007a).Therefore, given its role in GC initiation and growth, investigating the role of FZD7 in the context of GC metastasis is of great interest.

Table 1.5 Wnt pathway mutations in GC.

Wnt Component	Role in GC	Reference
Cytoplasmic		
APC	Mutated/deep deletion in GC patient datasets. Promoter hypermethylation in high grade gastric adenomas.	(Wang et al., 2012c)
β-catenin	Endogenous nuclear expression seen in 13/15 GC cell lines with a subsequent increase in TCF/LEF transcriptional activity. Abnormal nuclear expression seen in high grade gastric adenomas.	(Nojima et al., 2007; Wang et al., 2012c)
AXIN2	miR-544a targeted protein downregulation in GC cells. 30% of MSI high GCs have a frameshift mutation.	(Flanagan et al., 2017b; Yanaka et al., 2015)
*Gsk3*β	Genetic deletion causes rapid gastric tumor formation in mice.	(Radulescu et al., 2013)
Wnt target genes		
MYC	Gene amplification in GC patient samples. GC cells and mouse adenoma show gene upregulation in an Fzd7-dependent manner.	(Flanagan et al., 2017b)
LGR5	Overexpression regulates GC cell proliferation, migration, and invasion.	(Wang et al., 2018)
Wnt ligands		
WNT1	Upregulated in human GC tissue. Overexpression accelerates gastric cancer stem cells.	(Mao et al., 2014)
WNT2b	Upregulated in GC tissue.	(Katoh et al., 2001)
WNT3a	Upregulated in gp130F/F gastric tumors.	(Flanagan et al., 2019a)
WNT5a	High protein expression in GC patient samples, positively associated with the depth of tumor invasion and degree of lymph node metastasis.	(Saitoh et al., 2002)
WNT6	Upregulated in GC patient samples and GC cell lines. Expression positively correlated with tumor stage and node status.	(Yuan et al., 2013)
WNT10a	Upregulated in GC cells and primary GC tissue.	(Kirikoshi et al., 2001b)

Wnt antagonists		
DKK1	Hypermethylated in GC patient samples.	(Wang et al., 2013a)
DKK2	Hypermethylated in GC patient samples + gene transcripts lower in GC patient samples.	(Wang et al., 2017a)
sFRP2	Concurrently hypermethylated with *DKK2* + gene transcripts lower in GC patient samples.	(Wang et al., 2017a)
Wnt receptors		
RYK co-receptor	High expression correlated with poor differentiation, high TNM stage and liver metastasis in GC patients.	(Fu et al., 2020)
RNF43	Truncating mutation in MSI GC tumors. Protein expression is significantly lower in GC cells than normal gastric epithelial cells.	(Niu et al., 2015; Wang et al., 2014b)
FZD2	Upregulated in GC cells (TMK1, MKN7, MKN28, MKN45, MKN74, and KATO-III) and in 4/10 primary GC tissue.	(Kirikoshi et al., 2001a)
FZD5	Upregulated in GC cells (MKN45).	(Kirikoshi et al., 2001a)
FZD7	Overexpression is seen in late-stage clinical GC, correlating with a decrease in survival time. Knockdown significantly reduces GC cell proliferation, migration, EMT, and expression of stem cell markers.	(Li et al., 2018)
FZD8	Upregulated in 4/10 primary GC tissue.	(Kirikoshi et al., 2001a)
FZD9	Upregulated in 2/10 primary GC tissue.	(Kirikoshi et al., 2001a)

1.7 Aims of thesis

The overarching aim of this thesis is to examine the function of the Wnt receptor, Frizzled-7 (FZD7), in the context of gastric cancer. These studies will provide valuable insight into the role of Wnt/FZD7 signalling in the growth and metastasis of gastric cancer and allow assessment of the therapeutic benefit of targeting Wnt signalling at the level of the receptor for treatment of advanced gastric cancer. The main research aims of this thesis are as follows:

1.7.1 Aim 1.

It has recently been demonstrated that FZD7 is the predominant Wnt receptor responsible for regulating stem cell function and maintaining gastric homeostasis. In addition, FZD7 is abundantly expressed in human gastric tumours. Therefore, in chapter 2 the functional role of FZD7 in the growth of gastric cancer cells will be examined both *in vitro* and *in vivo*.

1.7.2 Aim 2.

The majority of gastric cancer patients present with advanced metastatic disease with limited treatment options. In chapter 3 the role of FZD7 in the metastasis of gastric cancer will be investigated, both genetically and pharmacologically, to assess its potential as a target for the treatment of advanced disease in such patients.

1.7.3 Aim 3.

Although many aspects of Wnt signalling are well characterized, our knowledge of the whole pathway remains incomplete. FZD7 forms co-receptor complexes with a variety of extracellular receptors making the activation of Wnt signalling complex. Inactivating mutations to the extracellular E3 ligases RNF43/ZNFR3 are associated with driving proliferation in gastric tumours. However, the functional role of RNF43/ZNFR3 in gastric cancer has not been elucidated. Chapter 4 aims to investigate the functional significance of RNF43/ZNFR3 mutations in gastric cancer development by characterization of tumours from novel mouse models in which *RNF43/ZNFR3* are conditionally deleted in the gastric epithelium.

Chapter 2: Materials & Methods

2. Materials & Materials

2.1 Cell Culture

2.1.1 Cell lines

The human gastric cancer cell lines MKN45 and MKN28 were purchased from ATCC and were sent to Eurofins Medigenomics, who performed independent, off-site analysis using PCR-single-locus-technology to confirm their authenticity. All cell lines were maintained in RPMI supplemented with 10% foetal bovine serum (Invitrogen) and 1% Penicillin Streptomycin (Invitrogen) and incubated at 37°C and 5% CO2. Cells were grown in 5 mL for T25 or 10 mL for T75 tissue culture flasks (Nunc, Leics, UK) with growth media changed every 2-3 days and cells passaged regularly when confluent.

2.1.2 Passaging cells

When cells become 80-90% confluent they were split at a ratio of 1:5. Media was aspirated, and cells were washed with phosphate-buffered saline (PBS) (Invitrogen) to remove any remaining media. 1 mL of 0.25% trypsin/EDTA (Invitrogen) was added to the flask and left to incubate at 37°C for 5-10 minutes until the cells became detached. Cell detachment was confirmed by microscopic observation, before adding 4 mL of media to inactivate trypsin activity. Cells were then split at an appropriate ratio, with remaining cells carefully discarded, re-plated for cell-based assays, or harvested for analysis.

2.1.3 Long term storage

Cells were detached from culture flasks as previously described in section 2.1.2. Following trypsin inactivation cells were centrifuged at 1200 rpm for 5 minutes at room temperature. The supernatant was removed and resuspended in complete growth medium containing 10% dimethyl sulfoxide (DMSO; Sigma, UK) and aliquoted into 1 mL cryo-tubes (Nunc). Cells were then slowly frozen at -80°C in a cryo-freezing vessel containing isopropanol (Fischer Scientific) for 24 hours before being transferred to liquid nitrogen storage.

2.1.4 Raising cells from storage

Cells stored in liquid nitrogen were transferred into dry ice before being thawed in a water bath at 37°C until 90% thawed. Cells were then transferred to a 15 mL falcon tube (Nunc) containing 3 mL of complete growth medium and centrifuged at 1200 rpm for 5 minutes. The supernatant was then removed, with the pelleted cells resuspended in normal growth medium before being plated into a T25 culture flask.

2.1.5 Cell seeding

Following cell detachment, non-passaged cells were collected in a 15 mL falcon tube and pelleted at 1200 rpm for 5 minutes and then resuspended in 1 mL of growth media. Cells were counted using a haemocytometer, 10 µL of single cell suspension was added to the haemocytometer chamber and counted by eye. Cells were then diluted accordingly with growth media and seeded into appropriate culture plates depending on the assay being performed.

2.2 Transfection of cell lines

2.2.1 Plasmids

In order to knockdown *FZD7* in respective cell lines, gastric cancer cells were transfected with a retroviral construct designed to specifically knockdown endogenous *FZD7* expression. Short-hairpin RNA (shRNA) construct designed to knockdown *FZD7* has previously been described (Vincan et al., 2007b). Briefly, RNAi targeting sites in human *FZD7* coding sequence (accession number NM_003507) were selected using siRNA TEMPLATE DESIGN TOOL (Ambion, Austin, TX, USA) and verified by Blast search. shRNA coding oligos were designed and cloned into *BglII* and *HindIII* sites of the pRETROSUPER vector (gift from Reuven Agami, Amsterdam, The Netherlands). Plasmids were tested for efficiency of *FZD7* mRNA depletion and, of possible targeting sites that met the Ambion criteria. ShFZD7 targeting sequences are shown in Table 2.1. In order to overexpress *FZD7* in respective cell lines, gastric cancer cells were transfected with a plasmid that contained full-length FZD7 (a gift from Masaru lab). FZD7 cDNA (Figure 2.1) was first cloned into the cloning vector, pUC118 using PstI sites (Sagara et al., 1998) after which it was sub-cloned into the pCDNA3.1 expression plasmid. Scramble shRNA were used as controls.

2.2.2 Transient transfection

Plasmids were transiently transfected into gastric cancer cells seeded to be 70-90% confluent in 24-well plates using appropriate amounts of lipofectamine 3000, p3000 and Opti-MEM (Table 2.2). After 24 hours of incubation cells were harvested for assays or analysis. FZD7 remained knocked down for 10-12 days. shRNA was used instead of siRNA due to availability in the lab and budgets. The shRNA had been used for previous projects leading onto this one.

Table 2.1 shFZD7 targeting sequence, italics indicate the 9-bp hairpin

Sense Oligo	5'-GATCCCCGTACCTGATGACCATGATCTTC*AAGAGA*GATCATGGT CATCAGGTACTTTTTGGAAA-3'
Antisense Oligo	5'-AGCTTTTCCAAAAAGTACCTGATGACCATGATCTCTCTT*GAA*GA TCATGGTCATCAGGTACGGG-3'

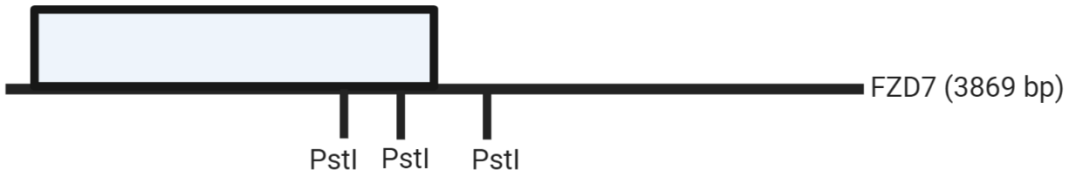


Figure 2.1 Schematic representation of FZD7 cDNA. The coding region is depicted as an open box, the noncoding region as a solid bar. FZD7 has 3 PstI sites.

Table 2.2 Transfection volumes

Transfection Reagent	Volume per well
DNA	500 ng
Lipofectamine 3000	1.5 µL
p3000 reagent	1 µL
Opti-MEM	50 µL

2.3 Cell Based Assays

2.3.1 CellTiter-Glo Assay

For growth assays, cells were plated in a 96-well plate format at 10,000 cells/well and treated with appropriate inhibitors for 24 hours. Cells were lysed with CellTiter-Glo® Luminescent Cell Viability Assay Reagent (Promega) and luminescence was read using a Clariostar plate reader (BMG Labtech). Percentage cell growth was calculated relative to DMSO treated control cells.

2.3.2 Soft Agar Colony Forming Assay

Human gastric cancer cells were transfected as described in section 2.2 or treated with 80 μg/mL OMP-18r5, 1 μM LGK-974 or vehicle. Following transfection (or wild-type cells for drug-treated cohorts) cells were washed in PBS, detached from the culture dish surface with 0.25% trypsin/EDTA and resuspended in RPMI as single cells. Single cells were counted with a haemocytometer and mixed in 0.5% agar/RPMI culture medium, pre-warmed to 37°C, for a final concentration of 500 cells/well and plated into 24-well tissue culture plates. Once the agar/cell suspensions solidified, cultures were overlaid with RPMI culture medium (containing drugs for drug-treated cohorts) and incubated at 37°C in 5% CO2 for 14 days. Drugs and media were refreshed every 7 days. Colonies (≥50 cells) were counted by eye and images taken on a dissecting microscope.

2.3.3 Migration Assays

Transwells with 8.0 μm pores were placed in 24-well culture dishes and single cells that had been incubated for 24 hours with 80 μg/mL OMP-18R5 or transfected with shFZD7 were resuspended in serum free RPMI media and seeded on the top of a Transwell insert (Figure 2.2). 5%-FBS-RPMI media was placed in the base of the well to act as a chemoattractant. Cells were again treated with 80 μg/mL OMP-18R5 (Drug experiments only; not shRNA experiments) and left to migrate for 24 hours. Experiments were performed in duplicates. After 24 hours, the Transwells were washed in PBS, fixed in 70% EtOH and stained with 0.2% crystal violet. The filter of the Transwell was then

carefully removed and mounted onto a microscope slide. Experiments were performed in duplicate and three random fields of view per replicate were counted (with ImageJ) and the average taken.

2.3.4 Invasion Assays

For invasion assays, the same protocol was followed with the addition of a 100 µL layer of Matrigel on top of the Transwell filter to mimic the extracellular matrix. The Matrigel was left at 37°C until set, the Matrigel was then rehydrated with serum-free media and GC cells seeded on top (Figure 2.2).

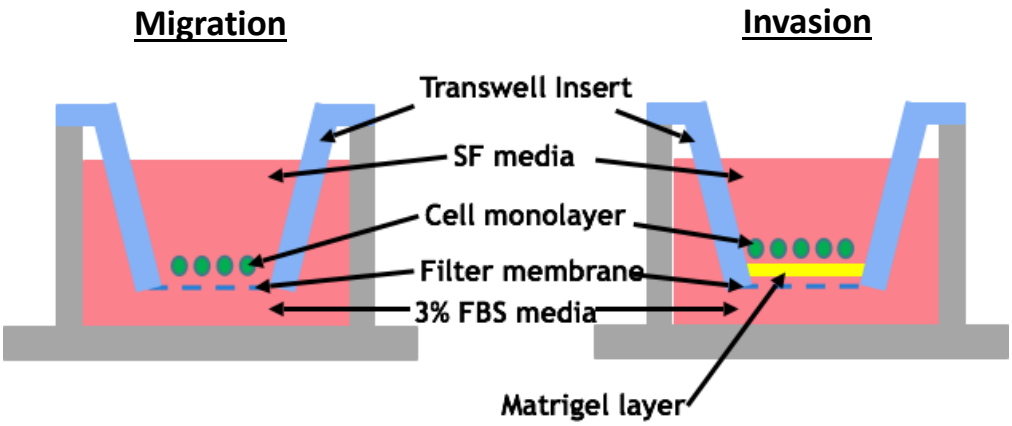


Figure 2.2 Experiment set up for migration and invasion Transwell assays.

2.3.5 EMT stimulation

Human gastric cancer cell lines were induced from an epithelial to a mesenchymal state using StemXVivo media supplement (R&D systems), which contains a mixture of recombinant proteins and neutralizing antibodies (Table 2.3). Cells were seeded into chamber slides (Nuch, Lab-Tek) at a concentration of 0.01x106 cells/mL in 200 µL of growth media. Attached cells were then treated with 2 µL of 100x StemXVivo to make a final 1x concentration. Cells were left to culture for 2 days before media was replaced with fresh EMT inducing supplement. After a further 3 days of culture cells were harvested for endpoint assays.

2.3.6 Fluorescence immunocytochemistry

Following treatment, cells were gently washed in PBS and fixed in 4% paraformaldehyde for 10 minutes. The fixative was removed, and the cells were washed with PBS. The cells were permeabilized with 200 µL of 0.2% Triton x100 PBS and left for 5 minutes at room temperature before being washed 3x with PBS. Cells were then blocked with 1% BSA in PBS for 30 minutes at room temperature. The blocking buffer was removed and primary antibody (Vimentin 1:300, EpCAM 1:100) was added and the cells were incubated overnight at 4°C in the dark. The following day the primary antibody was removed, and cells were washed 3x for 5 minutes in PBS. The secondary antibody (Alexa Fluor 488 or 594, 1:400) and DAPI (1:200) was added and cells were left to incubate for 1 hour in the dark. Cells were washed in PBS and mounted using Mowiol mounting solution. Cells were imaged using a fluorescent microscope.

Table 2.3 Components of StemXVivo media supplement.

StemXVivo media supplement contents
Recombinant human Wnt-5a protein
Recombinant Human TGF-beta1 protein
Anti-Human E-cadherin antibody
Anti-Human sFRP-1 antibody
Anti-Human Dkk-1 antibody

2.4 *In vivo* experiments

2.4.1 Gastric cancer xenografts

A total of $4x10^6$ human gastric cancer cell lines MKN45 or MKN28 in 100 µL of PBS were injected subcutaneously into the hind flanks of female 6-8-week-old nude mice (nu(ncr)-foxn1 nu/nu). 7 mice were used for each cohort which were treated with 20 mg/kg OMP-18R5 or vehicle control (2.5% DMSO + IgG) twice weekly via intraperitoneal injection. Xenografts were measured with calipers twice a week to monitor tumour growth. All animal work was conducted according to the UK Home Office regulations under valid personal and project licenses and in accordance with the Animal [Scientific Procedures] Act 1986. Experimental procedures were carried out in designated procedure rooms.

2.4.2 *In vivo* peritoneal dissemination model

$5x10^6$ MKN45 or $10x10^6$ MKN28 human GC cells suspended in PBS were injected into the peritoneal cavity of female athymic nude mice (nu(ncr)-foxn1 nu/nu), aged 6-8 weeks. Mice were treated daily with 1.5 mg/kg LGK-974, 3 mg/kg LGK-974 or vehicle control (DMSO + IgG) via oral gavage. After 4 weeks the mice were sacrificed and the tumour burden harvested and quantified by weight. All animal work was conducted according to the UK Home Office regulations under valid personal and project licenses and in accordance with the Animal [Scientific Procedures] Act 1986. Experimental procedures were carried out in designated procedure rooms.

2.4.3 Experimental Animals

Mice were interbred to generate compound mice with appropriate alleles. The RNF43-FLOX-ZNRF3-FLOX (background strain: C57BL/6J) mice were generated at the MRC Harwell Institute (Koo et al., 2012). The Claudin18CreER^{T2} mice (Unpublished data) were a gift from Nick Barkers' lab. To generate the Cldn18 knock-in, an IRES-CreER^{T2} cassette was inserted at the 3'UTR by homologous recombination in embryonic stem cells. To generate the construct Cldn18 genomic arms were cloned from BAC clones into pJet vectors. These

(5659 bp 5' arm & 5592 bp 3' arm) were sequentially inserted into a pBlueScript backbone with the IRES-Cre-ER^{T2} being targeted to the 3'UTR along with a TK Neomycin cassette (Figure 2.3). Plasmid electroporation, clone selection, microinjection, and screening of chimeras were performed by GenOway (Lyon, France). Mice were crossed to Zp3-Cre mice to delete the Neomycin cassette.

2.4.4 Colony maintenance & breeding

All animals were maintained on an outbred background and housed in a standard facility in accordance with institutional animal care guidelines and UK Home Office regulations. All animals were given access to RM3(E) standard diet (Special Diets Service UK) and fresh water *ad libitum*. Mice of 6 weeks of age or older and of known genotype were bred in trios of one male and two females. At approximately 4 weeks of age, pups were weaned and housed according to sex. Ear biopsies were taken for identification and genotyping purposes using a 2mm ear punch (Harvard apparatus).

2.4.5 Genetic Mouse Model

The novel transgenic mouse model used for this project was Claudin18CreER^{T2}; Rnf43flox; Znrf3flox; dTOM^{LSL}. This was created by crossing Claudin18CreER^{T2} mice with RNF43-FLOX-ZNRF3-FLOX mice. Claudin18CreER^{T2} driven Cre recombinase transgene was utilized to conditionally delete floxed *RNF43* and *ZNRF3* alleles (Koo et al., 2012) exclusively in the corpus epithelium of the stomach. Expression of the Cre recombinase was induced by administration of tamoxifen and mice were left for 140 days before being sacrificed.

2.4.6 Tamoxifen Administration

Induction of Cre recombinase activity in mice bearing Claudin18CreER^{T2} transgenes was controlled by tamoxifen binding to a mutated ER^{T2} receptor fused to the Cre recombinase protein. Stocks of 10 mg/mL tamoxifen (Sigma) were produced by dissolving tamoxifen in corn oil by shaking vigorously at 37°C in a foil-wrapped falcon tube for 4 hours. Tamoxifen was prepared fresh before use and kept in the fridge between injections but warmed to 37°C prior to

injection. To confirm recombination Clausdin18CreER^{T2} mice received 100 mg/kg of tamoxifen via intraperitoneal injection 3x in 1 day. For long-term induction Claudin18CreER^{T2}; Rnf43flox; Znrf3flox; dTOM^{lsl} received 100 mg/kg tamoxifen 4x, 1x daily.

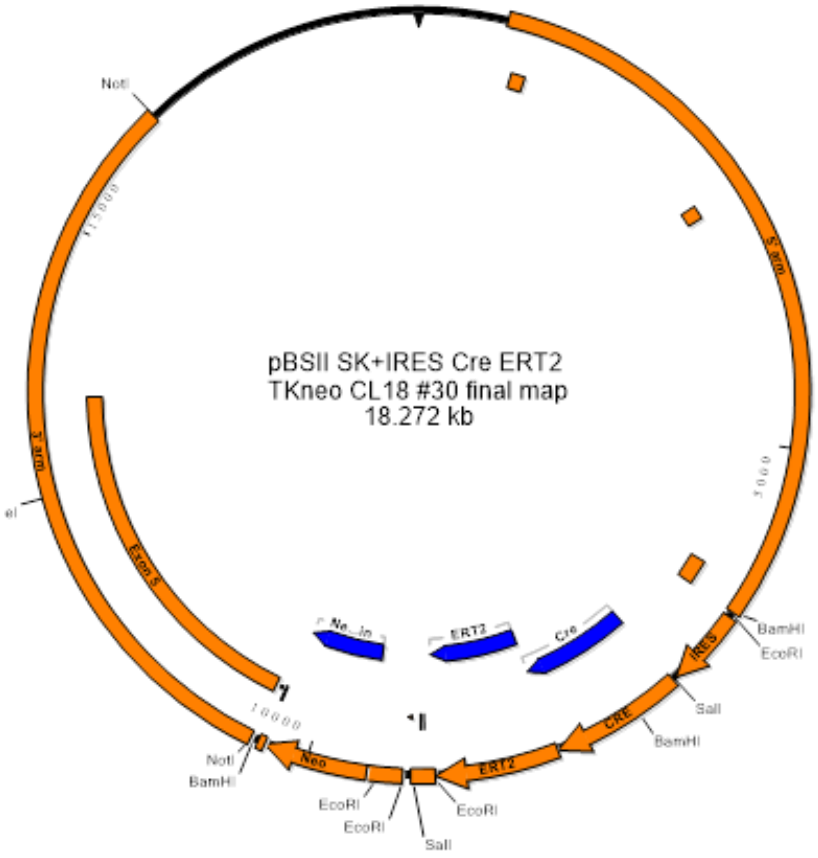


Figure 2.3 Claudin18 knock-in plasmid map. An IRES-CreER^{T2} cassette was inserted at the 3'UTR which was subsequently inserted into a pBlueScript backbone along with a TK neomycin cassette.

2.4.7 DNA extraction

Mouse ear biopsies were collected at weaning and temporarily stored at -20°C to prevent degradation. Each tissue sample was digested in 250 µL lysis buffer (VWR) containing 0.4 mg/mL proteinase K (Sigma), overnight at 42°C with agitation. The protein was precipitated by the addition of 100 µL of protein precipitation solution (VWR). The solution was mixed by inversion and protein and insoluble debris was pelleted by centrifugation at 13,000 rpm for 10 minutes. The supernatant was added to a fresh Eppendorf tube containing 250 µl of isopropanol (Thermo Fisher Scientific) to precipitate the DNA. The solution was mixed by inversion and centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded, and the pellet was left to air dry for an hour before resuspending in 250 µL of Milli-Q water. For short-term storage gDNA was left at room temperature or stored at 4C for longer-term storage.

2.4.8 Polymerase chain reaction (PCR) genotyping

PCR was performed on genomic DNA (gDNA) extracted (Section 2.4.7) from ear biopsies. PCR was performed in a 96-well semi-skirted straight side plates (Alpha labs). PCR reaction mixtures were made up according to table 2.4 containing an appropriate DNA polymerase and buffer (Promega GoTaq). A control well was made using the same PCR mix but with dH2O instead of the DNA. The 96-well plates were them sealed with aluminum foil seals (Star labs), and air bubbles were removed by tapping the plates on a hard surface. The reactions were run in a GS4 thermocycler (G strom). The reaction mixture and cycling time is outlined in Table 2.4 and 2.5 and primer sequences and annealing temperatures are shown in table 2.6.

2.4.9 Visualization of PCR products

PCR products were visualized by gel electrophoresis using 2% agarose gels. The gels were made by dissolving agarose (Eurogentec) 2% [w/v] in 1x Tris Borate EDTA (TBE) buffer (National Diagnostic) and heated in a microwave until boiling. The solution was then cooled under a running tap with agitation and 14 µl of Safe View fluorescent nucleic acid stain (NBS biological) was added per

400 mL. The gel solution was then poured into clean molds (Bio-Rad) and combs were added to create wells and left to set. Once set, the combs were carefully removed and the gels placed into an electrophoresis tank and covered with 1X TBE solution with Safe View (10 µl Safe View/100 mL 1X TBE). 5 µl of loading dye (50% Glycerol (Sigma), 50%dH2O, 0.1% bromophenol blue (Sigma)) was added to the PCR product samples and gently mixed by pipetting. 20 µl of the PCR samples were added to individual wells of the agarose gel and run alongside a molecular weight marker. The gel was run at 120 V for approximately 30 minutes or until the loading dye had run more than half way across the gel. The gel was then visualized for PCR products using a GelDoc UV Transilluminator (Bio-Rad) and images taken using the GelDoc software (Bio-Rad). PCR product sizes are outlined in table 2.6.

Table 2.4 Genotyping PCR reaction mixture

Component	Volume (µL)
5X colourless buffer	5
25 mM $MgCl_2$	1.25
10 mM dNTPs	0.5
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
Go Taq polymerase	0.25
DNA	3
dH_2O	13

Table 2.5 Genotyping PCR cycling conditions

Stage	Temperature	Time	
Initial denaturation	94	5 minutes	
Denaturation	94	60 seconds	X 35 cycles
Annealing	See table 2.6	60 seconds	
Extension	72	60 seconds	
Final Extension	72	10 minutes	
Hold	10	10 minutes	

Table 2.6 Genotyping PCR primer sequences and product sizes

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temp.(°C)	Product size (bp)
RNF43_Loxp	GAAGCAGACAATGAAGCGAAT	TAGTGCCCCACAGAGGACA	63	WT= 284, Mut= 405
ZNFR3_Loxp	CACACCCTGACCCTACGAA	TTACCACACCCATACCCAACT	60	WT= 324, Mut= 405
FZD7_Loxp	GCACCATCATGAAGCACG	CACAGTTAGCATCGTCCTGC	58	WT= 550, Mut= 630
Claudin18_KI	GCATGAAGTGCAAGAACGTG	GTAGACTCCCGTTGCTTTGG	58	450
Claudin18_WT	GTCAGTCCTACTAAACACACATGAA	Used in combination with C18_KI primers above	58	300
dTOM_WT	AAGGGAGCTCAGTGGAGTA	CCGAAAATCTGTGGGAAGTC	58	280
dTOM_Mut	CTGTTCCTGTACGGCATGG	GGCATTAAAGCATATCC	58	230

2.5 RNA Analysis

2.5.1 Epithelial cell extraction

To avoid interference from stromal and smooth muscle compartments in subsequent RNA analysis, the stomach epithelium was extracted. Stomachs were removed and flushed with PBS before being cut open along the greater curvature. The stomachs were then placed in digestion buffer (25 mM EDTA: For 100 mL, 98.9 mL PBS and 5 mL 0.5M EDTA) for 60-90 minutes in a falcon tube. After which, the digestion buffer was removed and 10 mL of cold PBS was added and the tube was vigorously shaken. The now detached muscle layer was removed and the tube was centrifuged at 1,500 rpm for 5 minutes. The supernatant was carefully removed and the pellet was resuspended in 1 mL of ice cold PBS. The resuspended samples were transferred to 2 Eppendorf tubes with 0.5 mL in each and centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was collected and frozen in liquid nitrogen and stored at -80°C until use.

2.5.2 Tissue homogenization

Gastric tissue or epithelial cell extracts were removed from storage and placed on dry ice to prevent defrosting. The tissues were placed in 1 mL Trizol (Invitrogen) in homogenizing lysing matrix D tubes (MP Biomedicals). Tissues were homogenized using a precellys 24 homogenizer (Bertin Technologies) at 6,000 rpm for 2 cycles of 30 seconds.

2.5.3 RNA extraction

Human gastric cancer cells were resuspended and homogenized in Trizol (Invitrogen), 1 mL per 10 cm^2 of culture dish area. Tissue samples were homogenized in 1 mL of Trizol using a homogenizer. Samples were incubated for 5 minutes at room temperature and 200 µL of chloroform per 1 mL of Trizol solution was added and mixed vigorously. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C in order to separate the upper aqueous phase (nucleic acid containing fraction) from the lower organic phase (protein containing fraction). The aqueous phase was transferred to a clean micro-centrifuge tube

and an equal volume of isopropanol was added, the solution was vortexed for 10 seconds and then left to incubate for 10 minutes at room temperature. Samples were centrifuged at 12,000 x g for 8 minutes at 4°C to precipitate the RNA. 1 mL of 75% EtOH per 1 mL of Trizol was added to wash the pelleted RNA and the samples were centrifuged at 7,500 xg for 5 minutes at 4°C. The EtOH was carefully removed and the RNA pellet was left to air dry for 5 minutes. The RNA was then resuspended in 25 µL of DEPC treated water. RNA samples were quantified using a DNA/RNA nanodrop spectrophotometer.

2.5.4 cDNA synthesis

1 µg of each RNA sample was reverse transcribed using modified MMLV reverse transcriptase and anchored oligo(dT) primers following the manufacturer's instructions (PCR Biosystems) in a final volume of 20 µL.

2.5.5 qRT-PCR

For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), master mixes were prepared using SyGreen Blue mix (PCR Biosystems) following the manufacturer's instructions, in final volumes of 10 µL in triplicate wells of a 96 well plate. RPL19 primers were used to normalize the data; for threshold cycle (CT) values, the $2^{-\Delta\Delta CT}$ method (Bustin, 2002) was used to calculate the fold change. Statistical analysis was performed on the ΔCT values. See table 2.7 for human primer sequences and table 2.8 for mouse primer sequences.

Table 2.7. qRT-PCR human primer sequences

Target	Forward sequence	Reverse sequence
FZD1	CAAGGTTTACGGGCTCATGT	TGAACAGCCGGACAGGAAAA
FZD2	GGCCACTGAAAACCGAACTT	CCAGAGGCGGAGGAGAACAA
FZD3	TGGGTTGGAAGCAAAAAGAC	CCTGCTTTGCTTCTTTGGTC
FZD4	GCCAATGTGCACAGAGAAGA	AGGTGGTGGAGATGAAGCAG
FZD5	CTGTGGTCTGTGCTGTGCTT	GGCCATGCCAAAGAAATAGA
FZD6	TCTGTGCCTCTGCGTATTTG	TCTCCCAGGTGATCCTGTTC
FZD7	GACCATCATGCCCAACCTTC	GGCCACTGAAAACCGAACTT
FZD8	CGGTTGTAGTCCATGCACAG	TTACATGCCCAACCAGTTCA
FZD9	TTTTCGGTAGCACAGGCTCT	AGTTTCCTCCTGACCGGTTT
FZD10	AGATTCCCATGTGCAAGGAC	AGTTGGGGTCGTTCTTGTTG
CCND1	TCGTGGCCTCTAAGATGAAGGA	TCGGGCCGGATAGAGTTGT
AXIN2	TCAAGACGGTGCTTACCTGT	TGCTGCTTCTTGATGCCATC
CD44	GTCTGCATCGCGGTCAATAG	GGTCTCTGATGGTTCCTTGTT
MYC	CAGCTGCTTAGACGCTGGATT	GTAGAAATACGGCTGCACCGA
LGR5	ACCCGCCAGTCTCCTACATC	GCATCTAGGCGCAGGGATTG
RPL19	AGCGAGCTCTTTCCTTTCG	GAGCCTCTTCTGAAGCCTGA
SNAI2	GGGGAGAAGCCTTTTTCTTG	TCCTCATGTTTGTGCAGGAG
SNAI1	CCTCCCTGTCAGATGAGGAC	CCAGGCTGAGGTATTCCTTG
TWIST1	GGAGTCCGCAGTCTTACGAG	TCTGGAGGACCTGGTAGAGG
CDH2	ACAGTGGCCACCTACAAAGG	CCGAGATGGGGTTGATAATG
VIMENTIN	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGGTGGCAATC
CDH1	TGCCCAGAAAATGAAAAAGG	GTGTATGTGGCAATGCGTTC

Table 2.8. qRT-PCR mouse primer sequences

Target	Forward sequence	Reverse sequence
FZD1	CAAGGTTTACGGGCTCATGT	TGAACAGCCGGACAGGAAAA
FZD2	CCGACGGCTCTATGTTCTTC	TAGCAGCCGGACAGAAAGAT
FZD3	TGGGTTGGAAGCAAAAAGAC	CCTGCTTTGCTTCTTTGGTC
FZD4	GCCAATGTGCACAGAGAAGA	AGGTGGTGGAGATGAAGCAG
FZD5	CTGTGGTCTGTGCTGTGCTT	GGCCATGCCAAAGAAATAGA
FZD6	TCTGTGCCTCTGCGTATTTG	TCTCCCAGGTGATCCTGTTC
FZD7	GCTTCCTAGGTGAGCGTGAC	AACCCGACAGGAAGATGATG
FZD8	TTACATGCCCAACCAGTTCA	CGGTTGTAGTCCATGCACAG
FZD9	AGTTTCCTCCTGACCGGTTT	TTTTCGGTAGCACAGGCTCT
FZD10	AGATTCCCATGTGCAAGGAC	AGTTGGGGTCGTTCTTGTTG
CCND1	TCGTGGCCTCTAAGATGAAGGA	TCGGGCCGGATAGAGTTGT
AXIN2	TCAAGACGGTGCTTACCTGT	TGCTGCTTCTTGATGCCATC
CD44	GTCTGCATCGCGGTCAATAG	GGTCTCTGATGGTTCCTTGTT
MYC	TAGTGCTGCATGAGGAGACA	GGTTTGCCTCCTCTCCACAG
LGR5	ACCCGCCAGTCTCCTACATC	GCATCTAGGCGCAGGGATTG
RPL19	AGCGAGCTCTTTCCTTTCG	GAGCCTCTTCTGAAGCCTGA

2.6 Histological Analysis

2.6.1 Tissue preparation

Freshly isolated stomachs were flushed with PBS and then cut along the greatest curvature. The stomachs were opened out and placed serosa side down onto a wax plate and pinned in place (Figure 2.4). The stomachs were fixed overnight at 4°C in 10% neutral buffered formalin (sigma). The formalin was removed and replaced with 70% EtOH at room temperature. Samples were stored in 70% EtOH in distilled dH_2O at 4°C until processing. After fixation, all tissues were placed in a cassette (Fisher) and processed using an automatic processor (Leica TP1050). The tissues were incubated in an increasing gradient of alcohols for dehydration (70% EtOH for 1 hr, 95% EtOH for 1 hr, 2 x 100% EtOH for 1 hr 30 mins and 100% EtOH for 2 hrs), then in xylene (2 x 2 hrs). The tissues were then placed in liquid paraffin for 1 hr and then twice more for 2 hrs. The samples were removed and embedded in paraffin wax by hand and allowed to harden. Paraffin embedded tissue was sectioned at 5 um using a microtome (Leica RM2135) and placed onto Poly-L-Lysine (PLL) coated slides and baked at 58°C for 24 hours.

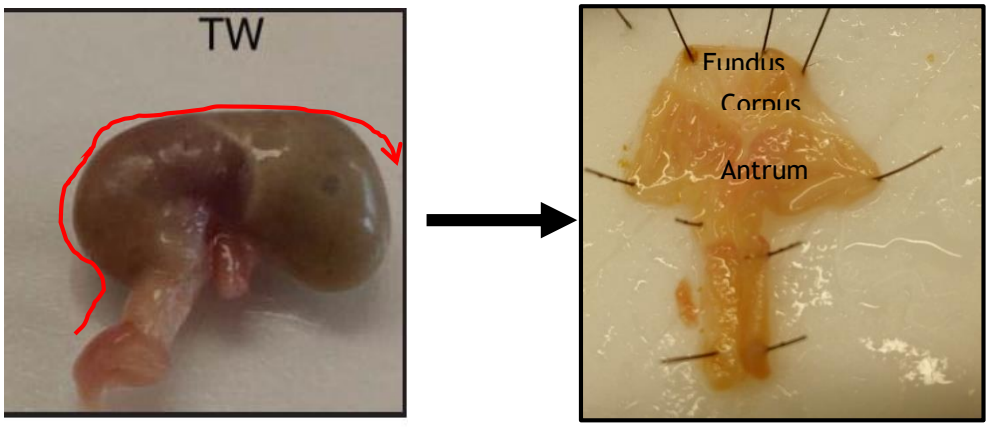


Figure 2.4 Stomach dissection. Preparation of stomach tissue for histology.

2.6.2 Freezing tissue

Sections of stomach were placed into individual lockable microtubes and placed on dry ice until frozen. The samples were then stored at -80°C until required.

2.6.3 Immunohistochemistry

Sections were de-waxed by serial immersions in xylene (2x 5 minute washes) and rehydrated by serial immersions in 100% EtOH (2x 2 minutes), 95% EtOH (2 minutes), and 70% EtOH (2x 2 minutes), before being transferred to dH_2O. Antigen retrieval was performed (See table 2.9 for specific treatment for each antigen) and slides were allowed to cool to room temperature. Endogenous peroxidase activity was blocked by treating slides with a hydrogen peroxide solution (3% hydrogen peroxide (v/v) in MilliQ water) for 10 minutes at room temperature, then washed 2x for 5 minutes in dH_2O. Non-specific binding of antibodies was then blocked by incubating sections for 30 minutes at room temperature in 10% normal goat serum in TBS/T. After incubation the slides were washed 2x for 5 minutes in TBS/T and once in dH_2O. Primary antibodies (Table 2.9), made up in blocking diluent, were added to sections and incubated overnight at 4°C. Sections were washed in TBS/T (3x 5 minutes) then incubated with secondary antibody (polymer horse-radish peroxidase conjugated mouse/rabbit/goat) for 30 minutes at room temperature. Following incubation slides were washed 3x for 5 minutes in TBS/T. Bound peroxidase was detected and developed by the addition of 3,3'-Diaminobenzidine substrate (DAB) at room temperature for 5-10 minutes or until slides turned brown. Slides were then washed for 2x 5 minutes in dH_2O. Slides were then counterstained with Mayers haemotoxylin (R.A. Lamb) for 30 seconds and run under cold water until the water became clear. Slides were then dehydrated through soaking increasing concentrations of EtOH (30 seconds in 70% EtOH, 30 seconds in 95% EtOH and 2x 30 second washes in 100% EtOH) followed by 2x 2-minute washes in xylene before being mounted immediately using DPX mounting solution (Sigma).

Table 2.9 IHC conditions.

Target	Antigen retrieval method	Primary antibody used
Nuclear ß-catenin	Boiled in 50 mM TRIS pH 9.5 for 1 hour	Mouse anti- ß-catenin (1:300) (BD Biosciences, 610154)
PCNA	Boiled in 5 mM EDTA Ph8.0 buffer for 15 minutes	Rabbit anti-PCNA (1:300)(BD Biosciences, 610665)
H^+/K^+ ATPase	Boiled in 50 mM TRIS pH 9.5 buffer in pressure cooker at 900Watts for 10 minutes	Rabbit anti- H^+/K^+ ATPase (1:400) (Santa Cruz, 84304)
Cleaved Caspase-3	Boiled in citrate buffer in pressure cooker at 900Watts for 15 minutes	Rabbit anti- Cleaved Caspase-3 (1:1000) (Cell signalling, 9661L) * Incubated for 48 hours
Red Fluorescent Protein	Boiled in DAKO pH 9 antigen retrieval buffer in pressure cooker at 900 Watts for 30 minutes	Rabbit anti-RFP (1:500) (Rockland, 600-401-379S)

2.6.4 IHC quantification

Three random fields of view were selected, per slide. 1000 cells were counted (using cell counter function on Zeiss software) per field of view. The percentage of positive cells to total cells was calculated.

2.7 Gastric Organoid Culture

2.7.1 Human tissues

Human gastric cancer and normal gastric tissues were obtained from patients who underwent surgery at the University Hospital of Wales, Cardiff with informed consent after the approval of the ethical committee (Wales Cancer Bank). Only samples from patients who had not received chemotherapy were selected.

2.7.2 GC tissue preparation

Small pieces (~1cm^2 x 4 mm thick) of tumour and adjacent non-neoplastic gastric mucosal tissue were taken from the fresh gastrectomy specimen. Samples were placed in ice-cold PBS for transport to the laboratory prior to culture. Normal and tumour tissue were processed using an adapted version of a previously published protocol (Bartfeld and Clevers, 2015), which is explained in detail in sections 5.3.1. In brief, samples were washed vigorously washed with PBS, minced into small fragments, and washed with PBS, before being placed in an enzymatic digestion solution.

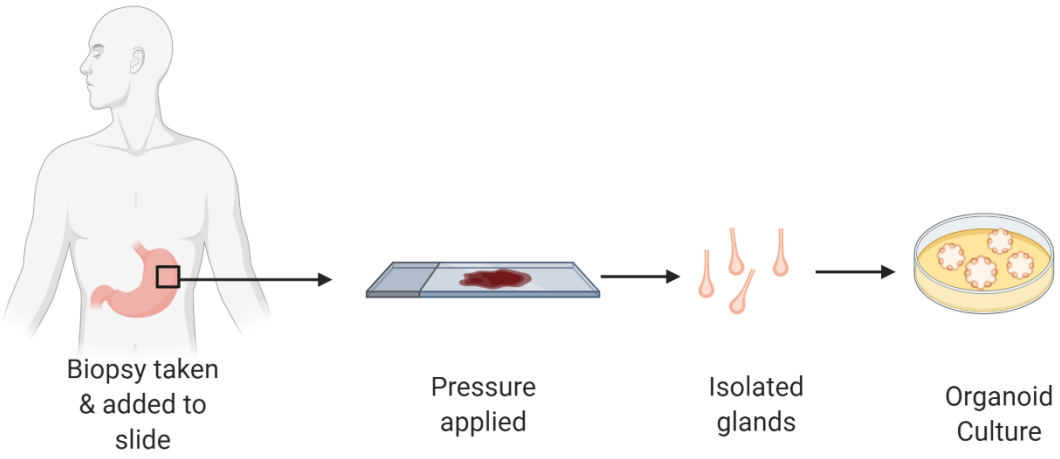
2.7.3 Normal gastric tissue preparation

Gastric tissue was twice washed in PBS containing 1x penicillin/Streptomycin (P/S), followed by the careful removal of the muscle and mucus layer using a scalpel. Inefficient removal of mucus from samples resulted in less viable organoids. Tissue samples were then minced into small fragments and placed into freshly prepared chelating buffer (10 mM EDTA, 0.5 mM DL-dithiothreitol, 1% P/S and 1 µg/µL Primocin) on ice for 45 minutes without agitation. Any agitation damaged the gastric glands. The digested tissue fragments were transferred to a sterile 10 cm dish and as much liquid as possible was removed. To release the glands, a glass microscopy slide was placed on top of the tissue and gentle even pressure was applied until the appear around the tissue appeared cloudy, representing the successful release of glands into solution (Figure 2.5). Gastric glands were collected, counted, resuspended in Matrigel and seeded approximately 100 glands per 50 µL

Matrigel per well of a 24-well plate. The plate was then gently inverted and the Matrigel allowed to solidify; the inversion ensured that the glands settled at the top of the Matrigel drop and had close access to the overlaid growth media (Table 2.10)

2.7.4 Human organoid culture

Human gastric cancer and normal gastric organoids were cultured according to the steps outlined in section 2.7.2 and 2.7.3. In brief, released gastric glands were resuspended in matrigel and plated in 24-well plates with growth media containing various growth factors outlined in table 2.10. Media was changed once every 7 days.



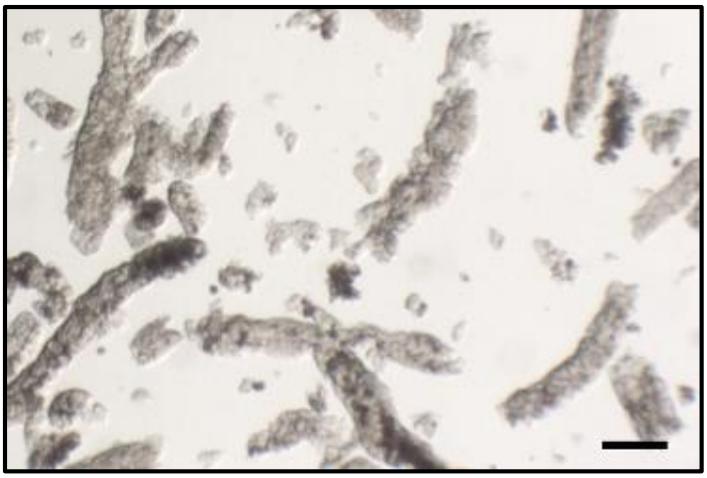


Figure 2.5 Normal Human gastric organoid culture expansion. Schematic of the gland isolation process and representative image of isolated glands from the gastric tissue sample.

Table 2.10 Gastric organoid culture media

Reagent Name	Stock solution	Final Concentration	Volume for 1 mL
Advanced DMEM/F12	NA	NA	450 uL
GlutaMAX	100x	1x	N/A
HEPES	1 M	10 mM	N/A
P/S	100x	1x	N/A
N2 supplement	100X	1X	10 ul
B27 supplement	50X	1X	20 ul
Mouse recombinant EGF	500 ug/ml	50 ng/ml	0.1 uL
Mouse recombinant Noggin	100 UG/ML	100 NG/ML	1 UL
R-Spondin1 conditioned media	10x	1x (10% of final volume)	1 UL
Recombinant FGF10	100 UG/ML	100NG/ML	1UL
Wnt3a conditioned media	2X	1X (50% of final volume)	500 ul
Gastrin	100 UM	10 NM	1 UL
N-Acetylcyteine	500 mM	1 mM	2 ul
Y-27623*	100mm	10um	1
A83-01	500 um	500nm	1ul

* Only add Y-27623 during initial culture or after passaging (then remove after 3 days).

2.7.5 Production of R-Spo1-condtioned media

1.5-2 x 10 ^{6} HEK293-R-Spo B8 clone cells were plated in a T175 flask in 35 mL of pre-warmed growth media (Advanced DMEM supplemented with 10% FBS, GlutaMAX and 150 µg/ml Zeocin). Cells were expanded by passaging when cells reached 75% confluency by the addition of 3 mL TrypLE for 2 minutes at 37˚C. 5 mL of growth media was added to inhibit the dissociation reaction. Cells were pooled and reseeded at 1.5 x10^{6} per T175 flask in 35 mL of growth medium. When cells had been expanded to 20-30 flasks and cells were at 75% confluency, the growth media was changed to harvest media (Advanced DMEM/F-12 supplemented with 1% penicillin/streptomycin, 1% GlutaMAX and HEPES 10 Mm). Cells were incubated in harvest media for 1 week. After this, media was removed into 50 mL tubes and centrifuged for 5 minutes at 500 x g at 8˚C to remove any cells. The media was filter using 500 mL filter cups and divided into 5 mL aliquots.R-Spo-1 medium was stored at -20˚C for up to 6 months.

2.7.6 Production of Wnt3a-condtioned media

1.5-2 x 10 ^{6} HVB-Wnt3a cells (gifted from Clevers lab) were plated in a T175 flask in 35 mL of pre-warmed growth media (Advanced DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, GlutaMAX and 300 µg/ml Zeocin). Cells were expanded by passaging when cells reached 75% confluency by the addition of 3 mL TrypLE for 2 minutes at 37˚C. 5 mL of growth media was added to inhibit the dissociation reaction. Cells were pooled and reseeded at 1.5 x10^{6} per T175 flask in 35 mL of growth medium. When cells had been expanded to 20-30 flasks and cells were at 75% confluency, the growth media was changed to harvest media (Advanced DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and GlutaMAX). Cells were incubated in harvest media for 1 week. After this, media was removed into 50 mL tubes and centrifuged for 5 minutes at 500 x g at 8˚C to remove any cells. The media was filter using 500 mL filter cups and divided into 15 mL aliquots. R-Spo-1 medium was stored at -20˚C for up to 6 months.

2.7.7 Validation of Wnt & R-Spo1-condintioned media

The TOPFLASH assay, a luciferase reporter assay, was used to monitor the concentration of both Wnt3a and R-Spo1 in cell culture media. Two luciferase expressing plasmids, firefly-expressing TOPFlash (Contains 8 TCF binding sites) and renilla-expressing plasmid (pRL, to normalize) were transfected into HEK293 cells in 24-well plates, 1.25 x 10^5 cells per well with 0.5 mL of medium and 400 ng TOPFlash and 25 ng pRL. These cells were then exposed to medium containing either Wnt-3a or R-Spo1 alone or in combination. After 48 hours of induction, firefly and renilla luciferase activity is read (Dual-Glo® Luciferase Assay System (Promega) & Clariostar plate reader (BMG Labtech)). Firefly counts were normalized with renilla counts. FOPFlash (Mutant TCF sites) was used as a control and data was further normalized to this basal control and titrated by comparing to a known source of each growth factor (de Lau et al., 2011). For Wnt3a-conditioned medium, adequate activity was a TOP/FOP value >25. For R-Spo1-condtioned medium, TOP/FOP assay results were valid if the results were 5-10 fold higher in the presence of R-Spo1 + Wnt3a as compared with Wnt3a only.

2.7.8 Human organoid passaging

Organoids were passaged every 2 weeks. Old media was removed and 1 mL of cold Advanced-DMEM/F12 (ThermoFisher Scientific) was added per well to wash the matrigel. A p1000 pipette was used to break up the gel which was transferred to a 15 mL falcon tube containing plain DMEM media. The organoid-matirgel-media mixture was centrifuged for 5 minutes at 300 g at 4˚C to pellet the gastric glands. The supernatant was carefully aspirated off. A p200 pipette was used to break up the pellet, it was then centrifuged for 5 minutes at 300 x g at 4˚C. The supernatant was removed, and the pellet was resuspended in an appropriate volume of Matrigel. 50 µL drops were added to each well of a 24-well plate. The plate was then gently inverted and the Matrigel allowed to solidify in a 37˚C incubator for 30 minutes. 500 µL of gastric growth media, with the addition of ROCKi, was added to each well.

2.7.9 Human organoid cryopreservation

For long-term storage established organoids were frozen in freezing media: 10% DMSO in Advanced-DMEM/F12 + Glutamax + HEPES + Pen/Strep + N2B27. Old media was removed from the organoids and they were washed with standard DMEM. Organoids were gently broken up by pipetting no more than 4 times up and down. Collected organoids were centrifuged at 300 g for 5 minutes at 4˚C after which the supernatant was removed. The pellet was resuspended in freezing media and 1 mL was aliquoted to a cryo-vial. Vials were stored in a -80˚C freezer overnight and then transferred to liquid nitrogen for long-term storage.

2.7.10 Raising human organoids from storage

Organoids retrieved from the liquid nitrogen were thawed at 37˚C in a water bath. Thawed organoids were added to a falcon tube containing 10 mL of Advanced-DMEM/F12. Organoids were centrifuged for 5 minutes at 300 x g at 4˚C. The pelleted organoids were resuspended in 100 µL of Matrigel and a 50 µL drop placed in the center of the well of a pre-warmed 24-well plate. Once solidified, the Matrigel dot was overlaid with 500 µL of pre-warmed gastric growth media with the additional supplement of ROCKi.

2.8 CRISPR cloning strategy

2.8.1 *In silica* design strategy

The specificity of the Cas9 nuclease is determined by the 20-nt guide sequence within the sgRNA. We utilized a plasmid containing an *S.pyogenes* Cas9, this system requires the target system to immediately precede a 5'-NGG PAM sequence. The 20-nt guide sequence base pairs with the opposite strand to mediate Cas9 cleavage at ~3 bp upstream of the PAM (Ran et al., 2013). While the sgRNA sequence must be upstream of a PAM site and be specific to the target gene, off-target activity must also be taken into consideration.

To increase the chance of effective cleavage to achieve full knockout of the FZD7 gene, two sgRNAs were designed. sgRNA targeting FZD5 were also

designed to be used as a control in future experiments. sgRNA was designed for both mouse and human species so that we have a full complement of constructs for use in human cultures and animal models. sgRNA target sequences were chosen that targeted either exon 1 or exon 2 of the gene and were present across all splice variants to ensure efficient knockout. Once a PAM site had been identified in either of these exons, the target sequence was checked for off-target activity by using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). To allow for cloning into the px458 (obtained from addgene and sgRNA oligos from sigma) Cas9 vector (Figure 2.6) 4-nt overhangs compatible with BbsI restriction sites were added to the sgRNA target sequence (Figure 2.6). The px458 vector contains a U6 promoter upstream of the sgRNA insertion site, therefore a G-bp was added (if not already present) to the 5'-end of the sgRNA target sequence. The sgRNA target sequences for the FZD5/7 knockout constructs can be seen in table 2.11 and the sgRNA target sequences for the various APC truncated constructs can be seen in table 2.12.

Forward primer: 5'-CACCGNNNNNNNNNNNNNNNNNNN-3'

Reverse primer: 5'-AAACnnnnnnnnnnnnnnnnnnnC-3'

Figure 2.6 sgRNA oligos with BbsI overhangs. "N" and "n" represent complementary base pairs. Red font represents the overhangs. PAM sequence is not included in the sgRNA sequence. The U6 promoter requires a G-bp at the transcription start site (underlined font). This should be added to the start of your sgRNA at the 5' site if one is not already present.

Table 2.11 Human and mouse sgRNA targeted to *FZD7*

Human FZD7_01 Forward	5'-CACCGGTGCGGGCGAGATCTGCGT-3'
Human FZD7_01 Reverse	5'-AAACACGCAGATCTCGCCCGCACC-3'
Human FZD7_02 Forward	5'-CACCGATGATCGTCGGCATCACCAC-3'
Human FZD7_02 Reverse	5'-AAACGTGGTGATGCCGACGATCAC-3'
Human FZD5_01 Forward	5'-CACCGCGCTCGTCGGCACTGAAGGA-3
Human FZD5_01 Reverse	5'-AAACCCAGCATTGTGGTGGCCTGC-3'
Human FZD5_02 Forward	5'-CACCGCAGGCCACCACAATGCTGG-3'
Human FZD5_02 Reverse	5'-AAACCCAGCATTGTGGTGGCCTGC-3'
Mouse FZD7_01 Forward	5'-CACCGCGAGAAAGGCATCTCGGTAC-3
Mouse FZD7_01 Reverse	5'-AAACGTACCGAGATGCCTTTCTCGC-3'
Mouse FZD7_02 Forward	5'-CACCGATGATCGTGGGCATCACTAC-3'
Mouse FZD7_02 Reverse	5'-AAACGTAGTGATGCCCACGATCATC-3'
Mouse FZD5_01 Forward	5'-CACCGTCTCCACAAGCGGCCAGAAT-3'
Mouse FZD5_01 Reverse	5'-AAACATTCTGGCCGCTTGTGGAGAC-3'
Mouse FZD5_02 Forward	5'-CACCGGTAGCGGCTTGTGGTAGTC-3'
Mouse FZD5_02 Reverse	5'-AAACGACTACCACAAGCCGCTACC-3'

Table 2.12 Human and mouse sgRNA targeted to *APC*

Human APC_01 Forward	5'- CACCGTCCATCCTTTCCCTGAAATC -3'
Human APC_01 Reverse	5'- AAACGATTTCAGGGAAAGGATGGAC -3'
Human APC_02 Forward	5'- CACCGTACTTTATTACATTTTGCCA -3'
Human APC_02 Reverse	5'- AAACTGGCAAAATGTAATAAAGTAC -3'
Human APC_03 Forward	5'- CACCGTAATGAAGAGAAACGTCATG -3'
Human APC_03 Reverse	5'- AAACCATGACGTTTCTCTTCATTAC -3'
Human APC_04 Forward	5'- CACCGTCAGCCATTCATACCTCTC -3'
Human APC_04 Reverse	5'- AAACGAGAGGTATGAATGGCTGAC -3'
Human APC_05 Forward	5'- CACCGGATCTGTATCAAGCCGTTC -3'
Human APC_05 Reverse	5'- AAACGAACGGCTTGATACAGATCC -3'
Mouse APC_01 Forward	5'- CACCGCCGACTCAGAAAATTTTGAC -3'
Mouse APC_01 Reverse	5'- AAACGTCAAAATTTTCTGAGTCGGC -3'
Mouse APC_02 Forward	5'- CACCGTCCATTCTGTCACTAAAGTC -3'
Mouse APC_02 Reverse	5'- AAACGACTTTAGTGACAGAATGGAC -3'
Mouse APC_03 Forward	5'- CACCGTAATGAAGAGAAACATCATG -3'
Mouse APC_03 Reverse	5'- AAACCATGATGTTTCTCTTCATTAC -3'
Mouse APC_04 Forward	5'- CACCGTCTGCCATCCCTTCACGTT -3'
Mouse APC_04 Reverse	5'- AAACAACGTGAAGGGATGGCAGAC -3'
Mouse APC_05 Forward	5'- CACCGAAAATGTCCCTTCGCTCCTA -3'
Mouse APC_05 Reverse	5'- AAACTAGGAGCGAAGGGACATTTTC -3'

2.8.2 Restriction enzyme digestion

To linearize the px458 backbone and allow insertion of sgRNA, a single restriction digestion was carried out according to table 2.13. The reaction was incubated at 37˚C for one hour.

2.8.3 DNA gel extraction

DNA fragments were electrophoresed on an agarose gel and were excised, weighed, and solubilized using binding buffer (ThermoFisher Scientific) at a ratio of 1:1 and the solution was incubated at 60˚C for 10 minutes. The solubilized gel solution was transferred to a GeneJET purification column (ThermoFisher Scientific) and centrifuged for 1 minute. The flow through was discarded and 100 µL of binding buffer was added to the column and centrifuged for 1 minute. The flow through was discarded and 700 µL of wash buffer was added to column and centrifuged for 1 minute. The flow through was discarded and the empty column was centrifuged for an additional 1 minute to remove residual wash buffer. The column was transferred to a clean 1.5 mL microcentrifuge tube and 50 µL of elution buffer was added to the center of the purification column membrane and centrifuged for 1 minute. The purified DNA was stored at -20˚C.

2.8.4 Preparation of sgRNA oligo inserts

To phosphorylate and anneal the top and bottom strands of oligos for each sgRNA design, the top and bottom strands of oligos were first resuspended to a final concentration of 100 µM. The mixture for phosphorylating and annealing the sgRNA oligos can be found in table 2.14. The reaction mixture was run in a thermocycler for 30 minutes at 37˚C; 5 minutes at 95˚C; ramped down to 25˚C at 5˚C/minute. The phosphorylated and annealed oligos were diluted 1:200 with dH_2O.

2.8.5 Cloning the sgRNA oligos into px458 backbone

A ligation reaction for each sgRNA was set up as described in table 2.15. The addition of Quick ligase was last to the reaction mix. A no-insert, px458-

only negative control for ligation was also set up. The ligation reactions were left at room temperature for 10 minutes

Table 2.13 BbsI single restriction digestion

Px458	1 µg
FastDigest BbsI (Fermentas)	1 µL
FastAP (Fermentas)	1 µL
10x FastDigest Buffer	2 µL
ddH2O	X µL
Total volume	20 µL

Table 2.14 Preparation of sgRNA oligo inserts

sgRNA oligo top (100 µM)	1 µL
sgRNA oligo bottom (100 µM)	1 µL
10X T4 ligation buffer (NEB)	1 µL
T4 PNK (NEB)	0.5 µL
ddH2O	6.5 µL
Total volume	10 µL

Table 2.15 Ligation reaction

BbsI digested plasmid	50 ng
Oligo duplex (1:200)	1 µL
2x Quickligation Buffer (NEB)	5 µL
Quick ligase (NEB)	1 µL
ddH2O	X µL
Total volume	11 µL

2.8.6 Transformation of competent cells

A vial of One-Shot® TOP10 chemically competent *E.coli* (Invitrogen) was thawed on ice. The ligated plasmid and insert (3 µL) was added directly into the vial of competent cells and mixed by tapping gently. The vials were incubated on ice for 30 minutes. Next, the vial was incubated for exactly 30 seconds in a 42˚C water bath and then placed on ice for 2 minutes. 250 µL of pre-warmed SOC medium was added to each vial under sterile conditions. The vials were then shaken at 37˚C for one hour at 225 rpm in a shaking incubator. 100 µL from each transformation vial was spread onto a separate LB agar selective (Ampicillin) plate. The remaining transformation mix was stored at 4˚C. The LB agar plates were inverted and incubated overnight at 37˚C.

2.8.7 Colony selection and overnight bacterial culture

Once the LB-ampicillin agar plates had been incubated overnight, colonies were picked and inoculated with 5 mL of LB-ampicillin broth and left overnight with constant shaking at 37˚C

2.8.8 Plasmid purification

Following the overnight culture, 2 mL of the culture was centrifuge at 12,000 x g. The pellet, containing the plasmid, was purified using a GeneJET plasmid Miniprep kit (ThermoFisher Scientific) according to the manufacturer's instructions.

2.8.9 Sequence verification

To confirm successful insertion of sgRNA into the backbone, the plasmid DNA was sequenced from the UG promoter (upstream of the sgRNA insert) using the U6-Forward primer (5'-GACTATCATATGCTTACCGT-3'). Sequencing was performed by eurofins.

2.9 Statistical Analysis

Data are expressed as mean ± SEM, where mean represents number of mice (n≥3 per genotype) or number of independent experiments (n≥3). Normality of data was checked by plotting a histogram. An unpaired student's t-test was used to determine statistical differences between normally distributed datasets where p values of ≤ 0.05 were considered significant. To detect statistical differences between non-parametric data sets the Mann Whitney U two-tailed test was performed where p values of ≤ 0.05 were considered significant. All statistical analysis was performed using Graphpad prism.

Chapter 3:
The Role of Frizzled-7 in Gastric Cancer Growth

This chapter is based on:

Flanagan, D., Barker, N., Di Costanzo, N. S., Mason, E. A., Gurney, A., Meniel, V. S., Koushyar, S., **Austin, C. R**., Pearson, H. B., Boussioutas, A., Clevers, H., Phesse, t. j. & Vincan, E. 2019. Frizzled-7 is required for Wnt signaling in gastric tumors with and without Apc mutations. Cancer Research, canres.2095.2018

3.1 Introduction

Aberrant Wnt pathway activation has been linked to many different forms of cancer. Mutations to the tumour suppressor gene, APC, a key negative regulator of Wnt/ β-catenin signalling or mutations to other Wnt signalling components such as β-catenin are observed in between 40-60% of gastric tumours (Wang et al., 2014b)

Whilst canonical Wnt signalling is generally considered to drive the proliferation and neoplastic transformation of cells, non-canonical Wnt pathways have been shown to play pivotal roles in tumour progression such as angiogenesis and metastasis (Anastas and Moon, 2013). As such, Wnt5a, considered a classical non-canonical Wnt ligand, is overexpressed in numerous cancers, including GC (Nam et al., 2017). Targeted inhibition of Wnt5a significantly reduces tumour growth and invasion (Hanaki et al., 2012). Thus, deregulation of canonical and/or non-canonical Wnt signalling components leads to aberrant induction of Wnt signalling and gene transcription, which can rapidly transform cells to promote gastric tumorigenesis.

Not only are intracellular components of Wnt signalling implicated in GC but more recently it has been shown extracellular secreted Wnt antagonists (sFRP and Dkk) are frequently epigenetically silenced through promoter hyper-methylation (Cheng et al., 2007; Nojima et al., 2007; Wang et al., 2012b). Critically, exogenous re-introduction of Wnt pathway inhibitors, such as sFRP1/2/5 and Dkk1, or reversing promoter methylation can significantly limit and reduce in vitro tumourgenicity and tumour xenograft burden of Wnt-pathway activated GC models by means of attenuating Wnt/β-catenin signalling (Cheng et al., 2007; Nojima et al., 2007; Wang et al., 2013a). These studies demonstrate that activated Wnt signalling is sufficient to drive gastric tumorigenesis and provides proof-of-principal that modulation of extracellular/upstream signaling components can impact on Wnt pathway output, irrespective of intracellular/downstream mutations that would otherwise drive positive Wnt signalling. Moreover, the fact that sFRPs and Dkks

act by inhibiting Wnt/FZD signalling implies an intrinsic role for Wnt/FZD. Indeed, FZD receptors, specifically FZD7, are commonly upregulated in GC leading to abnormal Wnt pathway activation (Zhao et al., 2014). FZD7 is particularly unique as it is one of the few FZD receptors that transmit both canonical and non-canonical Wnt signals (Figure 3.1), which have both been shown to play critical roles during tumorigenesis and tumour progression (Asad et al., 2014; Ueno et al., 2009; Vincan et al., 2007b).

FZD7 has attracted particular focus due to its upregulation in several different cancers, with inhibition of FZD7 successfully blocking growth in colorectal cancer, breast cancer and hepatocellular carcinoma (Phesse et al., 2016). Furthermore, FZD7 has recently been shown to be the predominant FZD receptor transmitting Wnt signalling to regulate stem cell function in the gastric (Flanagan et al., 2017a) and intestinal epithelium (Flanagan et al., 2015b). Lgr5 was first identified as a Wnt target gene and a marker of highly proliferative stem cells located at the base of intestinal crypts (Barker et al., 2007). It has since been confirmed as a stem cell marker in the antral (Barker et al., 2010b) and corpal (Leushacke, 2017) stomach and is able to generate all cell lineages of the gastric epithelium. In addition, these cells demonstrate a high dependency and sensitively to Wnt/β-catenin signalling for this maintenance. However, non-Lgr5 expressing stem cell populations have also been implicated in the homeostasis of the gastric epithelium (Arnold et al., 2011; Powell et al., 2012; Stange et al., 2013). *In vivo* lineage tracing from cells located within the isthmus that express either Sox2 or Lrig1 are also self-renewing and multipotent. These data suggest the co-existence of multiple stem cell populations within the epithelium that work in concert to maintain the integrity of the gastric epithelium. It has been demonstrated that FZD7 is the predominant Wnt receptor in regulating homeostasis in the intestinal epithelium, in which deletion of FZD7 in either the whole epithelium or specifically in the Lgr5+ intestinal stem cells, triggers rapid repopulation (Flanagan et al., 2015b). Similarly, the deletion of *FZD7* throughout the antrum

of the gastric epithelium also triggered repopulation, indicating that FZD7 regulates a population of stem cells in the antrum (Flanagan et al., 2017a). Therefore, it was assumed that FZD7 would regulate Lgr5+ cells in the gastric epithelium much like in the intestine. However it has recently been shown for the first time that deletion of FZD7 did not inhibit the capacity of Lgr5+ cells to lineage trace full gastric glands (Flanagan et al., 2019b). This demonstrates that the function of Lgr5+ cells in the gastric epithelium do not require FZD7 *in vivo,* illustrating a substantial difference for FZD7 in regulating Lgr5+ stem cells in the stomach compared to the intestine.

Despite compelling evidence implicating FZD7 in GC, its functional role in GC has not been elucidated and its therapeutic potential remains unknown. This project seeks to reveal the therapeutic benefit of inhibiting FZD7 in GC both *in vitro* and *in vivo*. As FZD7 belongs to a family of closely related genes it is not possible at present to pharmacologically target FZD7 in isolation, without also inhibiting other closely related FZD proteins. However, our industrial collaborators Oncomed Pharmaceuticals developed an antibody to block a subset of FZD receptors (FZD-1, 2, 5, 7 & 8) (Gurney et al., 2012), which will be used to support genetic experiments and determine the therapeutic benefit of targeting FZD7 in GC.

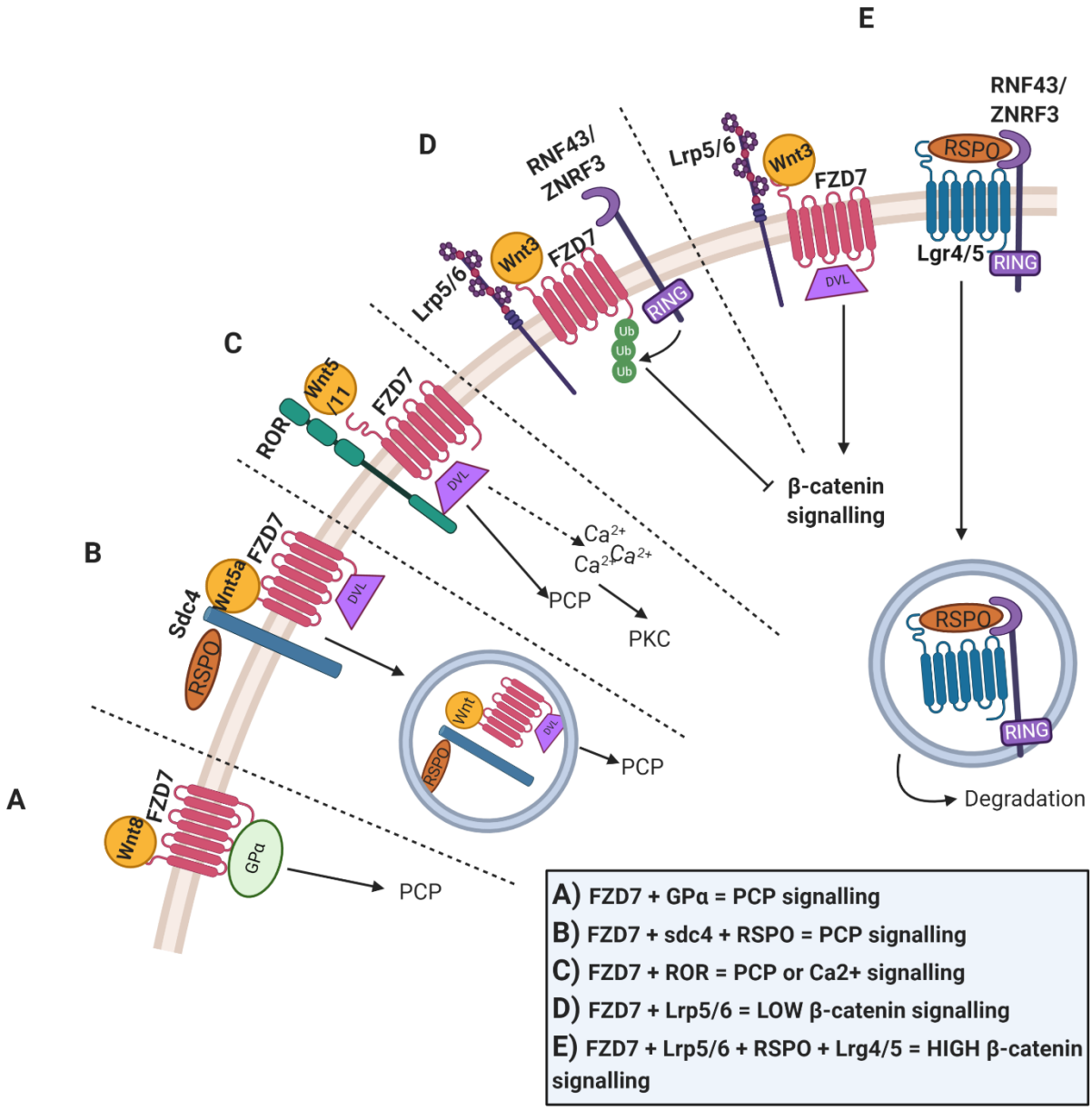


Figure 3.1 FZD7 receptor complexes and signalling output. (**A**) FZD7 can associate with G protein α (GPα) to transmit signals from Wnt8 via the PCP pathway; (**B**) FZD7 can associate with Syndican4 (Sdc4) and R-Spo to transmit Wnt5a signals via internalization of the whole receptor complex and ligand which then activates PCP signalling; (**C**) FZD7 can associate with Ror2 to transmit non-canonical Wnt signals from ligands including Wnt5 or Wnt11, either via PCP or Ca^{2+}; (**D**) FZD7 can associate with Lrp5/6 to transmit canonical Wnt signalling from ligands including Wnt3. However, RNF43/ZNFR3 ubiquitylates FZD7 and targets it for internalization and proteasomal degradation, thus resulting in turn-over of the receptor complex and low canonical Wnt signalling; (**E**) In the presence of Lrg4/5, and R-Spo, it is RNF43/ZNFR3 which is internalized and degraded and this FZD7/Lrp5/6 remains on the cell surface to transmit Wnt signalling and canonical signalling output is high.

3.2 Results

3.2.1 Gastric cancer cells require cell intrinsic Wnt signalling for growth

FZD receptors are known to be overexpressed in many cancers, including GC. Like many malignancies, GC is genetically heterogeneous, which complicates identifying non-redundant signalling pathways suitable for targeted therapy. To investigate the expression of FZD receptors which transmit oncogenic Wnt signals, we performed qRT-PCR for all 10 mammalian FZD genes on two human GC cell lines: MKN28 (intestinal-type) and MKN45 (diffuse-type). Several FZD receptors were abundantly expressed, including FZD7 (Figure 3.2). FZD2 was also highly expressed in both cell lines but not as highly as FZD7. FZD6 displayed high expression relative to the house-keeping gene. This suggests FZD receptors might be attractive therapeutic targets for GC.

The pan-FZD monoclonal antibody OMP-18R5 (Vanticumab) binds to 5 of the 10 FZD receptors: FZD1, FZD2, FZD5, FZD7, and FZD8 (Gurney et al., 2012). This directly blocks the ability of Wnt to interact with FZD, thus, preventing signal activation and pathway transduction. OMP-18R5 has shown efficacy in several solid cancer types, however, its therapeutic potential in GC has not been explored. To assess the ability of OMP-18R5 to inhibit the growth of GC cells we first performed colony forming assays in soft agar for 2 weeks. MKN28 and MKN45 GC cells treated with OMP-18R5 formed significantly fewer anchorage-independent colonies compared to vehicle control treated cells (Figure 3.3). It should be noted that MKN28 and MKN45 cells grown as a conventional 2D monolayer do not show growth inhibition following OMP-18R5 treatment (Figure 3.4). This suggests that OMP-18R5 does not inhibit the general viability of GC cells, but rather the more specific cancer stem cell characteristics of anchorage independent colony forming.

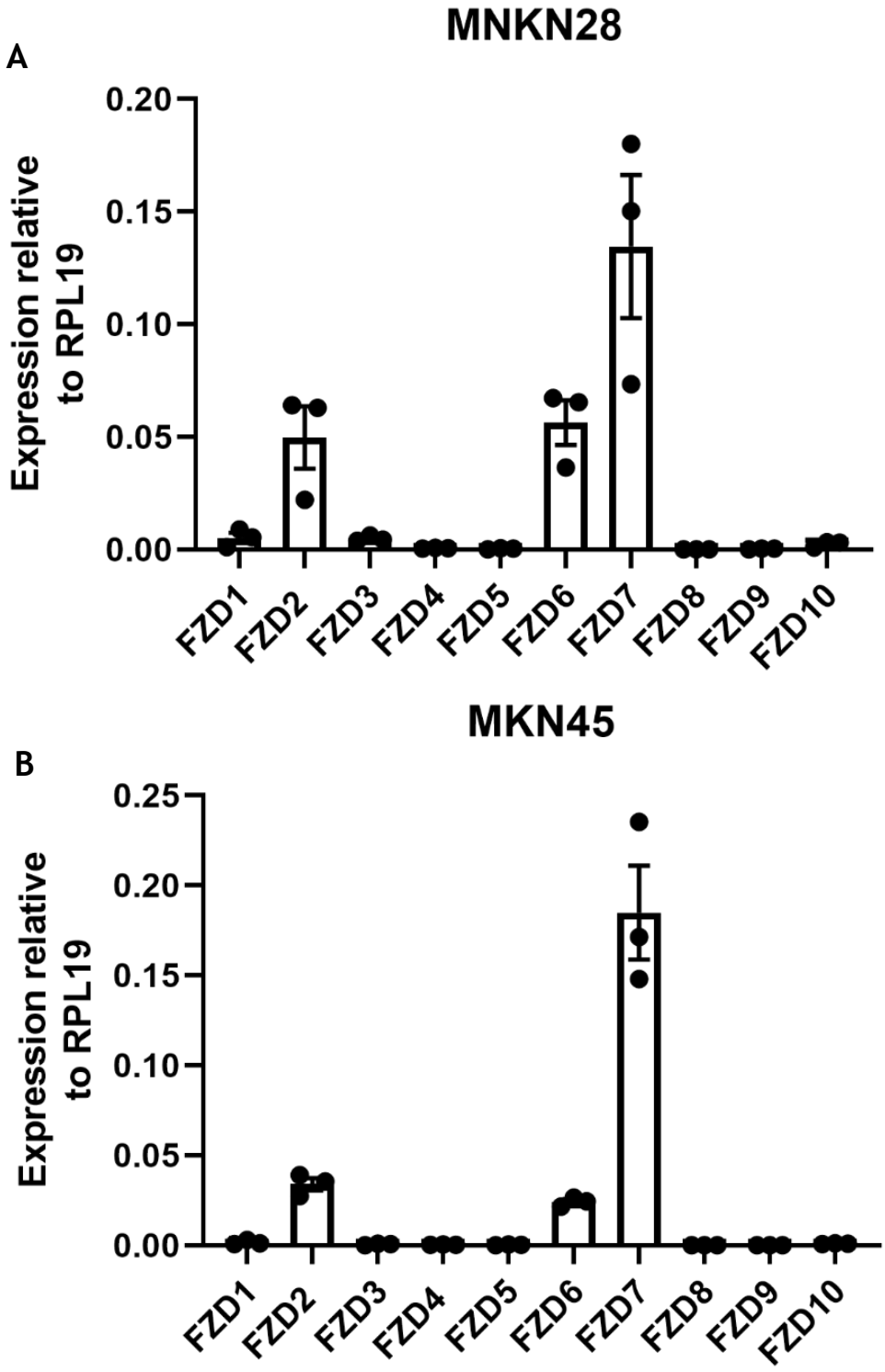


Figure 3.2 FZD7 abundantly expressed in GC cell lines. Relative expression of FZD receptors in **(A)** MKN28 and **(B)** MKN45 GC cells quantified by qRT-PCR. Expression shown relative to housekeeper (RPL19). Data represented as mean ± SEM, n=3.

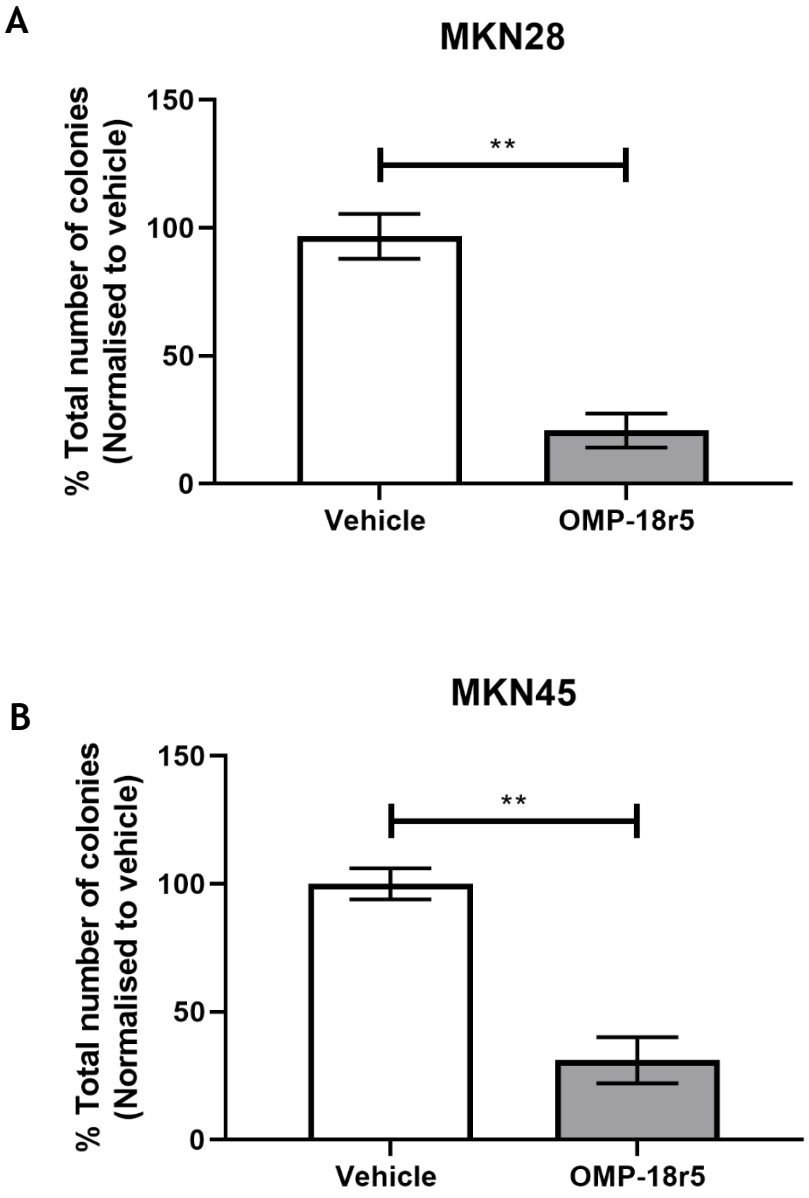


Figure 3.3 FZD inhibition reduces GC initiation. Quantification of cell colonies (>50 cells) from (**A**) MKN28 and (**B**) MKN45 GC cells grown in soft agar for 2 weeks treated with 80 μg/mL OMP-18R5 or vehicle control. Treatments were replaced every 4 days for the duration of 2 weeks. OMP-18R5 showed a reduction in number of colonies compared to control. The absolute number of colonies was normalized to the vehicle control. (**= $p \leq 0.01$, mean ± SEM, n=3, t-test).

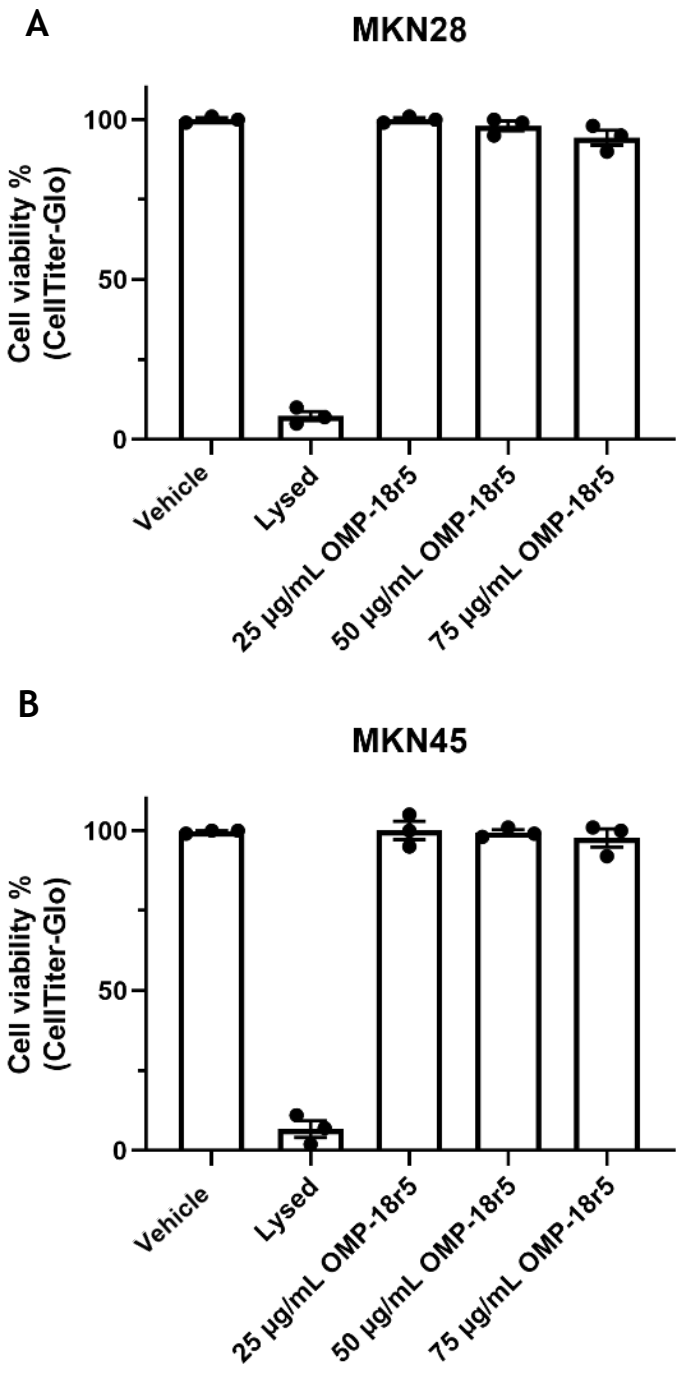


Figure 3.4 OMP-18R5 does not show efficacy in 2D setting. (**A**) MKN28 and (**B**) MKN45 GC cells were grown as 2D monolayer cultures in 96-well cell culture plates and treated with increasing concentrations of OMP-18R5, vehicle control or DMSO (to induce lysing of cells). Cell growth was assessed by Celtiter-Glo assay and cell viability percentage was calculated relative to DMSO treated cells. (mean ± SEM, n=3).

This suggests that cell intrinsic Wnt ligands are required for 3D-growth of GC cells. This was confirmed by treatment of GC cells with LGK-974 which prevents Wnt secretion through inhibition of the palmitoylation of the Wnt ligand by PORCN (Liu et al., 2013). MKN28 and MKN45 GC cells treated with LGK-974 formed significantly fewer anchorage-independent colonies compared to vehicle control treated cells (Figure 3.5). These data demonstrate cell intrinsic secretion of Wnt ligands and FZD receptor availability are required for the sustained growth and cancer stem cell properties of GC cells.

To confirm OMP-18R5 and LGK-974 treatment had reduced Wnt signalling associated with the reduced growth observed I had planned to perform TOPflash assays. However, due to restricted lab time due to the COVID19 pandemic these could not be completed.

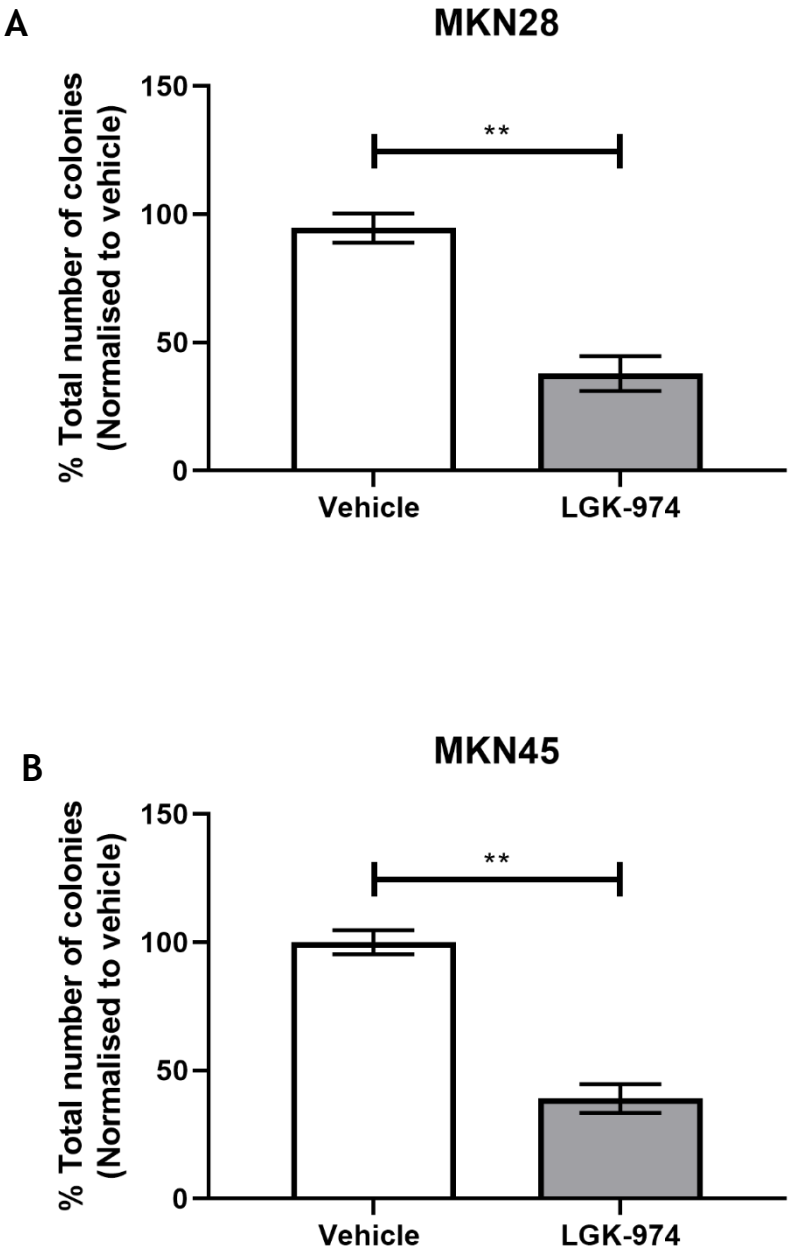


Figure 3.5 Inhibition of Wnt secretion reduces GC Initiation. Quantification of cell colonies (>50 cells) from (**A**) MKN28 and (**B**) MKN45 GC cells grown in soft agar for 2 weeks treated with 5 μM LGK-974 or vehicle control. Treatments were replaced every 4 days for the duration of 2 weeks. LGK-974-treated cells showed a reduction in number of colonies compared to control The absolute number of colonies was normalized to the vehicle control. (**= $p \leq 0.01$, mean ± SEM, n=3, t-test).

3.2.2 Pan-FZD inhibition reduces gastric cancer tumour burden *in vivo*

To determine if FZD inhibition could also reduce the growth of GC cells *in vivo* $10x10^6$ MKN45 or MKN28 GC cells were injected subcutaneously into the hind flanks of 6 week-old nude mice and allowed to develop into palpable gastric tumours. Growth of the tumours were measured twice weekly with calipers. To inhibit Wnt signalling, the mice were treated with 5 mg/kg OMP-18R5, twice a week for the duration of the experiment. This dose has been previously used successfully in publications by Oncomed (Gurney et al., 2012) . Gastric tumours were significantly smaller in OMP-18R5 treated mice compared to vehicle control treated mice in both the MKN45 and MKN28 cohorts (Figure 3.6). MKN45 tumours grew faster, reaching the tumour size limit by 19 days compared to 34 days for the MKN28 gastric tumours (Figure 3.6). The MKN45 group grew consistently in size whereas the MKN28 group grew slowly for the first 27 days before suddenly and significantly increasing in size. As previously reported (Gurney et al., 2012), no toxicity was observed in OMP-18R5-treated mice, which displayed consistent bodyweight and no signs of morbidity or the duration of the treatment (Figure 3.7). These data demonstrate that FZD inhibition is sufficient to block the initiation and progression of human GC cells.

Characterization of the GC xenografts showed a reduction in β-catenin staining (surrogate marker of active Wnt signalling) in OMP-18R5 treated mice compared to vehicle-treated control in both MKN45 and MKN28 GC cell lines (Figure 3.8). qRT-PCR analysis showed a significant decrease in the expression of Wnt target genes in the OMP-18R5 treated tumours in both MKN45 and MKN28 GC cells (Figure 3.9). This demonstrates that treatment with OMP-18R5 is able to inhibit Wnt signalling in gastric tumours *in vivo*. We performed qRT-PCR for all 10 mammalian FZD genes which demonstrated that no other FZD receptor was compensating for inhibiting FZD1, FZD2, FZD5, FZD7, and FZD8 with the OMP-18R5 antibody (Figure 3.10). The significant reduction in expression of FZD7 is due to its role as a Wnt target gene rather than a direct inhibition by OMP18R5 since this is a monoclonal antibody which targets FZD antigens rather

than gene transcription. These data show that OMP-18R5 is able to inhibit Wnt signalling in GC xenografts and strongly suggests that FZD receptors are rate limiting for growth of gastric tumours *in vivo*.

Tumours would have been further characterized by staining for PCNA and cleaved-caspase3 if not for restricted lab time due to the COVID19 pandemic.

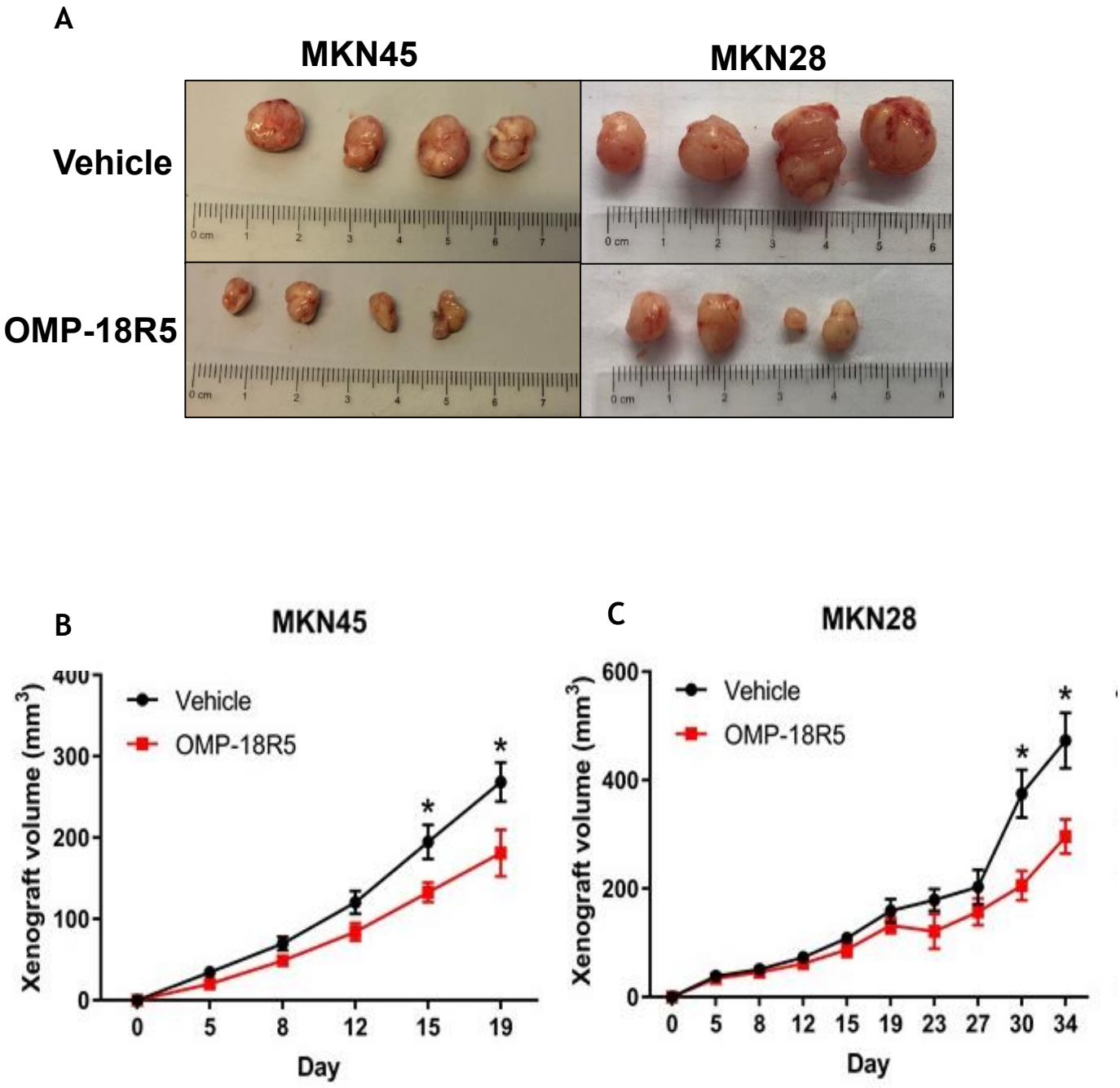


Figure 3.6 Pan-FZD inhibition reduces GC growth *in vivo*. (**A**) Representative images of GC cell xenografts treated with vehicle or OMP-18R5. GC xenograft growth overtime in (**B**) MKN45 and (**C**) MKN28. Tumour growth in both cell lines was reduced in the OMP-18R5 treated cohort, (*= $p \leq 0.05$, mean ± SEM, t-test, n=6, mice per cohort).

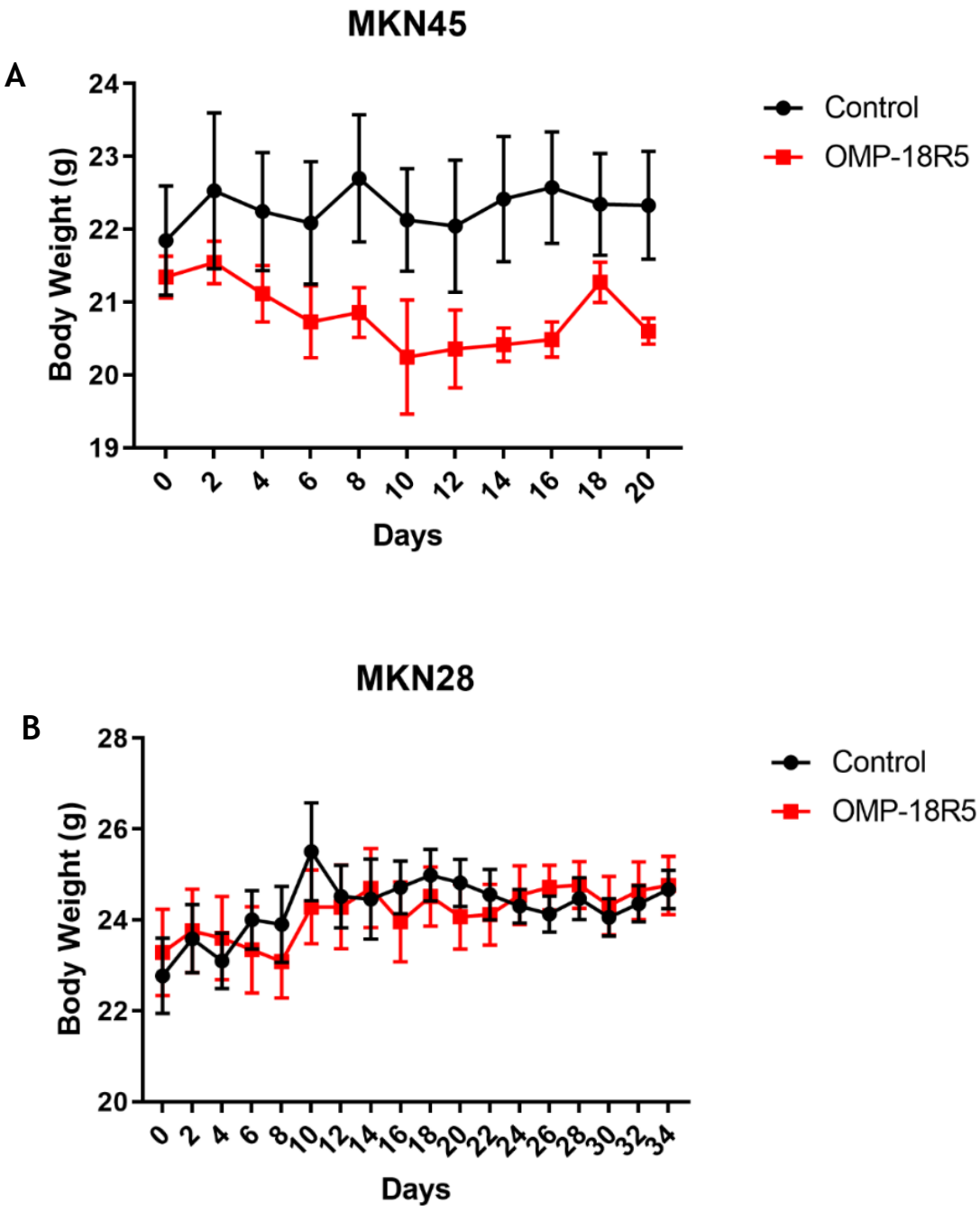


Figure 3.7 OMP-18R5 did not lead to increased morbidity. Body weight (grams) over time of mice treated with 5 mg/kg OMP-18R5 or vehicle control in (**A**) MKN45 and (**B**) MKN28. (mean ± SEM, t-test, n=6, mice per cohort).

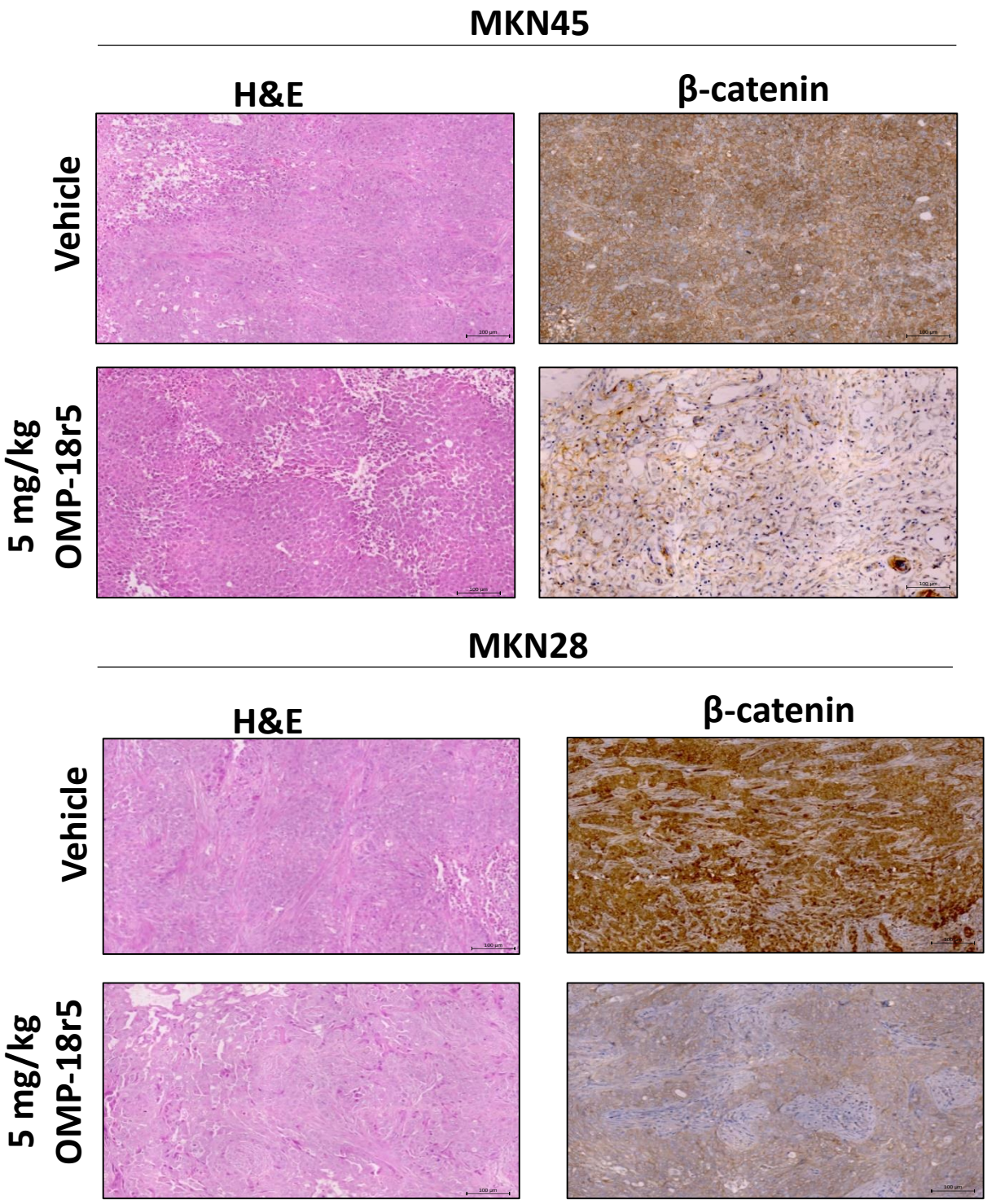


Figure 3.8 Pan-FZD inhibition reduced B-catenin expression in xenografts. Hematoxylin and eosin (H&E) staining and ß-catenin immunostained (IHC) sections from OMP-185R-treated and vehicle control-treated GC xenografts. OMP-185R treated xenografts showed a reduction in ß-catenin staining compared to control. (Scale bars = 100 μM).

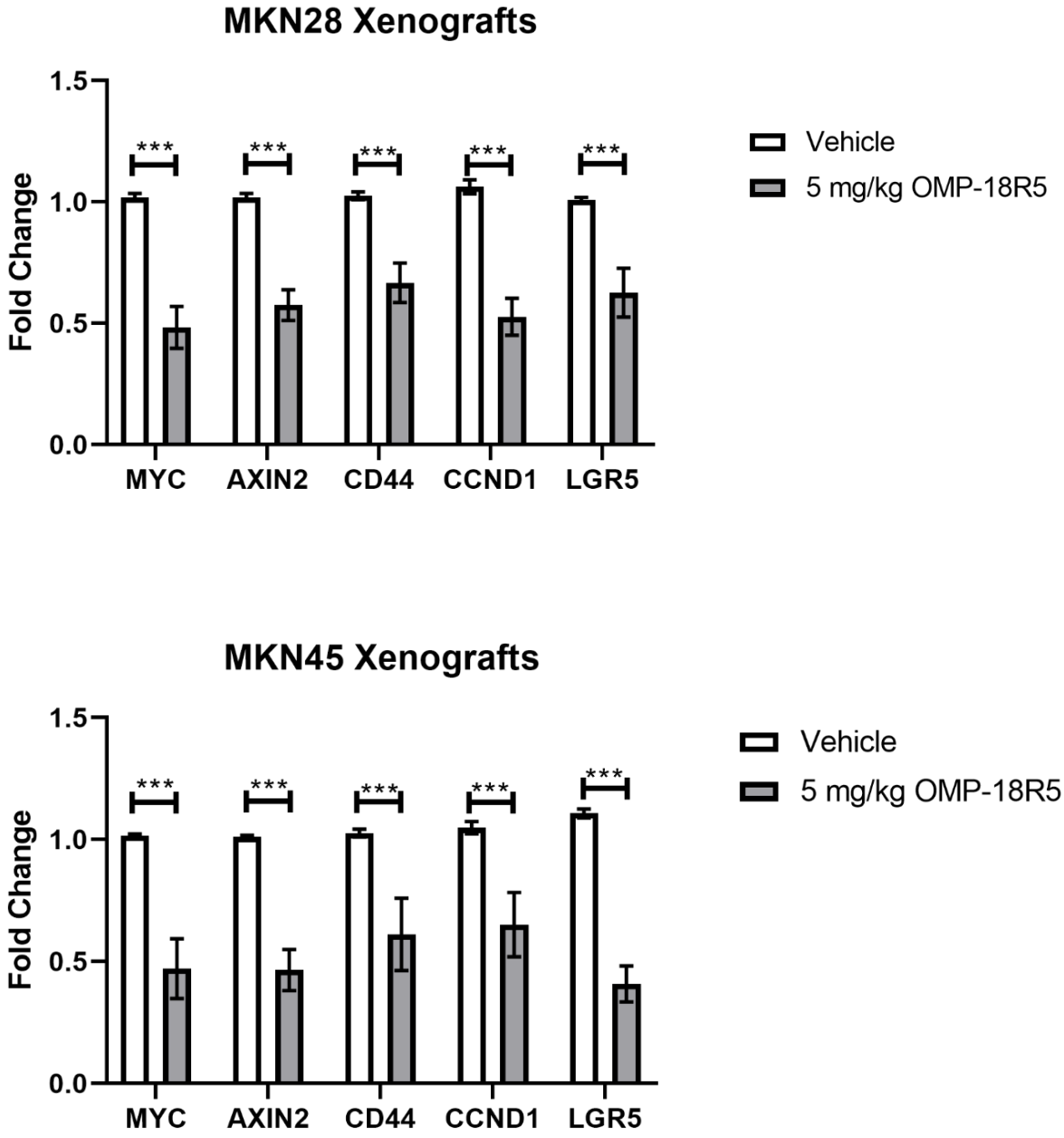


Figure 3.9 Pan-FZD inhibition reduced Wnt signalling in GC cells *in vivo*. qRT-PCR for Wnt target genes on GC xenografts from OMP-18R5 treated or vehicle control treated mice. All Wnt target genes had significantly decreased expression in the OMP-18R5 treated cohort compared to control. Normalized to RPL19 (***= $p \leq 0.001$, mean ± SEM, t-test, n=6 mice)

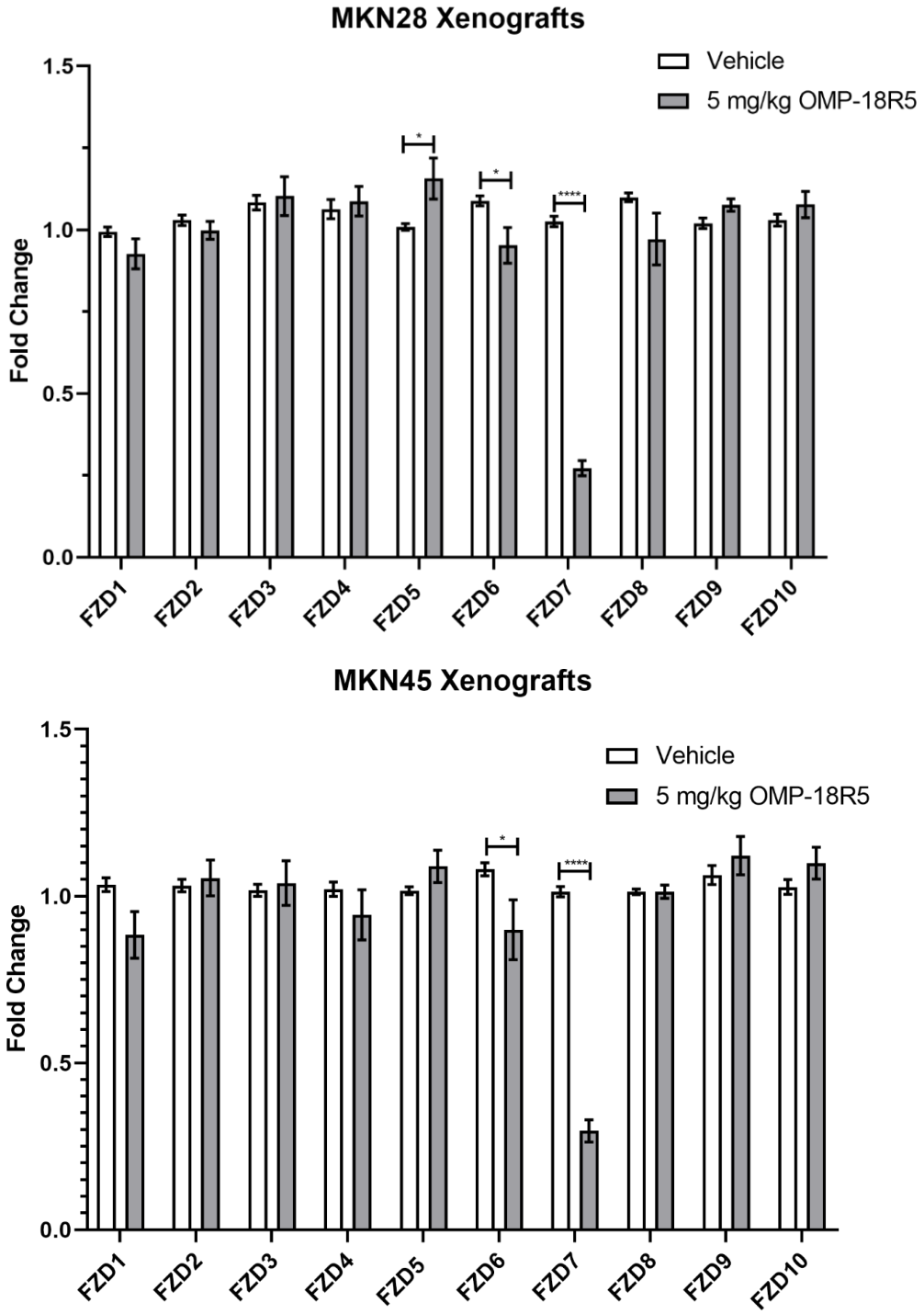


Figure 3.10 FZD receptors do not compensate when a subset of receptors are inhibited. qRT-PCR for FZD receptor genes on GC xenografts from OMP-18R5 treated or vehicle control treated mice. No FZD receptor was significantly increased in expression due to the inhibition of 5 out 10 mammalian FZD receptors. FZD7 has reduced expression due to its role as a Wnt target gene. Normalized to RPL19 (*= $p \leq 0.05$, ****= $p \leq 0.0001$, mean ± SEM, t-test, n=6 mice)

3.2.3 Targeted FZD7 knockdown reduced gastric cancer colony formation

Inhibition of GC cell growth following treatment with OMP-18R5 suggests that one of the five FZD receptors targeted by OMP-18R5 (FZD1, 2, 5, 7, and 8) is responsible for transmitting Wnt signals in GC cells. Gene expression analysis narrows this down to FZD2 and/or FZD7, as FZD1, FZD5, and FZD8 are undetectable in these cell lines (Figure 3.2). It has previously been shown that FZD2 is unable to compensate for the loss of FZD7 in the intestinal epithelium (Flanagan et al., 2015b), this may indicate a predominant role for FZD7 in Wnt signal transduction in gastric tissue. Indeed, FZD7 is upregulated in GC and is associated with poor clinical outcomes (Phesse et al., 2016).

To determine the specific requirement of FZD7 for the growth of human GC cells we performed colony forming assays on GC cells transfected with shFZD7 (Vincan et al., 2007a). To first confirm the FZD7-targted shRNA (shFZD7) was specific to FZD7, it was transfected into GC cell lines, MKN45 and MKN28, and the expression of Wnt target genes and FZD receptors analyzed by qRT-PCR. MKN45 and MKN28 cells displayed a significant reduction in expression of FZD7, with relative mRNA expression fold change of ≤ 0.5. Importantly, the gene expression of FZD2 was not reduced following FZD7-knockdown in either cell line (Figure 3.11). This confirms that despite similar homology and expression in GC tissue, our shFZD7 is specific to FZD7 and FZD2 is not compensating for its loss. Expression of Wnt target genes were significantly reduced in both MKN45 and MKN28 following FZD7-knockdown compared to control (Figure 3.11). This demonstrates that targeted knockdown of FZD7 in GC cells leads to a reduction in Wnt signalling and therefore has potential to be the predominant FZD receptor transmitting oncogenic Wnt signals in GC.

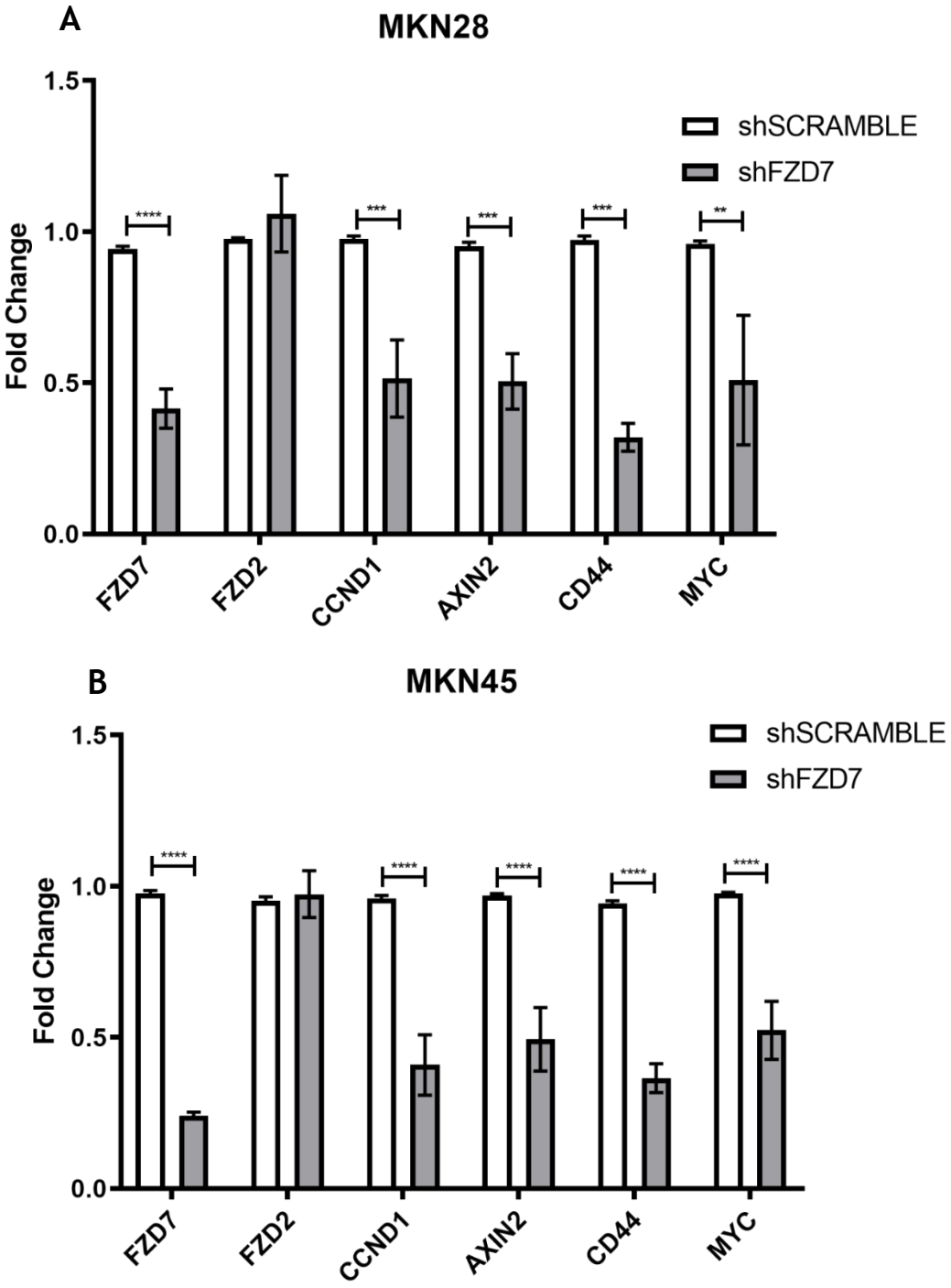


Figure 3.11 FZD7-knockdown reduces Wnt target genes in GC cell lines. qRT-PCR for Wnt target genes in GC cell lines (**A**) MKN28 and (**B**) MKN45 following FZD7-knockdown. Both cell lines showed a significant decrease in expression of Wnt target genes, including FZD7, in the shFZD7 cells compared to shSCRAMBLED control. FZD2 did not show a reduction in expression following FZD7-knockdown (**= $p \leq 0.01$, ***= $p \leq 0.001$, ****= $p \leq 0.0001$, mean ± SEM, t-test, n=3)

To investigate the role of FZD7 in the growth of GC cells, cells were transfected with shFZD7 and grown as anchorage-independent colonies in soft agar. MKN45 and MKN28 GC cells transfected with shFZD7 had a marked decrease in colony growth, compared to those transfected with scrambled shRNA (shSCRAMBLED) control (Figure 3.12). Importantly, growth inhibition following FZD7-knockdown was rescued by co-transfection with a full-length FZD7 expression construct (Figure 3.12). This observation was confirmed by qRT-PCR analysis of FZD7 expression in the cells (Figure 3.13). MKN28 and MKN45 cells with FZD7 knocked down had a decrease in expression of FZD compared to shSCRAMBLED control. FZD7 expression returned to similar levels as the control when co-transfected with shZFD7 and the FZD7 overexpression construct. This demonstrates the specificity of shRNA and that FZD7 regulates growth in human GC cells.

The reduction in colony forming ability in MKN45 and MKN28 cells transfected with shFZD7 was associated with decreased Wnt signalling (Figure 3.13). qRT-PCR analysis demonstrated that MKN28 and MKN45 transfected with shFZD7 had significantly decreased expression of Wnt target genes compared to shSCRAMBLED control with levels returning to control levels when co-transfected with shFZD and the FZD7 overexpression construct. These data suggest that FZD7 is the predominant Wnt receptor transmitting oncogenic Wnt signalling to regulate growth and cancer stem cell qulaities in GC cells and that targeting FZD7 may be an attractive therapeutic target to prevent initiation/growth of gastric tumours.

TOPflash assays would have been used to confirm FZD7-knockdown leads to a reduction in Wnt signalling. However, due to restricted lab time due to the COVID19 lockdown these could not be completed.

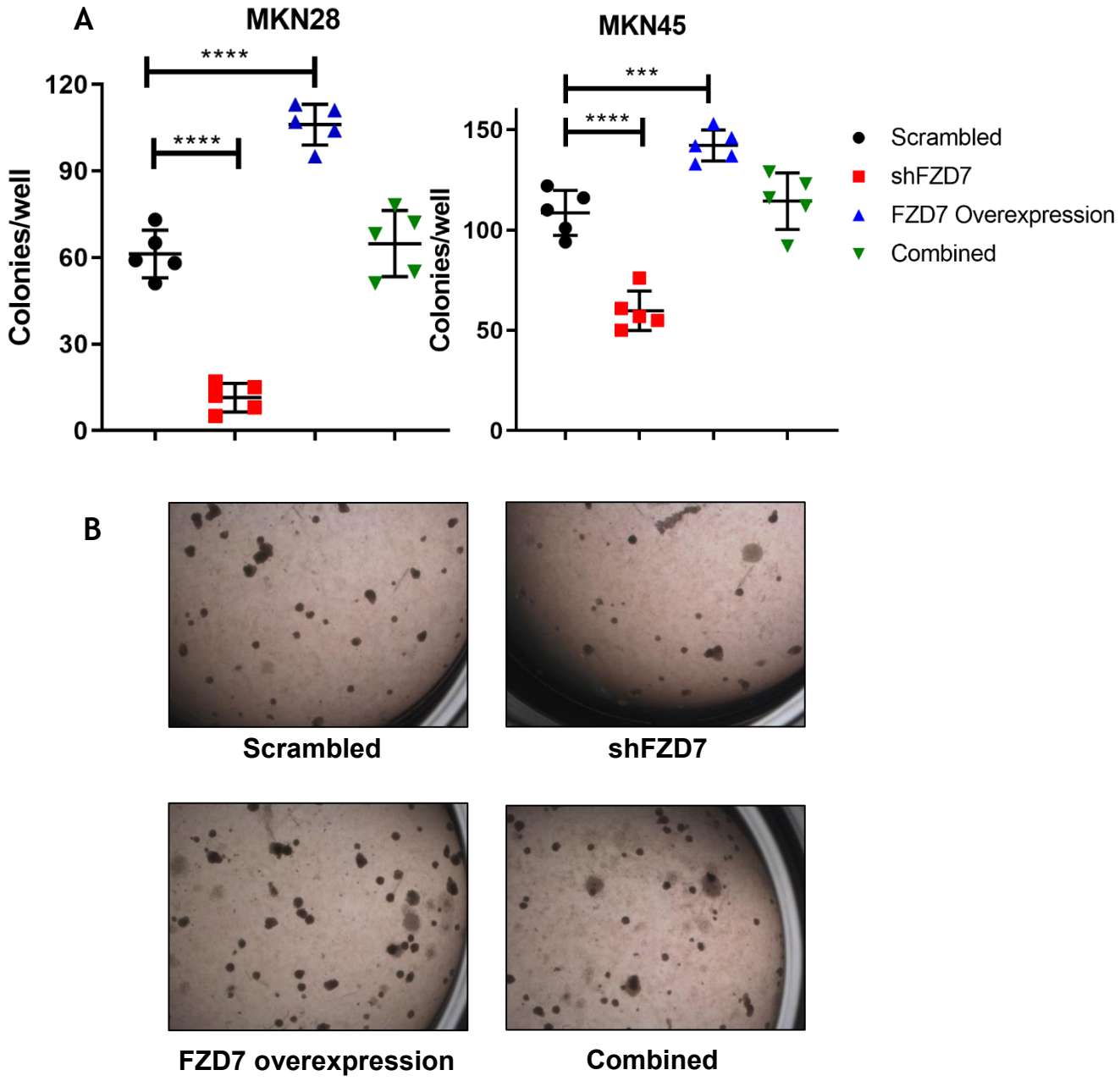


Figure 3.12 FZD7-knockdown reduces GC cell growth. (**A**) Quantification of cell colonies (≥50 cells) from MKN45 or MKN28 GC cells grown in agar for 2 weeks following transfection with shFZD7 alone, FZD7-overexpression construct, shSCRAMBLED control or combination of shFZD7 and overexpression construct. Both cell liens displayed a reduction in growth in cells transfected with FZD7 alone compared to control. This reduction in growth was rescued by co-transfection with FZD7-overexpression construct, returning the number of colonies per well to levels similar to control cells. (**B**) Representative images of MKN28 grown as colonies. (***=p≤0.001, ****= p ≤0.0001, mean ± SEM, t-test, individual experiments repeated 3 times).

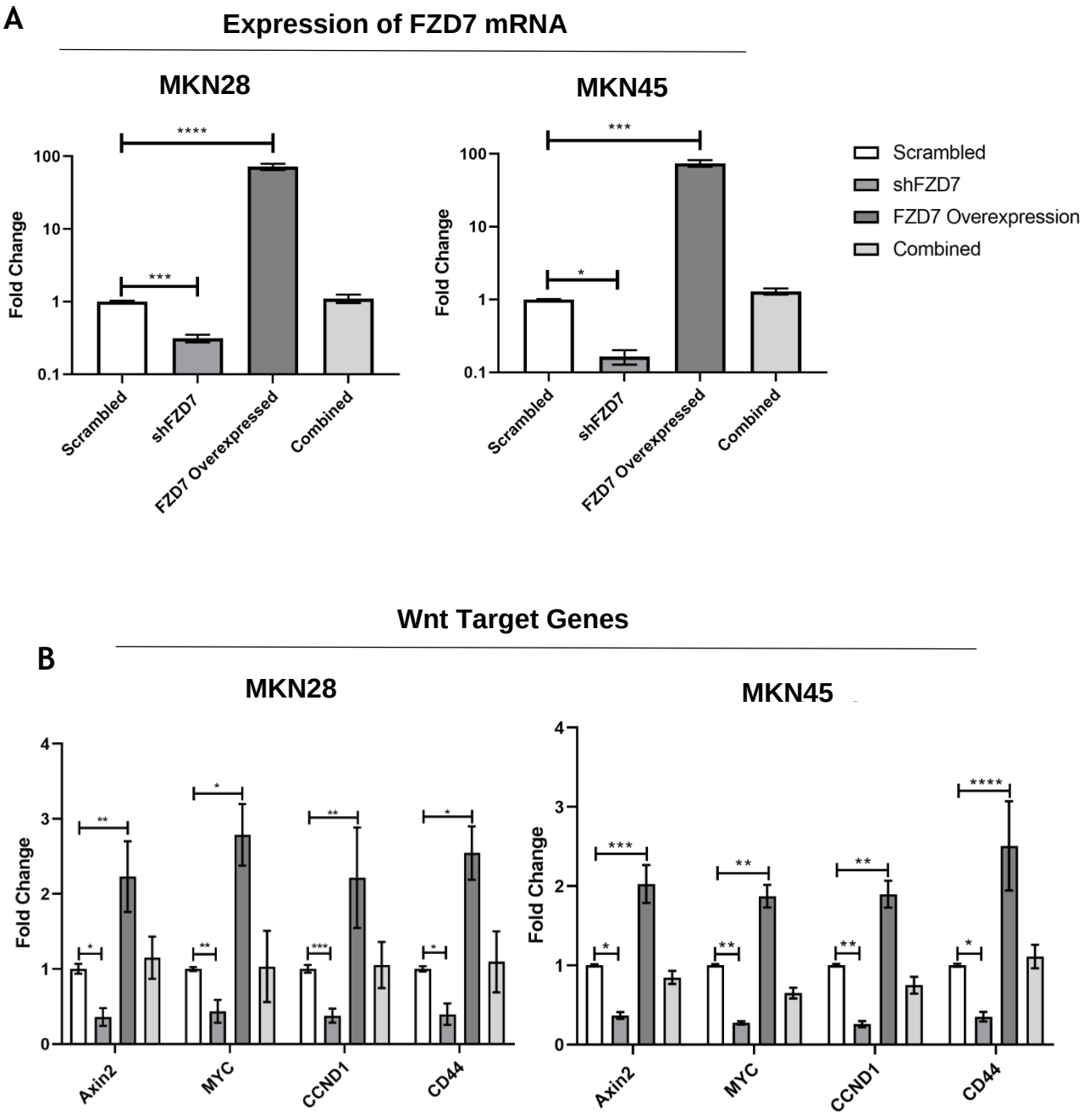


Figure 3.13 FZD7-knockdown reduces Wnt signalling. qRT-PCR analysis of cells described in figure 2.12. (**A**) Analysis of FZD7 expression in both cell lines confirm the phenotype observed in colony forming assays. (**B**) qRT-PCR analysis of Wnt target genes shows a decrease in all Wnt target genes in both GC cell lines in the shFZD7 group compared to control. Expression levels are returned to control levels upon co-expression with FZD7-overexpression construct. (*=p ≤0.05, **= p ≤0.01, ***= p≤0.001, ****= p ≤0.0001, mean ± SEM, t-test, n=3

3.3 Discussion

Aberrant regulation of Wnt signalling is a frequent theme in cancer biology. Given the wide array of cellular processes that are regulated by Wnt signalling during development and continued through to adulthood, it is unsurprising that some of the same cell processes that become compromised during carcinogenesis are due to deregulated Wnt signalling (Polakis, 2012). Our best understanding of the involvement of Wnt signalling in cancer comes from investigations into its well-known role in the initiation and progression of colorectal cancer (CRC) (Kinzler and Vogelstein, 1996; Sansom et al., 2004). Nearly all of CRCs, sporadic and familial, harbour mutations to components of the Wnt pathway, leading to pathway activation (Kinzler et al., 1991). However, unlike CRC, mutations to Wnt signalling pathway components in GC are modest in comparison (Clements et al., 2002; Koushyar et al., 2020). In fact, compared to CRC, mutations driving GC are still somewhat unclear. Efforts in genome sequencing of patient biopsies have provided insight into high risk pre-disposing environmental and genetic factors (Cristescu et al., 2015; Wang et al., 2014b), but these genomic studies are yet to be followed up with thorough functional investigations. Since it was demonstrated that Wnt activity could be regulated in CRC and GC, irrespective of downstream pathway-activating mutations (Caldwell et al., 2004; Cheng et al., 2007; Suzuki et al., 2004; Vincan et al., 2007a; Vincan et al., 2005; Zhao et al., 2014) the over-expression of certain upstream signaling components in GC has received increased research attention. This provides a novel avenue to investigate the therapeutic potential to treat deregulated Wnt signalling in GC via manipulation of the accessible cell surface FZD receptors.

This chapter has shown that FZD receptors are rate-limiting for the growth of GC *in vitro* and most importantly *in vivo*. Furthermore, it has elucidated, through use of targeted FZD7 knockdown, that FZD7 is likely the predominant Wnt receptor transmitting cell-intrinsic Wnt signals in human GC cells. Limiting the availability of FZD7 can reduce the activity of Wnt signalling, to levels that

cause a reduction in cell proliferation, a property utilized and required by developing tumours (Hanahan and Weinberg, 2011). Furthermore, the colony forming assays utilized in this chapter investigate the capacity of disaggregated single cells for form colonies and is therefore an assay for cancer stem cell (CSC) properties. Therefore, our results that show a decrease in colony forming ability in cells following FZD7 inhibition suggest that FZD7 is required in this process and that FZD7 is likely regulating gastric CSCs and thus potentially tumourigenesis.

This hypothesis is further supported by additional recently published evidence generated alongside this project. *Fzd7* was conditionally deleted in the gastric adenomas of Cre^{+} ;$gp130^{F/F}$;$Fzd7^{fl/fl}$ mice (Flanagan et al., 2019a), which allows robust recombination in these adenomas (Thiem et al., 2016). Tamoxifen induced mice developed significantly smaller and fewer gastric adenomas than Cre-negative mice. Furthermore, when *Fzd7* was co-deleted with *Apc* it was found sufficient to almost completely block the development of gastric tumours (Flanagan et al., 2019a). This supports our *in vitro* experiments demonstrating FZD7 inhibition is sufficient to block GC initiation. Taken together these data suggest that targeting FZD7 may lead to a potential therapeutic benefit in GC.

There are 10 mammalian FZD family members, which are classed into groups based on shared sequence and structural homology; FZD1,2 and 7, FZD3 and 6, FZD5 and 8, and FZD4,9 and 10 (Yu et al., 2012a). Interestingly, FZD belonging to the same class are often expressed on the same cell, meaning cancer cells can also display class-specific FZD expression. Specific FZDs were originally considered to only participate in distinct arms of the Wnt pathway, however, it is now generally accepted that the nature of FZD signaling (Canonical or non-canonical) is largely determined by the spatial and temporal ligand-receptor expression profile (Figure 3.1).

Within gastrointestinal cancers that show elevated Wnt activity, the FZD receptors commonly over-expressed are FZD1, 2 and 7 (King et al., 2012; Ueno et al., 2013). Therefore, it is unsurprising that FZD2 and FZD7 were abundantly

expressed in our human GC cell lines: not only are they commonly overexpressed in GIT cancers but share similar sequence and structural homology. It has been previously shown that FZD7 is commonly upregulated in hepatocellular, colon and gastric carcinomas and is associated with poor prognosis and survival (Merle et al., 2004; Schmuck et al., 2011; Vincan et al., 2007a). Additionally, FZD7 has been shown to be the predominant receptor regulating stem cell function in the gastric epithelium (Flanagan et al., 2017a). Furthermore, inhibition of FZD2 has been shown to reduce tumour growth and EMT phenotypes in GIT cancers (Gujral et al., 2014; Tomizawa et al., 2015).

Interestingly, FZD6 was also shown to be expressed in our GC cell lines. FZD6 has been shown to act as an inhibitor of the canonical pathways through activation of TAK1/NLK kinases, which reduce activation of ß-catenin target genes via phosphorylation of TCF/LEF transcription factors (Golan et al., 2004). Therefore, its abundant expression in our human GC cell lines may be in response to the increase in canonical signalling via upregulation of FZD7. Although FZD6 has been shown to regulate both canonical and non-canonical pathways, it has an emerging central role in the PCP signalling in cancer (Corda and Sala, 2017). Despite whether the PCP and non-canonical Wnt pathways are key players in oncogenic transformations still being a matter of discussion, there is evidence that GC cells hijack non-canonical signalling pathways to acquire the ability to metastasize through overexpression of Wnt5a (Kurayoshi et al., 2006). Wnt5a can bind to FZD6 forming a complex that drives migration and invasion in tumours where the WNT5A-FZD6 complex is overexpressed (Hirano et al., 2014; Kamino et al., 2011). However, this is contradicted by a recent paper where overexpression of FZD6 was able to suppress both proliferation and migration of GC (Yan et al., 2016). This clearly highlights the need for more functional experiments to decipher the roles of the other FZD receptors and identify which are viable as therapeutic targets in GC.

One of the most fundamental traits of a cancer cell is its ability to sustain proliferation. Under non-pathological conditions, mitogenic signals that control

proper cell-cycle entry and subsequent division are tightly regulated to ensure proper tissue homeostasis. However, cancer cells are able to deregulate these mitogenic and growth signals, which allow them to control their own fate. There are numerous ways in which cancer cells can achieve sustained proliferation such as producing growth factor ligands themselves, to which they can respond via the expression of cognate receptors, resulting in autocrine proliferative stimulation (Hanahan and Weinberg, 2011). Results presented in this chapter show that inhibiting FZD receptors led to a reduction in GC cell growth. Therefore, FZD receptors are essential in transmitting the oncogenic Wnt signalling that results in uncontrolled proliferative signalling. GC cells hijack this by overexpressing the receptors on their surface (Phesse et al., 2016). It should be noted that OMP-18R5 did not have efficacy on GC cells grown as a monolayer in a 2D environment. This highlights the importance of testing drug efficacy in conditions that better mimic tumour biology, such as organoids or mouse models and also suggests that Fzd7 regulates cancer stem cell activity and that the techniques used are not compromising the cell viability.

Our *in vitro* studies presented here and studies by others (Tomizawa et al., 2015) have shown that targeted inhibition of FZD is sufficient to block growth of GC cells. However, it is well documented that *in vitro* studies do not fully recapitulate the complex molecular and cellular interactions present in tumours (Hanahan and Weinberg, 2011). Our study demonstrated that functionally OMP-18R5 treatment triggered a reduced growth of human GC xenografts in mice. This supports previous work that demonstrates targeting multiple FZD receptors blocks the growth of several different cancers (Gurney et al., 2012). This can now be extended to GC. In other cancers OMP-18R5 has been used in combination with several standard-of-care chemotherapeutic agents, such as Taxol, Irinotecan and Gemcitabine, which showed strong synergy and significantly reduced cell proliferation and tumorigenicity (Gurney et al., 2012).Therefore, this antibody-based strategy of targeting FZDs may be beneficial to the treatment of a broad range of cancers, including GC. However,

the side effects of OMP-18R5 need to be further evaluated due its universality and non-specificity, therefore, a more specific drug target (I.E. a single FZD receptor) could lead to fewer side-effects. Consequently, it is important academically and clinically to elucidate the predominant FZD receptor transmitting oncogenic Wnt signalling in GC.

Of the 10 mammalian FZD receptors, FZD7 is one of the few FZDs that has a key role in relaying proliferative cues (via Wnt/ß-catenin signalling) to stem and progenitor cell populations, thereby contributing to tissue homeostasis (Flanagan et al., 2017a; Flanagan et al., 2015b). Targeted inhibition of FZD7 has been shown to be sufficient to significantly reduce cell differentiation in human embryonic stem and limb progenitor cells (Fernandez et al., 2014; Mei et al., 2014). As such, FZD7 is commonly overexpressed in a variety of cancers, including GC, where it can successfully transduce high levels of Wnt/ß-catenin signalling to cells and propagate cell proliferation to facilitate tumorigenesis. Targeted strategies to lower the availability of FZD7 have yielded robust decreases in tumour growth via decreasing cell proliferation (Asad et al., 2014; Merle et al., 2004; Simmons et al., 2014; Ueno et al., 2009; Vincan et al., 2007a). Results presented in this chapter support the findings of others as we too observe a significant reduction in GC cell growth following FZD7 knockdown along with a decrease in expression of Wnt target genes. Deletion of *Fzd7* in the normal gastric epithelium triggers repopulation (Flanagan et al., 2017a) which is a likely explanation for why FZD7-deficient GC cells and xenografts have reduced growth and why the reintroducing FZD7 into FZD7-deficient cells rescues the phenotype. Furthermore, our colony forming assays have demonstrated that FZD7 was required for gastric stem cell activity with additional support showing that deletion of Fzd7 prevents gastric tumourigenesis (Flanagan et al., 2019a). There is accumulating evidence of the close association between FZD7-regulated stem cells and cancer. For example, conditional deletion of FZD7 in adult intestinal epithelium, resulted in stem cell loss and organoid death. Furthermore, conditional deletion of FZD7 specifically

in the Lgr5+ intestinal stem cells in the crypts lead to impaired epithelial regeneration in transgenic mice (Flanagan et al., 2015a). Additionally, FZD7 is significantly elevated in human embryonic stem cells and has been used as a novel embryonic stem cell-specific surface antigen due to its involvement in embryonic stem cell self-renewal and pluripotent stat maintenance (Fernandez et al., 2014). It has been validated in breast cancer that FZD7-dependent enhancement of Wnt signalling promotes normal mammary stem cell activity (Chakrabarti et al., 2014). This implies that stem cells in normal and malignant tissues may share common molecular bases. This strengthens our findings that FZD7-mediated enhancement of Wnt signalling promotes gastric CSC and therefore enhances GC cells growth as FZD7 is known to regulate stem cell activity in normal gastric epithelium (Flanagan et al., 2017a) as well as being upregulated in human gastric tissue (Van Cutsem et al., 2016). These data support our findings of FZD7 regulating gastric CSCs and therefore playing a non-redundant role in transmitting oncogenic Wnt signalling in GC cells. Therefore, specifically targeting FZD7 for treatment of GC present a good therapeutic strategy. A recent study found a small molecular compound (SRI37892) that was able to target the transmembrane domain of FZD7 and block Wnt signal transmission. Treatment with SRI37892 inhibited LRP6 phosphorylation and downregulated the level of cytosolic free ß-catenin, and functionally repressed cell viability and colony formation in breast cancer (Zhang et al., 2017). This highlights the importance of understanding the interplay between the receptors, co-receptors and ligands at the cell surface, in order to develop more effective and specific drugs. This also confirms that targeting Wnt signalling at the level of the receptor is a good therapeutic strategy and supports our findings in GC.

dFz7-21, is a selective peptide of the FZD7 receptor subclass (FZD1, 2 and 7), it has been shown to inhibit Wnt signalling by binding to the FZD7 CRD subclass at a new site proximal to the lipid-binding groove. This alters the dimer interface and disrupts the formation of the Wnt-FZD-LRP complex which leads

to improper Wnt signalling (Nile et al., 2018). The anti-cancer effects of dFz7-21 have not been demonstrated; however, the peptide was able to block Lgr5+ stem cell function, giving it potential as a drug to block FZD7 in cancer.

The WNT-FZD-LRP signalosome is considered key to transmit canonical Wnt signalling in a cell (MacDonald and He, 2012). However, in the colon it has recently been found that Wnt ligands were redundant in this complex. It has been shown that LRP5 knockdown inhibits Wnt signalling in APC mutant CRC cell lines (Saito-Diaz et al., 2018), however, treatment with IWP-2 (Porcupine inhibitor) had no effect on Wnt signalling in the same cell lines. This suggests that Wnt is redundant in the Wnt-FZD-LRP signalosome and further research is required to determine which Wnt-driven cancers are sensitive to porcupine inhibitors and the molecular mechanisms driving oncogenic Wnt signalling at the level of the receptor. In contrast to the colon, our data showed that inhibiting Wnt secretion led to a reduction in GC cell growth, demonstrating that cell intrinsic secretion of Wnt ligands as well as FZD receptor availability are required for the sustained growth of GC cells. This highlights that despite their proximal locations and shared role in digestion, their molecular regulation is very different. It also strengthens the value of modulating Wnt signalling at the level of the receptor. The modulation of LRP5/6 needs to be investigated further in the context of GC.

Together our data suggests that FZD7 is the predominant receptor transmitting oncogenic Wnt signalling in GC and that inhibition of FZD7 and modulation of the WNT-FZD-LRP signalosome is a potential novel therapeutic strategy for GC. Interestingly, we observed the same results in both of our human GC cell lines. This is exciting as the key difference between cell lines is their APC mutational status; MKN45 are wild-type for APC whereas MKN28 have a truncating mutation in APC at codon 1450 (GGA to TGA, Arg to STOP) (Yokozaki, 2000). This demonstrates that inhibition of FZD7 is sufficient to block Wnt signalling in GC cells with mutant APC, which is consistent with similar studies done in CRC cells (Ueno et al., 2008; Vincan et al., 2005). Recently it was shown that APC^{KO} CRC

cells induced formation of the WNT-FZD-LRP signalosome, resulting in activated Wnt signalling. However, CRC with APC mutations treated with a porcupine inhibitor did not inhibit the activation of the signalosome, suggesting that Wnt ligands are dispensable in Wnt pathway activation resulting from APC truncation in CRC cells (Saito-Diaz et al., 2018). On the other hand, LRP6 deletion in CRC cells mutant for APC (SW480 and DLD1) did inhibit canonical Wnt signalling and have decreased cytoplasmic levels of ß-catenin. These data suggest that Wnt receptor signalosome is activated by mutant APC and can induce Wnt signalling independent of Wnt ligands. Recent work from Owen Sansom's group have showed that GTPases, Ra1A and Ra1B, are required for efficient internalization of FZD7 to activate Wnt signalling in intestinal stem cells (Johansson et al., 2019) supporting findings from Saito-Diaz *et al*., who also showed that rapid activation of Wnt signalling by the signalosome in APC mutant cells was due to internalization of the complex via clathrin-dependent endocytosis. This illustrates a conserved mechanism of internalization of the signalosome in WT and APC mutant Cells. It has demonstrated that CRC cells with a mutation in the mutation cluster region (MCR) of APC can respond to Tankyrase inhibition, suppressing oncogenic signaling in response to AXIN1/2 stabilization (Schatoff et al., 2019). Conversely, CRC cells containing an early truncating mutation (APC^{min}) were unresponsive to Tankyrase inhibition. This highlights the importance of the extent of APC truncation when considering therapeutic intervention as truncated APC can still be translated and transcribed leading to a functional or partially functional protein (Flanagan et al., 2019c). Our results demonstrating that GC cells with APC mutations are responsive to inhibitor at the level of the receptor had APC mutations that fall outside of the MRC. These data suggest a difference in how APC mutant GC and CRC cells respond to Wnt inhibitors, depending on the location of the mutation in the APC. Further investigations are required to understand the molecular mechanism underlying Wnt signaling in GC and how mutant APC modulates the response of GC cells to FZD7 inhibition.

Interestingly, approximately 37% of APC mutant gastric tumours are mutant for RNF43, demonstrating that FZD is deregulated in subset of APC mutant gastric tumours and therefore an attractive target (TCGA, 2014). However, RNF43 and APC mutations are mutually exclusive in colon tumours suggesting that CRC and GC cells preferentially select different Wnt mutations that confer optimal or 'just-right' levels of Wnt signalling required for tumour growth (Albuquerque et al., 2002; Lamlum et al., 1999). This confirms that modulating upstream of Wnt pathway component mutations is a viable strategy for treating GC cancer.

3.4 Conclusions

Many GCs arise via mutation to downstream effector proteins that facilitate the enabling hallmarks of cancer. Therapeutic targeting of these effector proteins is often challenging due to their molecular inaccessibility and associated pleiotropic effects. In this study, we have provided a route to reduce GC cell growth and cancer stem cell activity via targeted inhibition of Wnt receptor FZD7, both molecularly and pharmacologically. Collectively, we have demonstrated that targeted inhibition of Wnt receptors, specifically FZD7, is rate-limiting for the growth of human GC cells with and without APC mutations. Critically, this provides proof-of-principle that modulation of Wnt signalosome can further regulate Wnt signalling irrespective of downstream mutations that constitutively activate the pathway. This provides a broad scope for the application of this novel therapeutic strategy for the treatment of GC.

Chapter 4:
The Role of Frizzled-7 in Gastric Cancer Metastasis

4.1 Introduction

The findings presented in chapter 2 provide an exciting platform for targeting cancer stem cells via manipulation of FZD7 in GC. However, cancer is a complex disease comprised of other essential traits that must be considered, one such trait being metastasis (Hanahan and Weinberg, 2011).

Due the asymptomatic nature of GC, the majority of patients present with locally advanced and/or metastatic disease. The prognosis for patients with metastatic GC is very poor, with a median survival of 4 months (Van Cutsem et al., 2016). The current first-line therapy for GC patients with advanced disease is a combination of cisplatin/capecitabine with Transuzumab if the tumours are positive for HER2 expression However Transuzumab only yields a partial response and resistance usually develops (Koushyar et al., 2020). Although ramucirumab (targets vascular endothelial growth factor receptor-2) is approved for clinical use, there have been no reported benefits in GC (ElHalawani and Abdel-Rahman, 2015). Although the understanding GC etiology and pathophysiology have increased in recent years, identifying novel and reliable therapeutic targets has remained a challenge. Therefore, understanding the molecular aberrations that drive GC progression is key to identifying novel druggable targets to bring about treatment options for this disease.

It is well established that Wnt signalling not only drives the initiation of solid cancers but also contributes to the metastatic progression of the primary tumour. The reactivation of Wnt signalling in the cancer stroma favors cancer stem cell survival, whilst within the primary tumour reactivation aids the EMT of tumour cells, the migration and invasion of tumour cells and prevents dormancy at metastatic secondary sites (Nwabo Kamdje et al., 2017). Therefore, targeting Wnt signalling is an attractive therapeutic strategy for cancer metastasis.

Published investigations into oncogenic Wnt signalling in the context of GC metastasis have been limited. However, a recent study has identified, through gene set enrichment analysis, that *ADAM17* (TNF- α-converting enzyme) mediates GC cell migration through regulation of both the NOTCH and Wnt signalling pathways. ADAM17 was shown to be abundantly expressed in primary GC tissue, metastatic lymph nodes, and in metastatic GC cell lines. Furthermore, knockdown of *ADAM17* in a metastatic GC cell line suppressed canonical Wnt signalling via the downregulation of ß-catenin (Li et al., 2019b)

Evidence of the involvement of Wnt signalling in the induction of EMT in GC comes from studies into the microRNA, mir-544a. Overexpression of mir-544a induces the translocation of ß-catenin from the cytoplasm to the nucleus, therefore increasing canonical Wnt signalling in GC cells (Yanaka et al., 2015). In addition to the upregulation of canonical Wnt signalling, mir-544a overexpression downregulated the protein expression of the Wnt destruction complex protein, AXIN2 (Yanaka et al., 2015).

In regards to the migration and invasion capabilities of GC cells, the stem cell marker LGR5 has been found to promote these through the regulation of canonical Wnt signalling (Wang et al., 2018). GC cells treated with a porcupine inhibitor, to prevent Wnt secretion and therefore the Wnt-FZD-LRP complex, displayed a decrease in LGR5-induced proliferation and migration of GC cells. Whereas, Wnt3a-treated cells rescued the LRG5-induced phenotype. Additional evidence demonstrated that LGR5 overexpression induced the translocation of ß-catenin to the nucleus, and increased the gene expression of two Wnt target genes, AXIN2 and TCF1 (Wang et al., 2018).

GC cells with overexpressed LGR5 had an increased cell motility via a morphological change; cells became elongated with a fibroblast-like appearance, with this phenotype reversing when the GC cells were treated with a porcupine inhibitor. This suggests that LGR5 regulates cell migration through Wnt signalling (Wang et al., 2018). This is consistent with recent work showing that cytonemes are induced by autocrine Wnt8a binding to the Ror2 receptor

(Mattes et al., 2018). Activation of cytonemes mediates the transport of Wnt8a to surrounding cells which then trigger canonical Wnt signalling (Mattes et al., 2018). This highlights the cross-talk between non-canonical and canonical Wnt signalling in migrating cells, and gives support to investigating FZD7 as a receptor involved in GC progression due its unique ability to transduce signalling in both the canonical and non-canonical pathways and therefore it's potential as a therapeutic target.

Wnt5a-targeted knockdown in GC cell lines has been shown to reduce cell migration both in vitro and in vivo through inhibition of Rac1 and laminin ϒ2, both drivers of GC cell invasion. Suppression of Wnt5a using an anti-Wnt5a antibody prevented the clathrin-mediated rapid internalization of the Wnt5a-FZD2 receptor complex (Hanaki et al., 2012). Together, these studies identify Wnt signalling, either at the ligand/receptor level, or internalization of the receptor complex, as an important mechanism in driving GC metastasis, which therefore could contain some attractive therapeutic targets. Further investigations into the precise molecular mechanisms underlying Wnt signalling in GC progression is still required/ Therefore, this chapter aims to contribute to the understanding of Wnt signalling in the context of GC progression.

Due to the role of Wnt in cancer metastasis and our previous work highlighting the important role of FZD7 in GC growth, it is likely that this receptor will also be involved in GC progression. Therefore, this chapter aims to elucidate the role of FZD7 in gastric cancer metastasis through *in vitro* studies inhibiting FZD7, both molecularly and pharmacologically. FZD7 will be assessed for its role in the migratory and invasive capabilities of GC cells as well its role in the process of EMT; a key process in a cells ability to acquire a migratory phenotype The therapeutic benefit of inhibiting Wnt signalling at the level of the receptor in GC metastasis will also be investigated using an *in vivo* model to better recapitulate the tumour microenvironment. GC cells will be injected into the abdominal cavity to resemble GC peritoneal dissemination and mice treated with LGK-974 to block Wnt secretion. This will assess the requirement of Wnt

signalling for GC cells ability to survive in the peritoneal niche and to colonize a secondary site.

4.2 Results

4.2.1 Inhibition of the Wnt signalosome suppressed GC migration & invasion

The Wnt pathway has been implemented as a key signalling pathway driving cancer metastasis. As reported in chapter 2 (Figure 3.2) several FZD receptors are abundantly expressed in our two human GC cell lines, MKN28 and MKN45. To investigate the role of FZD receptors in the migration of GC cells the widely accepted transwell assays were employed. Transwell assays evaluate the ability of single cells to migrate through a porous membrane. GC cells were treated with the pan-FZD monoclonal blocking antibody, OMP-18R5 at a concentration of 80 μg/mL (as previously used(Gurney et al., 2012)) or vehicle control. MKN28 and MKN45 GC cells treated with OMP-18R5 migrated less through the pores of the filter membrane, demonstrating a significant reduction in the migratory ability of the cells compared to the vehicle-treated cells (Figure 4.1). This illustrates that FZD receptors regulate GC motility and migration.

Another key property of metastatic cancer cells is their ability to invade into tissue. Therefore, the ability of human GC cells to invade was investigated through Transwell invasion assays; this measures the invasion of cells through extracellular matrix, a process commonly found in cancer metastasis. This is achieved by the addition of a thin layer of Matrigel seeded onto the semi-permeable membrane. Both human GC cell lines treated with OMP-18R5 had a significantly decreased ability to invade through the Matrigel layer compared to vehicle-treated control (Figure 4.2) This demonstrates that FZD receptors are required for GC cells ability to invade.

To ensure the migratory and invasive ability observed in our GC cell lines was due the effect of OMP-185R blocking a subclass of FZD receptors and not due to drug toxicity, a cell viability assay was performed. In both MKN45 and MKN28 GC cell lines, increasing concentrations of OMP-18R5 did not reduce their viability (Figure 4.3). This confirms that the results observed in Figure 4.1 and Figure 4.2 are due the inhibitory action of OMP-18R5 on FZD receptors.

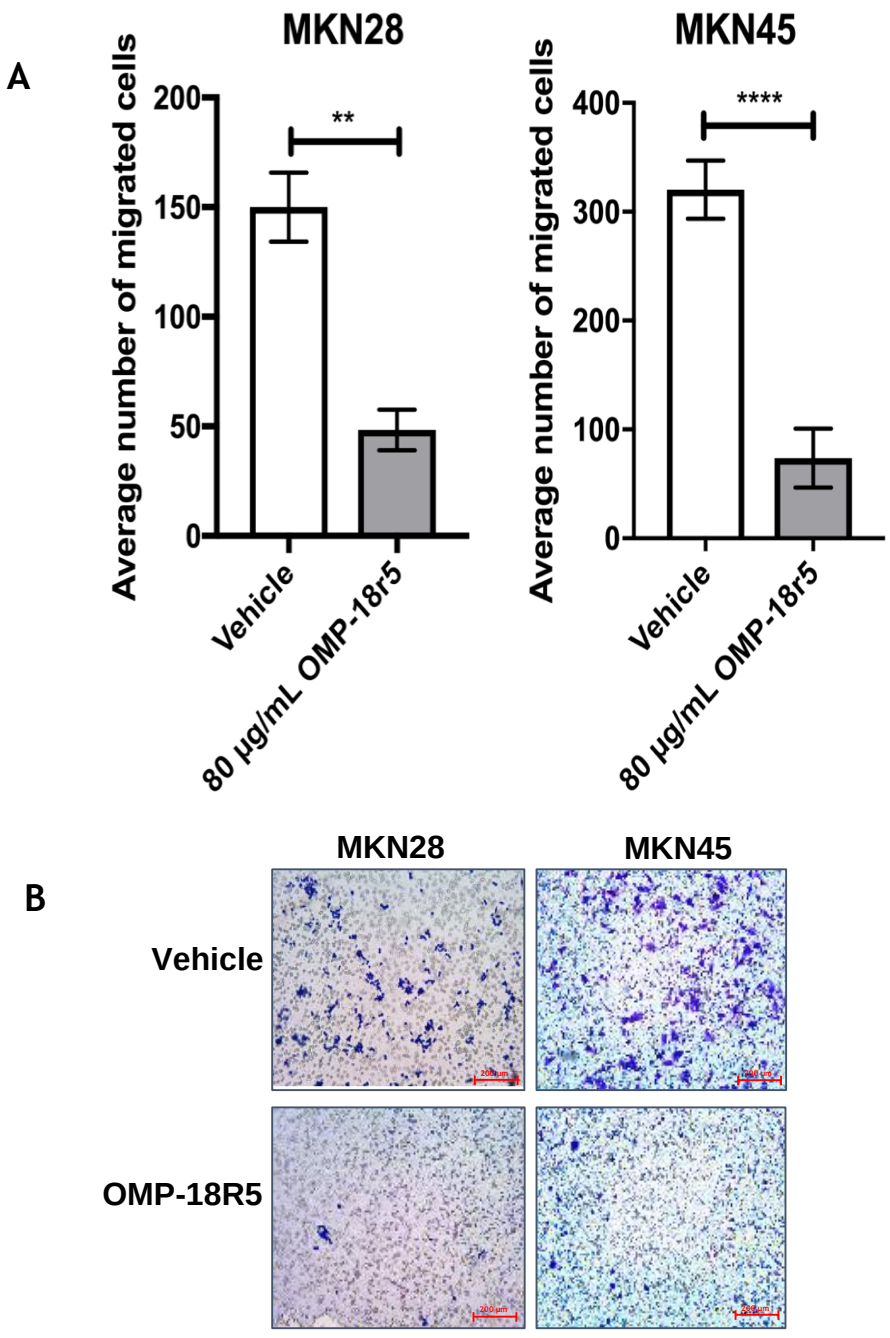


Figure 4.1 Pan-FZD inhibition reduced migratory ability of GC cells. (**A**) Treatment of MKN28 and MKN45 GC cells with 80 µg/mL OMP-18R5 for 24 hours significantly reduced their ability to migrate through the pores of a Transwell insert compared to vehicle-treated control. (**B**) Representative images of GC cells on the underside of the filter membrane stained with crystal violet. Experiments were performed in duplicate and three random fields of view per replicate were counted by eye and the average taken. (**= p ≤0.01, ****= p ≤0.0001, mean ± SEM, t-test, n=3)

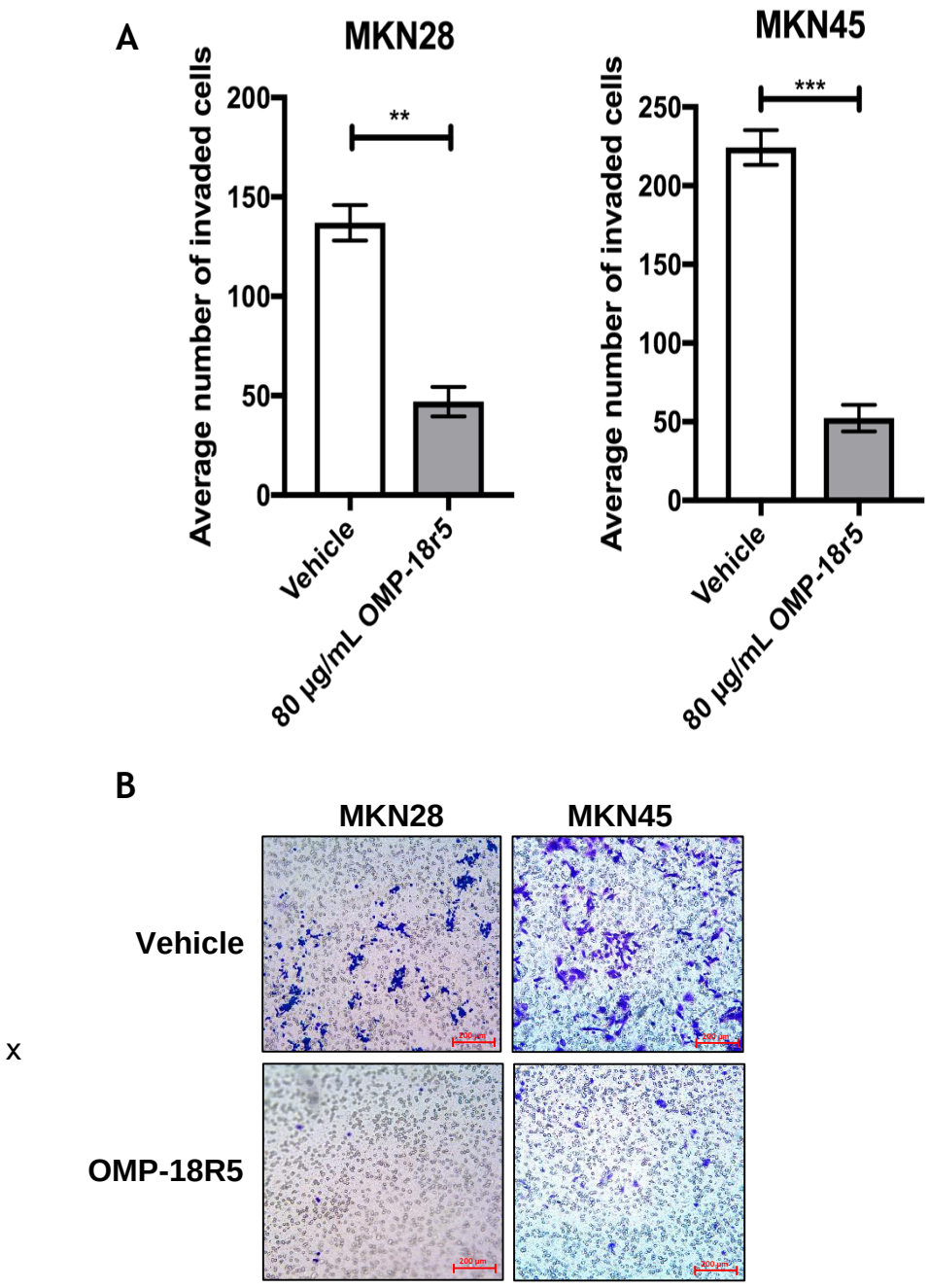


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Figure 4.2 Pan-FZD inhibition reduced invasive ability of GC cells. (**A**) Treatment of MKN28 and MKN45 GC cells with 80 µg/mL OMP-18R5 for 24 hours significantly reduced their ability to invade through a thin layer of matrigel in a Transwell insert compared to vehicle-treated control. (**B**) Representative images of GC cells on the underside of the filter membrane stained with crystal violet. Experiments were performed in duplicate and three random fields of view per replicate were counted by eye and the average taken. (**= p ≤0.01, ***= p ≤0.001, mean ± SEM, t-test, n=3)

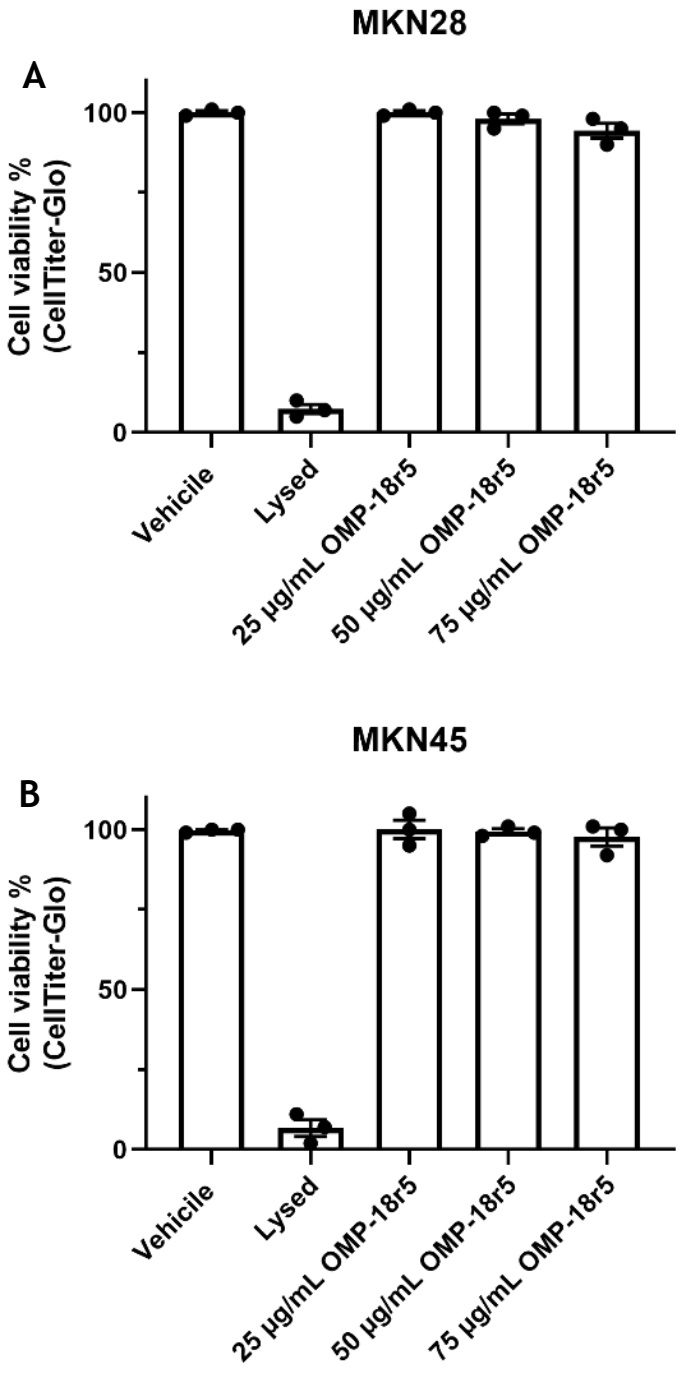


Figure 4.3 OMP-18R5 not toxic to GC cells. (**A**) MKN28 and (**B**) MKN45 GC cells were grown in 96-well cell culture plates and treated with increasing concentrations of OMP-18R5, vehicle control or DMSO (to induce lysing of cells). No drug toxicity was observed in either cell line. Cell viability was assessed by Celtiter-Glo assay and cell viability percentage was calculated relative to DMSO treated cells. (mean ± SEM, n=3).

These data suggested that cell intrinsic Wnt ligands were required for the migratory and invasive ability of GC cells. This was confirmed by treatment of GC cells with LGK-974 which prevents Wnt secretion through inhibition of the palmitoylation of the Wnt ligand by PORCN (Liu et al., 2013). MKN28 and MKN45 GC cells treated with LGK-974 had a significantly reduced ability to migrate through the filter membrane of the Transwell insert compared to vehicle-treated control (Figure 4.4). Human GC cell lines treated with LGK-974 also had a significantly inhibited ability to invade through the layer of Matrigel above the filter membrane of the Transwell insert (Figure 4.5). To confirm that drug toxicity was not responsible for the reduced number of GC cells observed on the underside of the Transwell filter membrane, GC cells were treated with increasing concentrations of LGK-974 and their cell viability assessed by CellTiter-Glo assay. Increasing concentrations of LGK-974 did not significantly reduce cell viability compared to vehicle-treated control (Figure 4.6). These data demonstrate that cell intrinsic secretion of Wnt ligands and FZD receptor availability are required for the migratory and invasive ability of human GC cells.

To confirm inhibition of Wnt signalling had led to a reduced migratory and invasive ability of GC cells, TOPflash assays would have been used on GC cells treated with LGK-974. However, due to restricted lab time due to the COVID19 lockdown these could not be completed.

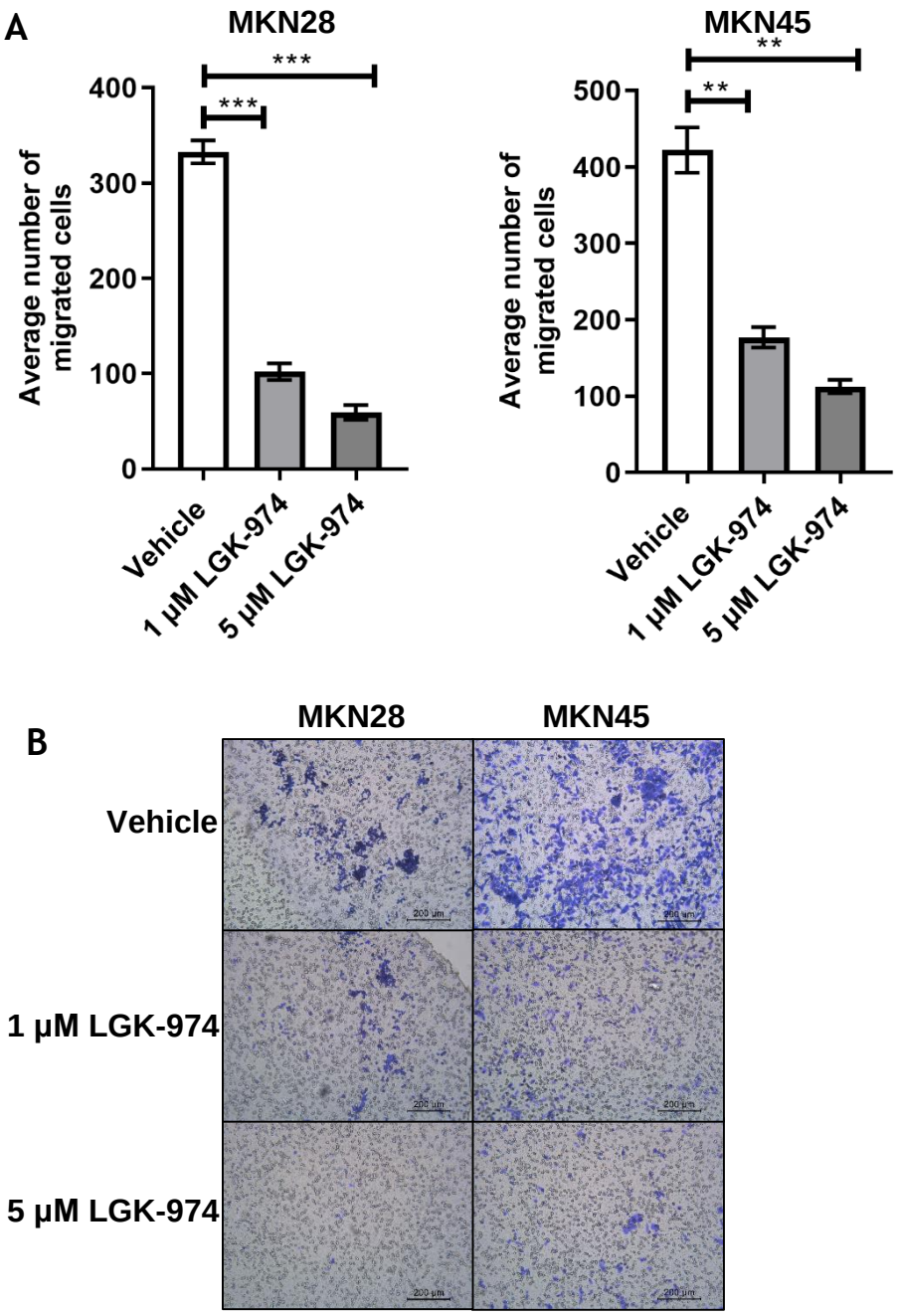


Figure 4.4 Wnt inhibition reduced migratory ability of GC cells. (**A**) Treatment of MKN28 and MKN45 GC cells with 1 μM and 5 μM LGK-974 for 24 hours significantly reduced their ability to migrate through the filter membrane in a Transwell insert compared to vehicle-treated control. (**B**) Representative images of GC cells on the underside of the filter membrane stained with crystal violet. Experiments were performed in duplicate and three random fields of view per replicate were counted by eye and the average taken. (**= p ≤0.01, ***= p ≤0.001, mean ± SEM, t-test, n=3)

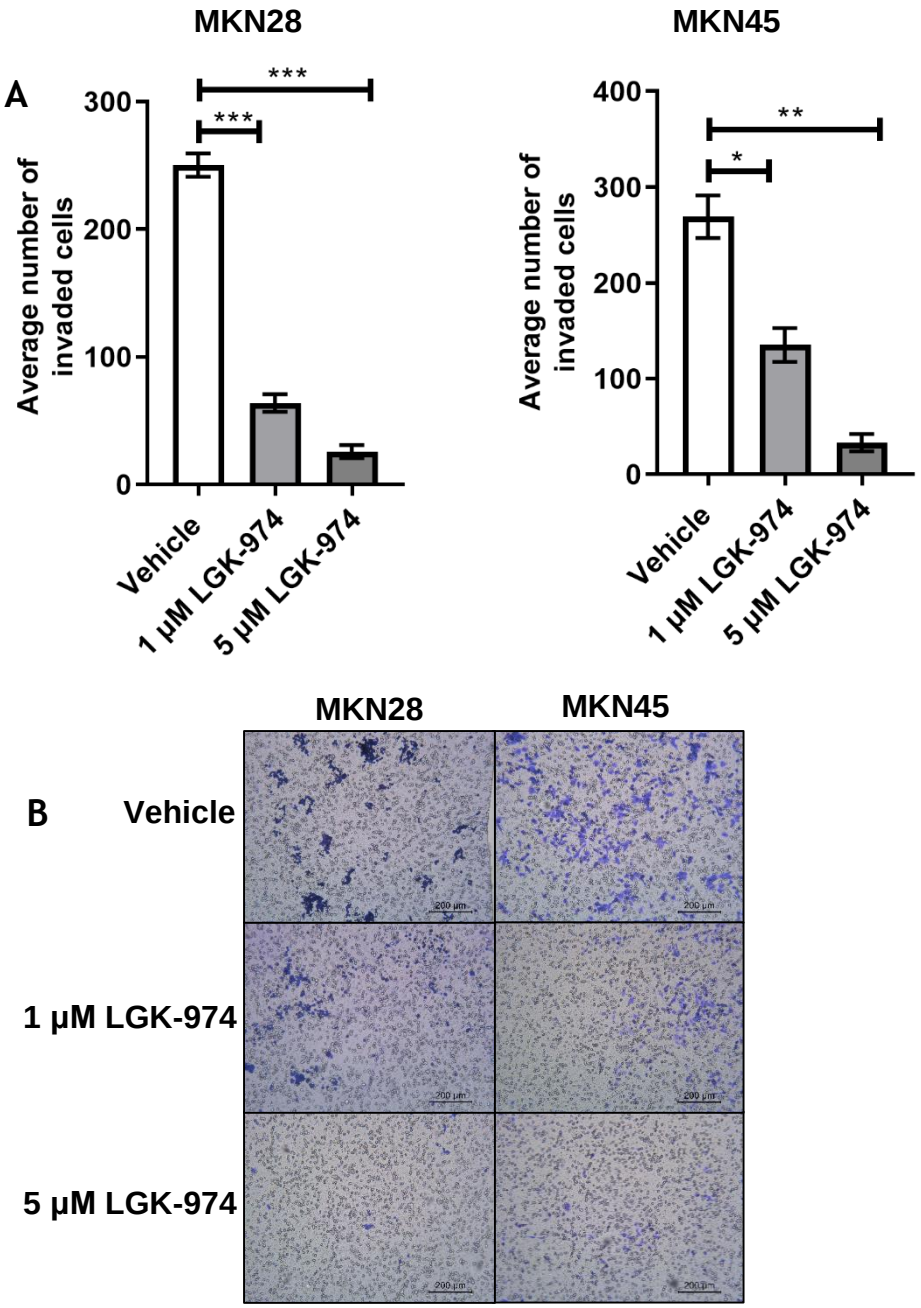


Figure 4.5 Wnt inhibition reduced invasive ability of GC cells. (**A**) Treatment of MKN28 and MKN45 GC cells with 1 μM and 5 μM LGK-974 for 24 hours significantly reduced their ability to invade through a thin layer of Matrigel in a Transwell insert compared to vehicle-treated control. (**B**) Representative images of GC cells on the underside of the filter membrane stained with crystal violet. Experiments were performed in duplicate and three random fields of view per replicate were counted by eye and the average taken. (*= p≤0.05, **= p ≤0.01, ***= p ≤0.001, mean ± SEM, t-test, n=3)

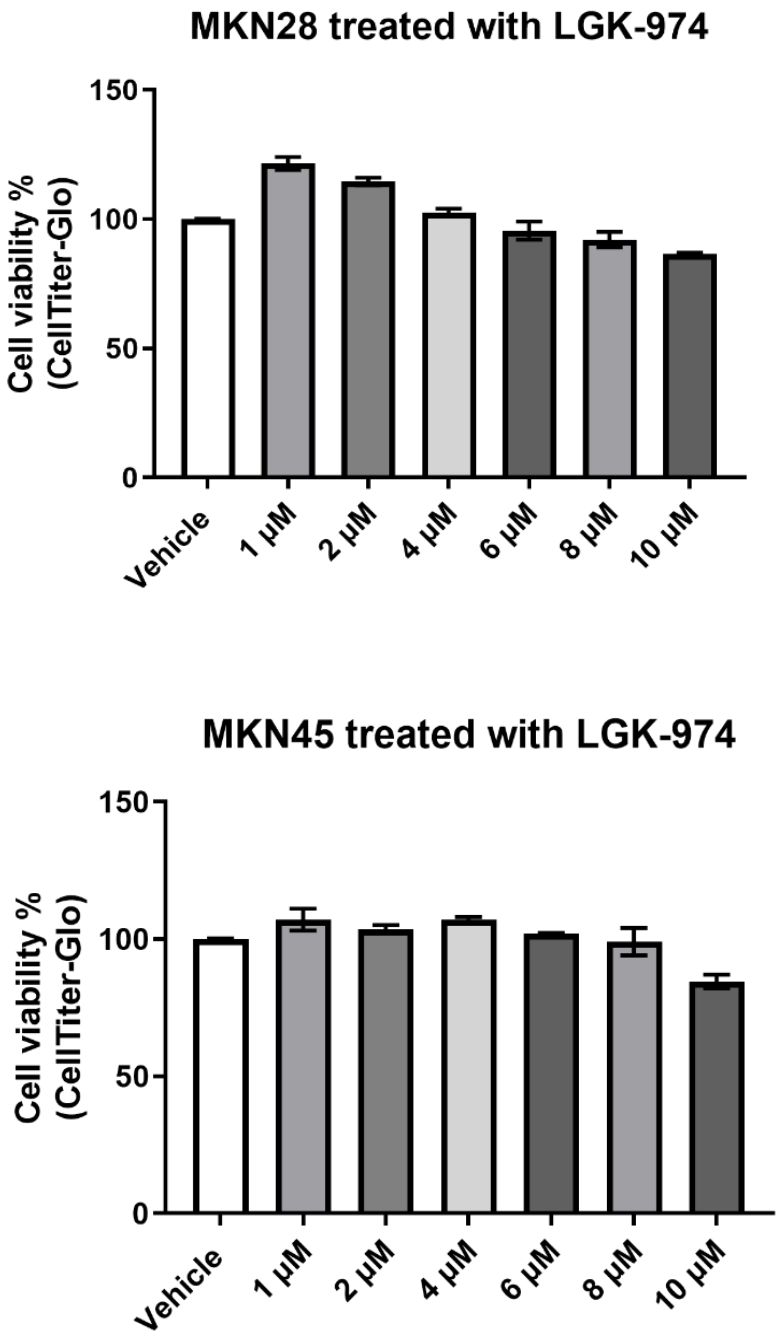


Figure 4.6 LGK-974 not toxic to GC cells. MKN28 and MKN45 GC cells were grown in 96-well cell culture plates and treated with increasing concentrations of LGK-974 or vehicle control. No drug toxicity was observed in either cell line. Cell viability was assessed by Celtiter-Glo assay and cell viability percentage was calculated relative to DMSO treated cells. (mean ± SEM, n=3).

4.2.2 FZD inhibition blocks EMT in human GC cells.

EMT is a vital process in tumour invasion and metastasis. There is increasing evidence demonstrating that activation of Wnt signalling can drive a transcriptional program and promote EMT in cancers (Basu et al., 2018). To determine the role of FZD receptors in transmitting the signals required to drive EMT in GC, fluorescent immunocytochemistry was performed. GC cells were treated with OMP-18R5 following the induction of the EMT state and then stained for epithelial and mesenchymal markers. Vehicle-treated MKN28 GC cells that were induced into EMT via the addition of StemXVivo supplement into their growth media were positive for the mesenchymal marker vimentin and negative for the epithelial marker, Ep-CAM. It is confirmed that these cells lost epithelial markers and gained mesenchymal markers due to EMT induction by the observation of positive staining for Ep-CAM and negative staining for vimentin in vehicle-treated cells that had not been induced to undergo EMT (Figure 4.7). MKN28 GC cells induced to undergo EMT and treated with OMP-18R5 (to inhibit a subclass of FZD) failed to upregulate Vimentin and retained expression of Ep-Cam indicating that blocking Fzd receptors prevented EMT. (Figure 4.7). OMP-18R5 treatment of non-induced MKN28 cells did not have any unexpected results and showed the same expression of markers as the vehicle-treated non-induced cells (Figure 4.7). This demonstrates that Wnt signalling, via the FZD receptors, is involved in the promotion of the EMT state in GC. To confirm it was Wnt/ß-catenin signalling pathway, it was planned that the cellular levels of ß-catenin and canonical Wnt pathway target genes were to be investigated by immunofluorescence staining, however, this could not be completed due to lab restrictions due to the COVID19 lockdown. The lockdown also prevented further analysis to investigate if the same result was observed in MET, by removing StemXVivo from the growth media. These experiments would have yielded a more complete understanding of the role of Wnt in the metastasis of GC.

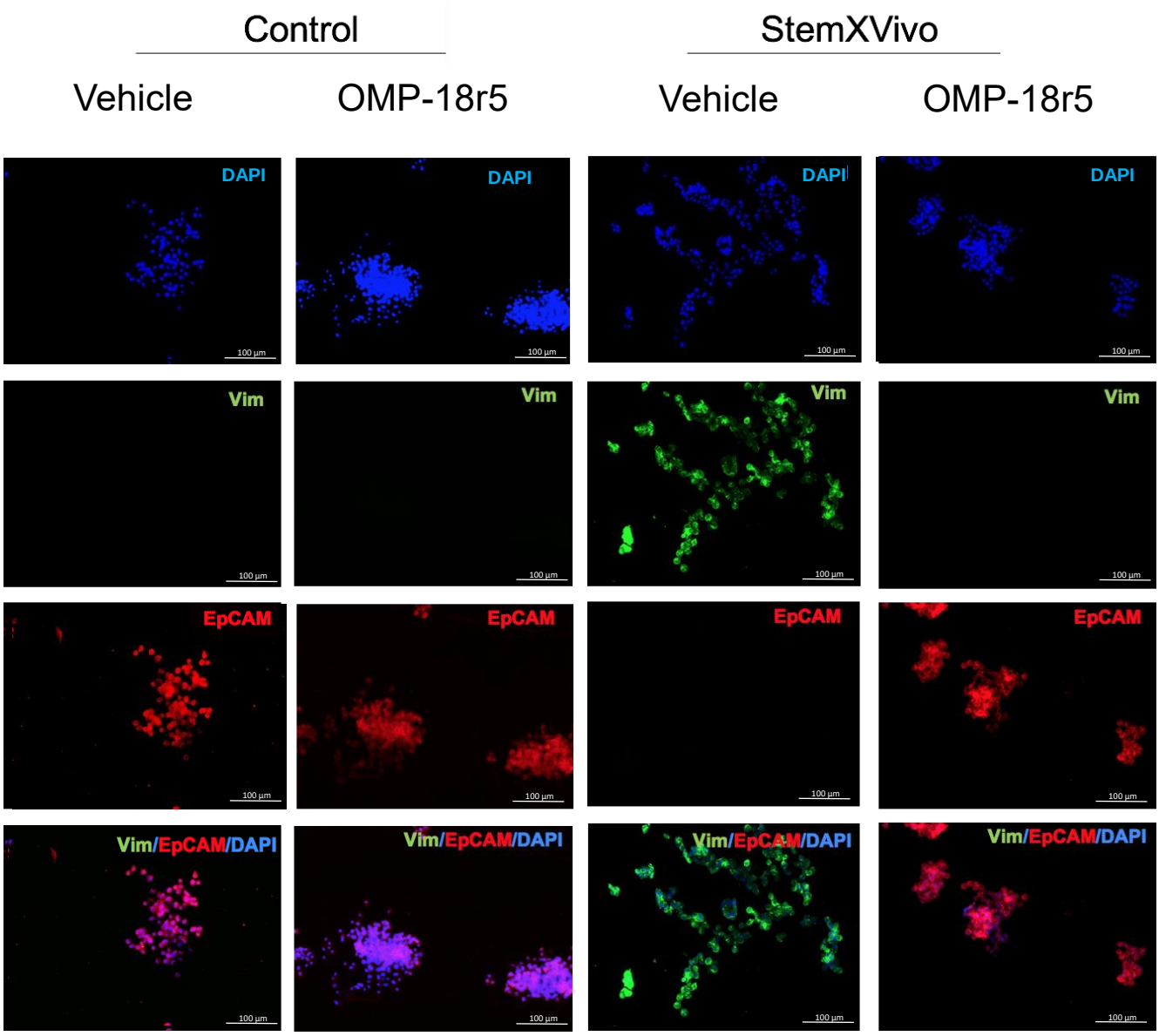


Figure 4.7 FZD inhibition blocks EMT in GC cells. Immunofluorescence assay of EMT-induced MKN28 GC cells showed treatment with 80 µg/mL OMP-18R5 blocked EMT compared to vehicle-treated EMT-induced control. Cells were stained for vimentin (green) and Ep-CAM (red) and counterstained with DAPI (blue). (Scale bars = 200 µM).

To quantify the expression mRNA levels of epithelial and mesenchymal markers following EMT stimulation and OMP-185R treatment, qRT-PCR was performed. Vehicle-treated MKN28 GC cells not induced to undergo EMT displayed a significantly decreased expression of mesenchymal markers, Slug (SNAI2), Snail (SNAI1), Twist1, N-cadherin (CDH2) and vimentin, and a significantly increased expression in the epithelial adhesion marker E-cadherin (CDH1) compared to EMT-induced vehicle-treated cells (Figure 4.8). This illustrates that the addition of StemXVivo to the growth media of MKN28 induces an EMT state resulting in the upregulation of mesenchymal markers. OMP-18R5-treated EMT-induced GC cells displayed a similar expression of mesenchymal and epithelia markers as the vehicle-treated non-EMT induced control cells (Figure 4.8). This illustrates that inhibition of FZD receptors, and therefore modulation of the Wnt signalosome, is sufficient to block the process of EMT in human GC cells.

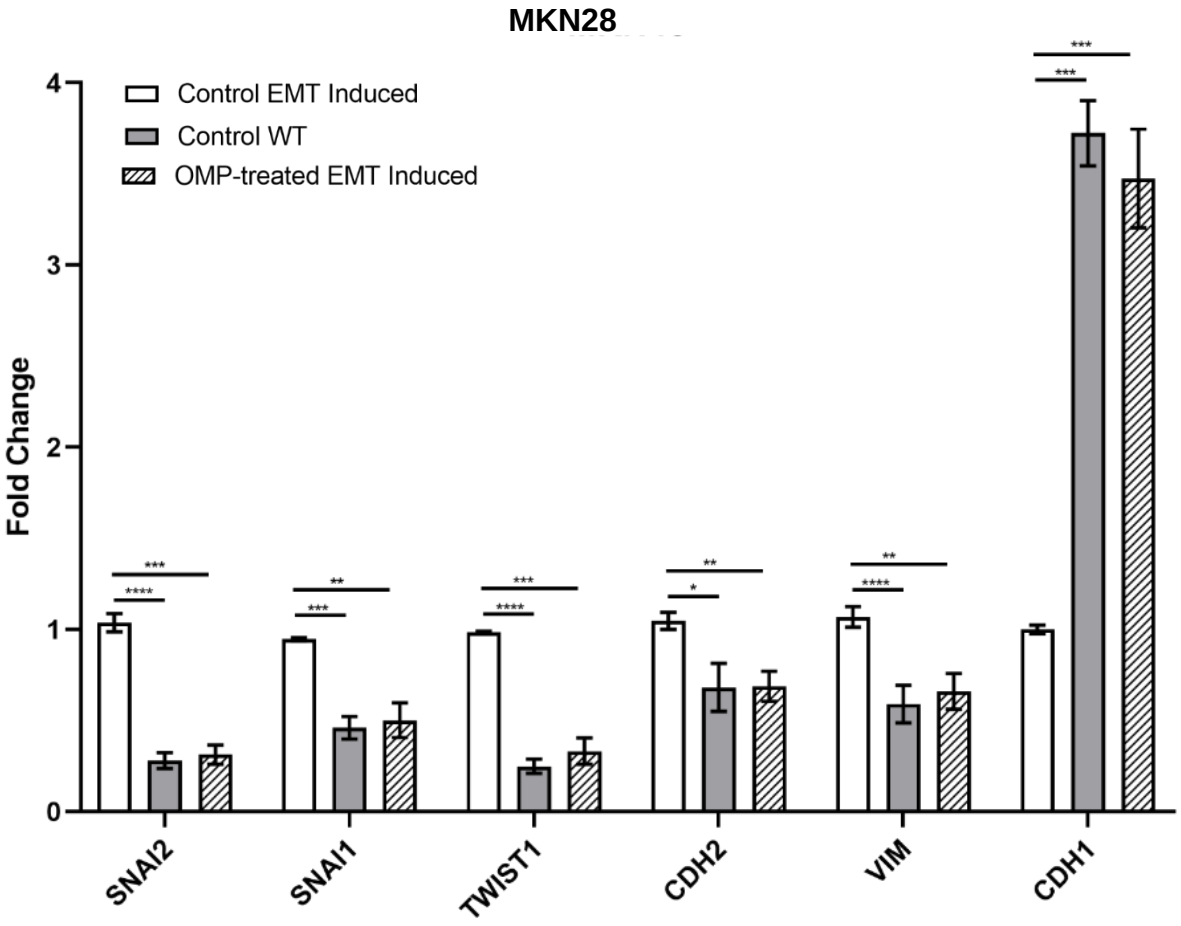


Figure 4.8. FZD inhibition blocks the EMT expression profile in GC cells. qRT-PCR analysis of mesenchymal and epithelial markers showed EMT-induced MKN28 GC cells treated with 80 µg/mL OMP-18R5 blocked the process of EMT compared to vehicle-treated EMT-induced control. (*= p ≤0.05, **= p ≤0.01, ***= p ≤0.001, ****= p ≤0.0001, mean ± SEM, t-test, n=3)

4.2.3 Wnt inhibition is not sufficient to block GC metastasis *in vivo*.

The process of cancer metastasis is extremely complex and is influenced by a multitude of intrinsic and extrinsic factors, such as immune cells, cancer-associated fibroblasts and stromal cells (Liu et al., 2017). Therefore, it is important to investigate metastasis in a context that recapitulates the tumour microenvironment. To achieve this an *in vivo* model of peritoneal dissemination (the most frequent form of metastasis in GC) was designed. Human GC cell lines, MKN28 and MKN45 were injected into the abdomen of nude mice and left to form micro-metastases in the peritoneal cavity. Unfortunately, OMP-18R5 was unable to be used due to the manufacturer being taken over and pause on production being issued. Therefore, LGK-974 was used as an alternative to inhibit Wnt signalling. Mice were treated with 1.5 mg/kg LGK-974, 3 mg/kg LGK-974 or vehicle control via oral gavage. After 4 weeks the mice were sacrificed and the tumour metastatic burden was quantified. No significant difference was observed in the metastatic tumour burden of mice treated with LGK-974 compared to vehicle-treated control (Figure 4.9). No toxicity was observed in LGK-974-treated mice, which displayed bodyweight consistent with tumour burden and no signs of morbidity or intestinal distress (diarrhea) for the duration of the treatment (Figure 4.10).

These data suggest that although LGK-974 is able to inhibit migration and invasion of GC in vitro, it is not able to prevent the formation of peritoneal tumours when GC are injected IP. This could be due to several factors which are addressed in the discussion at the end of this chapter.

The characterization of the tumours harvested from this experiment could not be completed due to lab restrictions in place due the COVID19 lockdown. Unfortunately, the effect of FZD inhibition on GC metastasis could not be investigated *in vivo* due to supply issues of OMP-18R5.

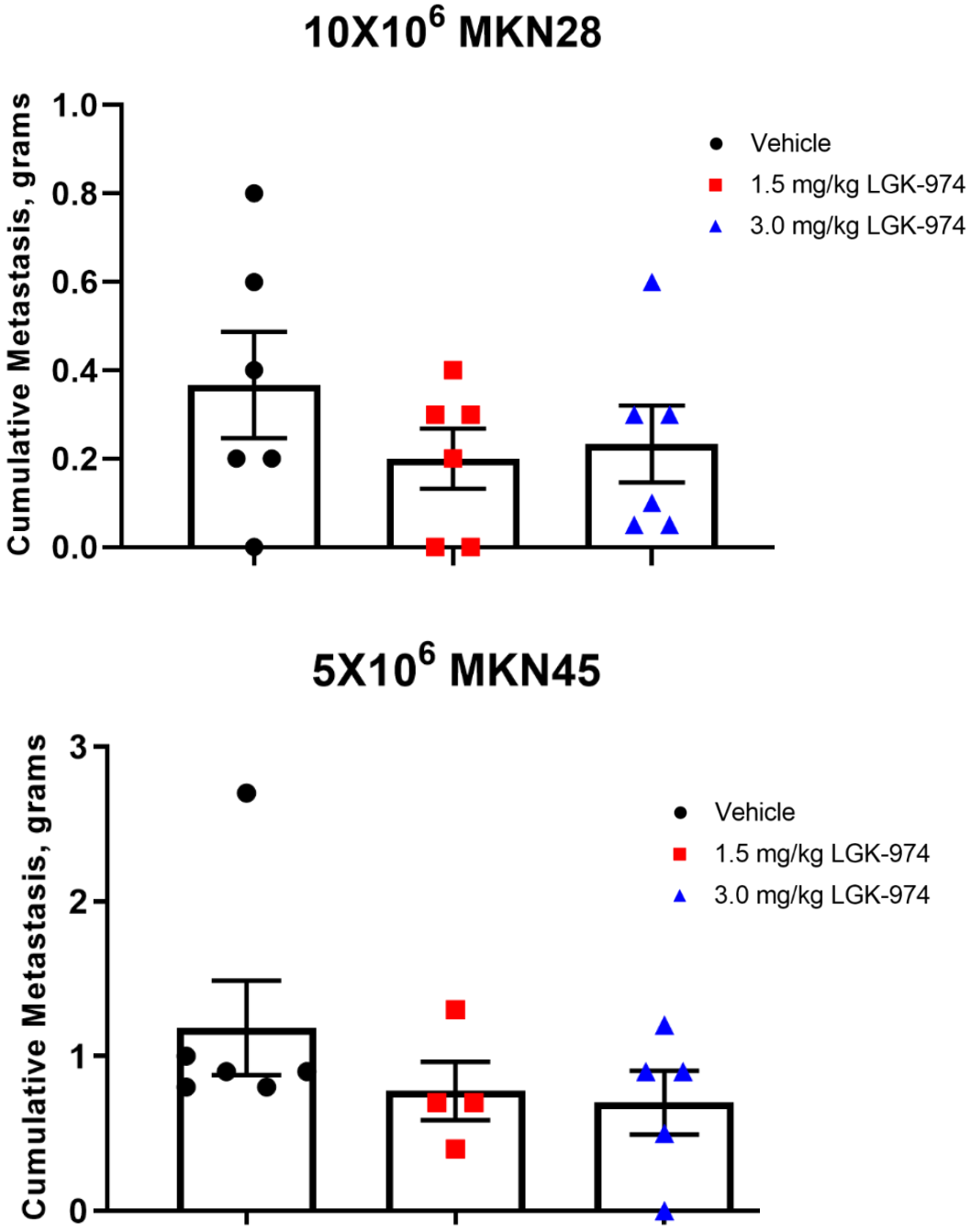


Figure 4.9. Wnt inhibition did not reduce tumour burden *in vivo*. Metastatic burden (weight in grams) of GC cells, MKN28 and MKN45, in injected into the peritoneal cavity of mice and treated with LGK-974 or vehicle. No significant difference was observed in LGK-974-treated mice compared to Vehicle-treated cohorts. (mean ± SEM, t-test, n=6 mice per cohort).

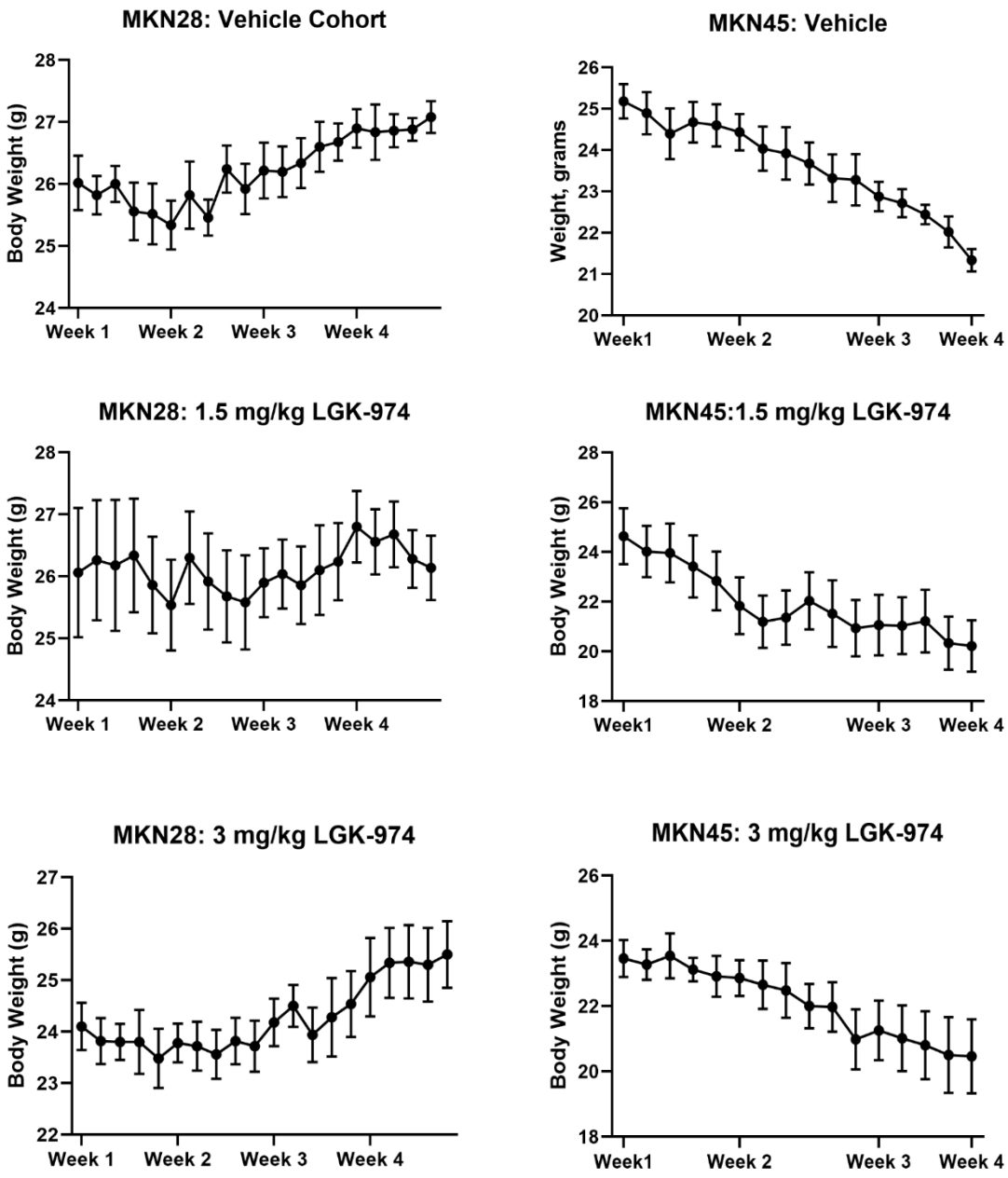


Figure 4.10. LGK-974 did not lead to increased morbidity. Body weight (grams) over time of mice treated with 1.5 mg/kg LGK-974, 3 mg/kg LGK-974 or vehicle control for both MKN28 and MKN45 cohorts. (mean ± SEM, t-test, n=6, mice per cohort).

4.2.4 Targeted FZD7 knockdown suppressed GC migration and invasion

Suppression of GC migration and invasion following treatment with OMP-18R5 suggests that one of the five FZD receptors targeted by OMP-18R5 (FZD1, 2, 5, 7, and 8) is responsible for transmitting Wnt signals in GC cells during these cellular functions. The results presented in chapter 2 demonstrated that FZD7 is the predominant FZD receptor transmitting oncogenic Wnt signalling, which regulates GC growth; specific knockdown of FZD7 produced very similar results in colony forming assays as inhibition of the secretion of all Wnt ligands, suggesting a non-redundant role for FZD7 in transmitting Wnt in GC cells These findings, together with the evidence that FZD7 upregulation in GC is associated with poor clinical outcomes (Phesse et al., 2016), strongly suggest that FZD7 may also play a key role in GC metastasis. To determine the specific requirement of FZD7 for the migration of human GC cells we formed *in vitro* transwell migration assays on MKN28 and MKN45 GC cells transfected with shFZD7 or shSCRAMBLED control. For both MKN28 and MKN45 cells, FZD7 knock-down cells displayed a significant reduction in their ability to migrate through the pores of the filter membrane (Figure 4.11). This illustrates that FZD7 is a key player in regulating the motility of GC cells.

Another key property of metastatic cancer cells is their ability to invade into tissue. Therefore, the ability of human GC cells to invade was investigated through transwell invasion assays. Both human GC cell lines transfected with shFZD7 had a significantly decreased ability to invade through the Matrigel layer above the filter membrane compared to shSCRAMBLED control (Figure 4.12). This demonstrates that FZD7 regulates GC cells ability to invade.

These data suggest that FZD7 is the predominant Wnt receptor transmitting oncogenic Wnt signalling that regulates GC cells ability to migrate and invade. Therefore, targeting FZD7 may be an attractive therapeutic target to treat metastatic GC.

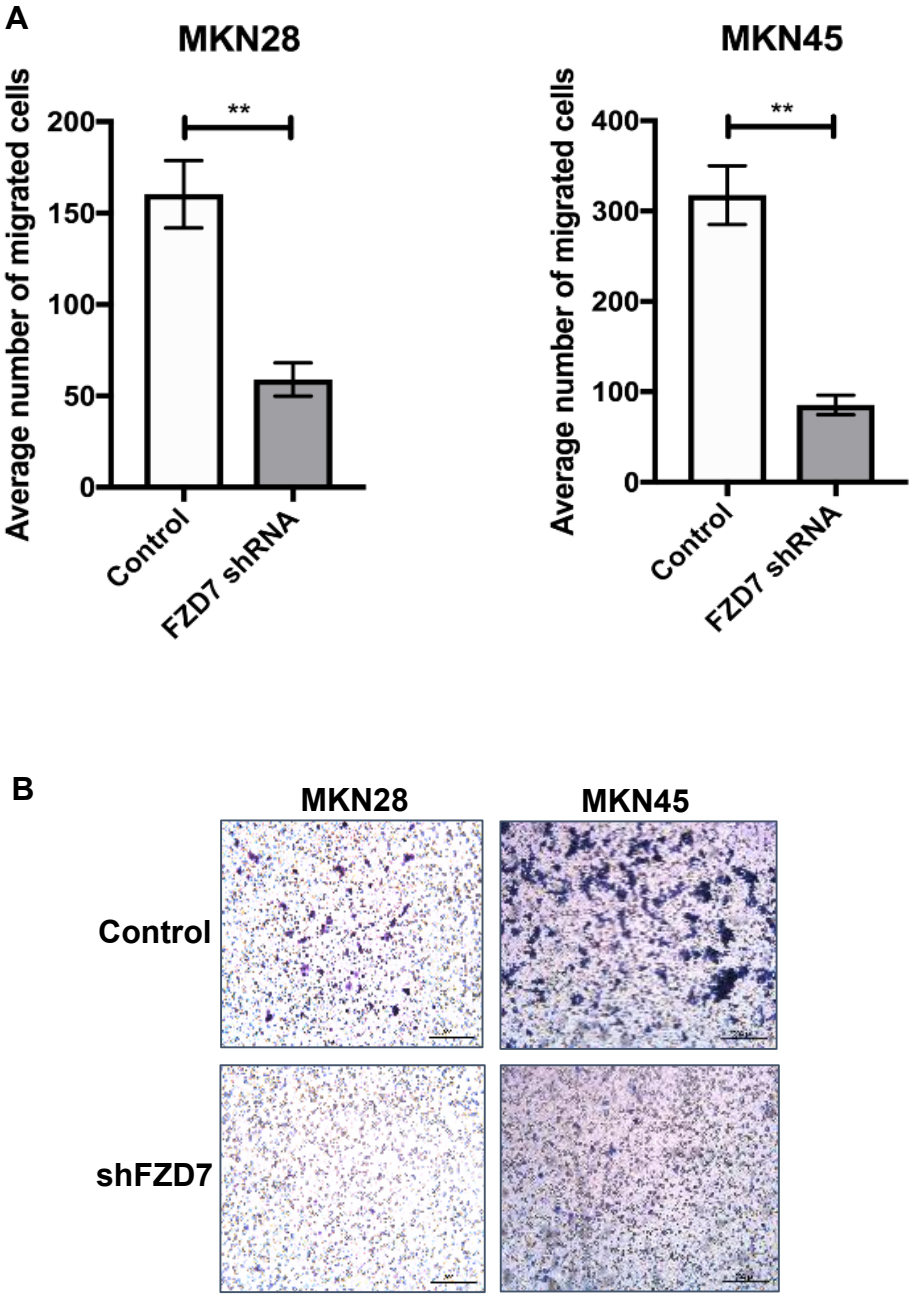


Figure 4.11 FZD7-knockdown suppresses GC migration. (**A**) Knock-down of FZD7 in both MKN28 and MKN45 GC cells significantly reduced their ability to migrate *in vitro* compared to control. (**B**) Representative images of GC cells, stained with crystal violet, on the underside of the filter membrane. Experiments were performed in duplicate and three random fields of view per replicate were counted by eye and the average taken. (**= p ≤0.01, mean ± SEM, t-test, n=3)

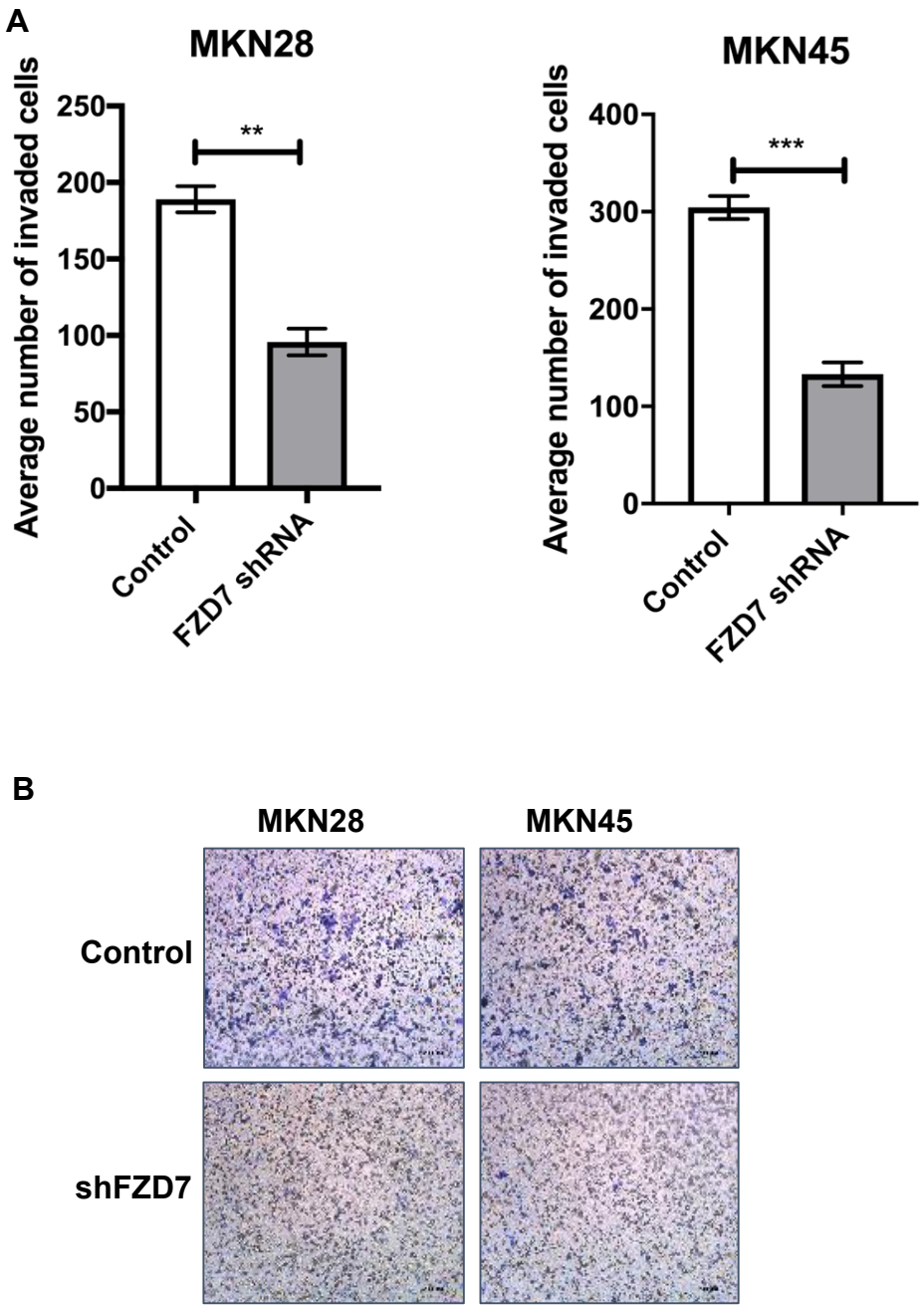


Figure 4.12 FZD7-knockdown suppresses GC invasion. (**A**) Knock-down of FZD7 in both MKN28 and MKN45 GC cells significantly reduced their ability to invade *in vitro* compared to control. (**B**) Representative images of GC cells, stained with crystal violet, on the underside of the filter membrane after invading through a Matrigel layer. Experiments were performed in duplicate and three random fields of view per replicate were counted by eye and the average taken. (**= p ≤0.01, mean ± SEM, t-test, n=3)

The following was planned but could not be completed due to COVID19 lockdown:

To determine if FZD7 was the predominant receptor regulating the process of EMT fluorescent immunocytochemistry was going to be utilized. GC cells deficient for FZD7 (via transfection with shFZD7) would have been stained for a range of mesenchymal and epithelial markers following induction of EMT. This could have complimented the results presented in chapter 3.

To assess if the FZD7-knockdown phenotype observed in the migration and invasion *in vitro* assays could be rescued by overexpression of FZD7. The assays were to be repeated with the additional conditions of: shFZD7 only, FZD7-overexpression plasmid and co-transfection of shFZD7 and FZD7-overexpression plasmid.

4.3 Discussion

Metastasis is a characteristic of late stage cancer and remains a major challenge to therapy. This is due to an incomplete understanding of this highly complex pathological process. Metastatic cancer cells are able to acquire four key hallmarks of metastasis that are essential for all metastases to develop: Motility and invasion, modulation of the microenvironment, plasticity, and ability to colonize (Welch and Hurst, 2019). Investigations into these key traits of metastatic cancer cells will lead to a better understanding of the process and push forward the development of therapeutic interventions.

This is especially important in GC as metastasis is the primary reason of death in GC patients, but the underlying mechanisms remain unclear and treatments are still limited. Therefore, it is urgent to investigate the molecular process of GC metastasis to lead to the identification of novel therapeutic targets to bring about a benefit for patients. As highlighted in chapter 2 the Wnt/ß-catenin signaling pathway is a potent pathway for GC pathogenesis and growth, with FZD7 acting as the predominant receptor transmitting oncogenic signals. Wnt signalling has also been implemented in GC metastasis, with the upregulation of FZD7 associated with poor prognosis in advanced cases (Li et al., 2018) although there are currently limited studies on how Wnt signalling drives metastatic GC.

Recently, cancer stem cell (CSC) hypotheses are attracting increasing attention with research suggesting that CSCs contribute to tumour aggressiveness, metastasis and relapse (Yu et al., 2012b). CSC possess the ability to self-renew and differentiate into multiple lineages (Dalerba et al., 2007) and have been identified in several types of solid cancer including GC (Takaishi et al., 2009). It has been shown that FZD7 regulates a population of CSC in GC, although unlike the intestine, this exact population of CSC has not been identified (Flanagan et al., 2019b). Our results from chapter 2 support the hypothesis that FZD7 is regulating a population of gastric CSCs as knockdown of FZD7 in GC cell lines resulted in a reduced colony forming ability; this illustrates that FZD7 is

likely regulating the CSC activity. Therefore, the role of FZD7 in regulating a population of stem cells with the ability for self-renewal, and therefore metastatic potential, make it an enticing target for treating metastatic GC.

The role of FZD7 in metastasis has been highlighted in many other cancers. For example, it has been shown to promote tumour metastasis via the Wnt pathway in esophageal squamous cell carcinoma (Cao et al., 2017) and down-regulation of FZD7 was shown to decrease the metastatic capabilities of CRC cells (Ueno et al., 2009). However, the function of FZD7 in GC metastasis has not been thoroughly investigated. This chapter has shown that FZD receptors contribute to the migratory and invasive abilities of GC cells and elucidated that FZD7 is the likely Wnt receptor transmitting aberrant signalling in human GC cells, thus, enabling GC metastasis. It is important to highlight that specific knockdown of FZD7 gave similar effects to OMP-18R5 and LGK-974 treatment, this suggests a non-redundant role for FZD7. Furthermore, within our GC cell lines used only a small group of FZD receptors were abundantly expressed (FZD6 and 7) and of these, OMP-18R5 only blocks FZD7. We have also shown that FZD receptors play a key role in the regulation of the EMT process and could be an attractive therapeutic target in preventing GC cells ability to gain an invasive phenotype.

Accumulating evidence associates Wnt signalling with the regulation of cancer cell migration and invasion in many cancers (Nusse and Clevers, 2017). FZD2 has been shown to contribute to the migration and invasion of endometrial cancer cells, with overexpression promoting migration through the canonical Wnt pathway (Bian et al., 2016). Additionally, FZD2 blockage by siRNA reduced neuroblastoma cells motility and induced a less vascularized phenotype (Zins et al., 2016), demonstrating that cell migration can be modulated by regulation of the Wnt receptors. More recently, overexpression of FZD8 was shown to increase prostate cancer cell migration and invasion *in vitro* and *in vivo* by the activation of the Wnt/β-catenin signalling pathway (Li et al., 2017). Furthermore, shRNA-mediated knockdown of FZD7 has been reported to inhibit invasion and migration of cervical cancer cells (Deng et al., 2015) This evidence

in combination of our data supports a role for Wnt-regulated migration and invasion in GC. The results presented in this thesis strongly suggest that FZD7 is playing a functional role in the regulation of GC cells migration and invasion.

Our results demonstrating that inhibiting FZD7 suppressed migration and invasion were observed in both MKN45 (APC-WT) and MKN28 (APC-Mut) GC cell lines, highlighting that APC mutational status is not a rate-limiting factor for targeting Wnt ligands/receptors in metastatic GC. This further builds on the results presented in chapter 2 in confirming that modulating the Wnt signalling pathway at the level of the receptor in cells with downstream mutations is a viable therapeutic strategy for GC metastasis.

The process of EMT is a key step in metastasis that provides primary tumour cells with the properties required to invade their surrounding microenvironment and spread to distal sites and form micro-metastases. Wnt signalling has been reported to promote EMT through up-regulating mesenchymal transcription factors, such as Slug and Twist (DiMeo et al., 2009). Similarly, FZD receptors are known as critical factors for EMT processes. Wnt5a-FZD2-mediated non-canonical signalling has been demonstrated to drive EMT in liver, lung, colon and breast cancer cell lines (Gujral et al., 2014). FZD4 ablation induces active ß1-integrin and E-cadherin expression, confirming that FZD4 regulates EMT and cell adhesion in prostate cancer cells (Gupta et al., 2010). Similar to our EMT data presented in this chapter recent investigations have shown that down-regulation of FZD7 expression significantly inhibits cell invasion and migration, accompanied with decreased vimentin and snail, and increased E-cadherin in cervical and ovarian cancers (Asad et al., 2014; Deng et al., 2015). Despite the clear importance of the role of FZD receptors in mediating EMT, limited research has been undertaken in GC. Our study demonstrated that inhibition of FZD receptors, through treatment with OMP-185R, caused an increase of E-cadherin expression along with decreases of N-cadherin, snail, slug, twist, and vimentin. Snail expression has been shown to cause the stabilization of ß-catenin leading to the expansion of the stem cell

niche in CRC (Hwang et al., 2014). Together, these observations suggest that EMT induces stemness in invasive cancer cells. These findings provide evidence that FZD receptors can induce EMT and promote metastases in GC cells, even in cells with a mutation to intracellular APC. It is likely, given the gene expression analysis performed in chapter 3 (Figure 3.2) that of the 5 FZD receptors blocked by OMP-18R5, FZD7 is the predominant receptor transmitting Wnt signals to regulate EMT. Therefore, targeting FZD receptors/FZD7 to inhibit EMT or inducing mesenchymal-epithelial-transition (MET) might be an attractive therapeutic strategy.

There is also accumulating evidence showing the requirement of MET in the colonization and metastasis of carcinomas, for example, loss of the EMT transcription factor Prrx1 in breast cancer cells induces MET and leads to the establishment of a CSC niche which was required for metastasis (Ocaña et al., 2012; Stankic et al., 2013). In squamous cell carcinoma, Twist1-mediated EMT was necessary for primary tumour cells to invade local tissue, similar to the increase observed in Twist1 expression in our data. However, the silencing of Twist-1 and the re-acquisition of E-cadherin was necessary for the colonization in the distant tissue (Tsai et al., 2012). Therefore, targeting EMT alone might be counterproductive, unless the exact timing could be controlled which is unlikely, and inhibiting both EMT and MET could however be a more promising therapeutic strategy.

The *in vivo* data presented in this chapter highlight the importance of the tumour microenvironment (TME) and metastatic niches. The *in vitro* data demonstrated that inhibiting Wnt secretion by treatment of GC cell lines with LGK-974 was sufficient to suppress migration and invasion *in vitro*. However, this result was not observed when LGK-974 was used for *in vivo* experiments. This could be due to a multitude of inter-playing factors that capture the complexity of the metastatic process from vascular system to stromal cells. However, LGK-974 has been used to significantly reduce tumour burden *in vivo* in other Wnt-driven cancers, although not GC (Liu et al., 2013). We may not

see a result in GC due to a different requirement of Wnt signalling, echoing the "just right" model of Wnt (Albuquerque et al., 2002). Additionally, it has been shown in APC mutant CRC cells that Wnt signalling could be modulated at receptor level, via LRP5 knockdown, but treatment with a porcupine inhibitor (Similar to LGK-974) had no effect on Wnt signalling on the same cells (Saito-Diaz et al., 2018). This suggests that some Wnt-driven cancers can have ligand-independent activation of Wnt receptors and downstream signalling but still be modulated at the level of the receptor. Therefore, in the *in vivo* GC model, Wnt ligands may be redundant in the LRP5-FZD-WNT signalosome and this will need to be investigated further. Although we observed an effect of LGK-974 on our GC cells *in vitro*, the results observed in the *in vivo* model are more likely to be more representative of clinical GC due existence of a tumour microenvironment. It will be worth optimizing the in vivo model as although no significant difference between LGK-974-treated and vehicle-treated cohorts was observed, they seem to be a slight trend for smaller tumour burdens in LGK-974-treated cohorts. Therefore, adjusting the dosing regime and experiment length may be required to ensure full drug efficacy in an in vivo setting.

As LGK-974 has been shown to be effective in other cancers, it is possible there is something unique about the peritoneal niche for GC. There is very limited research into molecular alternations that facilitate intraperitoneal spread of GC, however, increased expression of connexin 43 (gap-junction protein) has been observed in GC cells that have metastasized into the peritoneal cavity. Overexpression of connexin 43 enhanced their intercellular communication with peritoneal mesothelial cells, which in turn accelerated the infiltration of GC cells into the peritoneal mesothelium for further colonization (Tang et al., 2013). This suggests that further investigations on GC cells seeding onto the lining mesothelial layer may be beneficial. Interestingly, a recent paper has shown that stromal Wnt is required for stem cell activity in the stomach in mice (Kim et al., 2020). Single cell transcriptome analysis identified conserved

stromal cell populations that expressed Wnt ligands. This highlights the importance of investigating the stoma in more details could suggest that Wnt may be deregulated in the stroma of gastric tumours in humans.

Other than the peritoneum, the liver is a common site of GC metastasis. A recent study has revealed a positive feedback loop between cancer-associated fibroblast and tumour cells in the liver metastasis niche of GC (Li et al., 2019a). This highlights the importance of understanding the complex cross-talk among the different cells involved in the TME, especially to better understand the role of TME in cells metastasis ability.

A major hurdle in the study of tumour metastasis is the lack of a mouse model with a competent immune system that can mimic the entire metastatic cascade. The most common model in the field is the experimental metastasis model, whereby, cancer cells are injected into the tail vein of mice to travel through the circulation and form metastases at distant organs, most commonly the lungs. While this model has provided valuable insights, it omits the first stages of the metastatic cascade: Detachment from the primary tumour and intravasation. For GC, this model is not well suited due to the most common form of metastasis being peritoneal dissemination (Mura and Verdelli, 2016) not hematogenous metastasis. Other groups have attempted to use orthotopic models, however the engraftment success of GC in mice is very low (Nguyen et al., 2017) and they are accurate representations are cells or PDXs are injected into the outer serosa layer of the stomach (Busuttil et al., 2018). This leaves only the thin outer serosa layer for the cells to invade through, whereas, in human GC the cells have to invade through all layers of the stomach wall in order to metastasize. Therefore, our model of GC peritoneal dissemination was the most accurate *in vivo* model available to us until better models of cancer metastasis are developed.

While the *in vivo* model involves directly injecting GC cells into the peritoneum, thus replicating the later stages of the metastatic cascade, the *in vitro* migration and invasion assays reflect the earlier stages. Therefore, the

migration and invasion regulation by FZD7 observed in these *in vitro* experiments may be most important in these earlier stages to allow GC cells to leave the primary gastric tumour. Whereas, the *in vivo* assay was focused at the end stage of metastasis when the cancer cells seed at a new site. Therefore, the difference in the *in vitro* and *in vivo* results may be reflected in the fact that Wnt regulation is different at the beginning of metastasis to that at the end and thus require different signalling. Our results could suggest that Wnt/FZD7 may regulate the very early stages of metastatic GC cells leaving the primary tumour. To investigate this, researchers require a new genetically engineered mouse model (GEMM) in which the primary tumour develops through to the invasive stages with mice developing metastasis to clinically relevant secondary sites. At present these GEMMs do not exist.

4.4 Conclusions

GC metastasis remains a huge public health burden and a number of fundamental questions concerning the mechanism of GC metastasis are still unanswered. The findings presented in this chapter have provided a path to reduce GC cell migration, invasion, and activation of EMT via targeted inhibition of Wnt receptors, both genetically and pharmacologically. We have demonstrated that modulating the Wnt signalosome can further regulate Wnt signalling irrespective of downstream mutations and, therefore, reduce GC cells ability to metastasis. We have also highlighted the urgent need to investigate the TME to understand the complex cross-talk enabling metastasis. These findings provide an attractive target, FZD7, as a novel therapeutic target for the treatment of advanced and metastatic GC.

Chapter 5: Characterization of $RNF43^{-/-}$ / $ZNRF3^{-/-}$

5.1 Introduction

Wnt-induced ß-catenin-mediated transcription is a well-established driving force for stem cell self-renewal during adult tissue homeostasis, with aberrant Wnt/ß-catenin signalling being a major player behind tumourigenesis. In the previous chapters we have shown that modulation of the Wnt signalosome can inhibit constitutively activated Wnt signalling regardless of the mutational status of downstream components such as APC. This chapter investigates how Wnt is deregulated at the level of the receptor/ligand, we have previously shown that FZD7 plays an important role in GC but how FZD7 is becoming deregulated is still not known. A key player involved in FZD regulation and located at the plasma membrane are the E3 ligases RNF43 and ZNRF3. RNF43 regulates FZD turnover on the plasma membrane and therefore this chapter aims to investigate the functional significance of RNF43/ZNRF3 loss of function in the context of GC.

In a non-pathological setting RNF43, and its functional homologue ZNRF3, act as a negative regulator of the Wnt pathway and play as role as a tumour suppressor (Koo et al., 2012); RNF43 and ZNRF3 share moderate sequence conservation of 39% identity between the two proteins (Zebisch et al., 2013). In the absence of R-spondin (agonist of the canonical Wnt signalling), activation of the Wnt signalling pathway results in activation of target genes, including RNF43/ZNRF3 (Hao et al., 2012). Upon their integration on the cell membrane, they ubiquitinate the Wnt receptor complex, FZD-LRP5/6, which leads to its internalization and lysosomal degradation (Figure 5.1) (Koo et al., 2012); this negative feedback loop functionally limits Wnt signalling. This was demonstrated by simultaneous knockout of RNF43 and ZNRF3 in the mouse intestine which resulted in strong proliferation of the stem cell compartment (Koo et al., 2012). Interestingly, loss of function (LOF) mutations to RNF43/ZNRF3 only lead to Wnt hypersensitivity in the respective cell, and therefore, the presence of Wnt is still essential to induce hyperproliferation. This is in contrast to LOF mutations to other Wnt pathway negative regulators

downstream of the receptor complex, such as APC, whose downregulation or complete knockout can autonomously promote cell proliferation (Hao et al., 2012; Koo et al., 2012), however this can still be modulated by activation/inhibition of upstream components such as Wnt ligands and FZD receptor availability(Flanagan et al., 2019c). Additionally, RNF43 can itself be targeted for removal from the cell surface through interaction with R-spondin, whereby it forms a tertiary complex with Lgr4/5 which induces ubiquitination and membrane clearance of RNF43. This results in increased cell surface level of FZD and the re-accumulation of Wnt at the cell surface with consequent enhancement of Wnt signalling (Serra and Chetty, 2018). Additionally, it has been shown that RNF43 physically interacts with TCF4 in cells and tethers TCF4 to the nuclear membrane, thus silencing TCF4 transcriptional activity even in the presence of constitutively active mutants of ß-catenin (Loregger et al., 2015). This demonstrates multiple mechanisms in which RNF43 can mediate Wnt signalling, both upstream and downstream of intracellular Wnt pathway components.

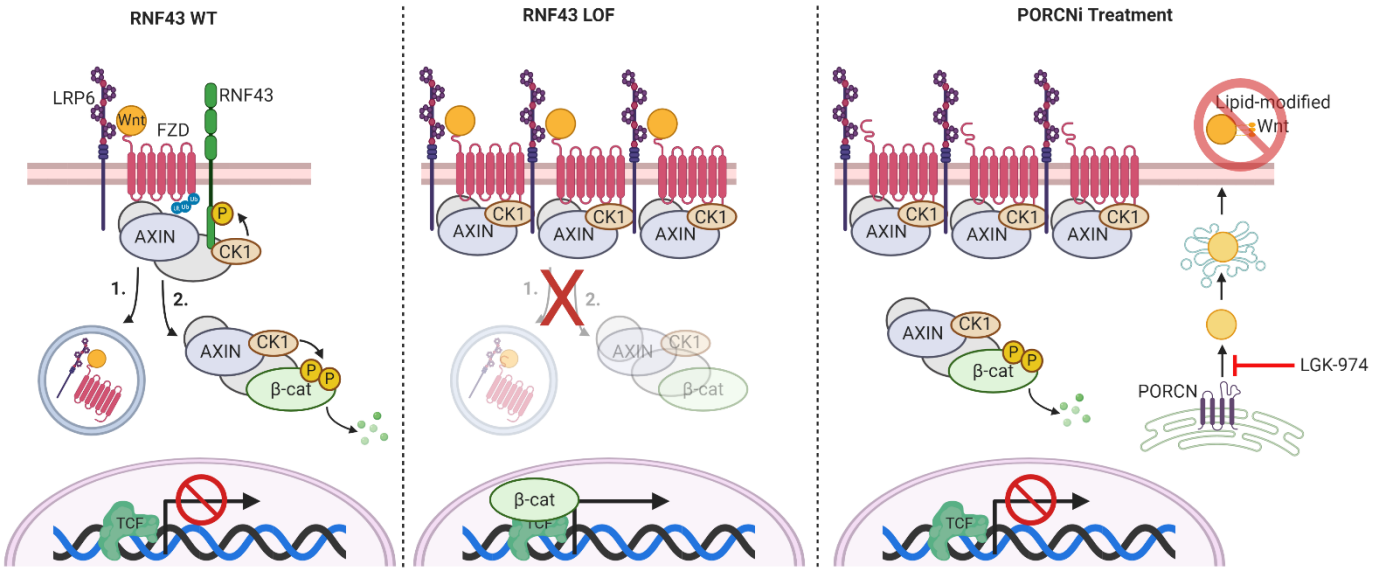


Figure 5.1 PORCNi mechanism of action in LOF RNF43 cells. (**Left**) RNF43 performs a bifunctional tumour suppressor role by 1. Targeting Wnt receptors for endocytosis and lysosomal degradation, and 2. By transiently interacting with the destruction complex to reconstitute its activity in the cytosol and re-establish Wnt pathway inhibition. (**Middle**) LOF mutations prevent RNF43 function at the plasma membrane, leading to Wnt receptor overexpression and consequently hypersensitivity of cancer cells to Wnt. (**Right**) Wnts are post-translationally palmitoleated by PORCN in the ER, which is crucial for their interation with Wntless that transports them to the plasma membrane. PORCNi (Such as LGK-974) prevent the palmitoleation of all Wnts, thus preventing their interation with Wntless and subsequently their transport. Therefore, despite overexpression of FZD receptors there are no availible Wnts to initiate Wnt signalling and Wnt target genes are not transcribed.

Mutations in *RNF43* have been reported in several solid cancers, such as colorectal (Eto et al., 2018; Lai et al., 2019; Yan et al., 2017a), gastric (Wang et al., 2014b), pancreatic (Jiang et al., 2013), ovarian (Ryland et al., 2013), and endometrial (Giannakis et al., 2014). Inactivation of RNF43 through *RNF43* mutation is one of the primary causes of permanent activation of the Wnt signalling pathway through enhanced FZD receptor expression (Serra and Chetty, 2018). Identified RNF43 mutations are most commonly truncating events: non-sense mutations and frame-shift mutations, consistent with the tumour suppressor role of RNF43. There are two recurrent hotspot mutations within *RNF43*: G659fs and R117fs, accounting for ~50% of RNF43 mutations in colon cancer (Giannakis et al., 2014). Since RNF43 are often in tumours with high mutational burdens, they may not be considered driver mutations, however due to their frequency in many cancers, especially GC, colorectal and endometrial, RNF43 mutations may confer a fitness advantage (Giannakis et al., 2014)

Whole-genome sequencing has revealed that RNF43 is frequently mutated and subsequently downregulated in GC (Wang et al., 2014b). The most common mutation hotspots for RNF43 are located close to the microsatellite instability (MSI) loci and may explain the high frequently of RNF43 mutations in GC, due to GCs commonly being deficient in DNA mismatch repair genes, MLH1 (Shen et al., 2018). This is reflected by observation that the MSI subtype of GC has a 10-fold higher mutation frequency in RNF43 than that of microsatellite stable (MSS) subtype (54.6% versus 4.8%). Additionally, 62.5% of RNF43 mutations in GC were truncating (Figure 5.2) (Wang et al., 2014b). These data and RNF43's role as a part of the Wnt/β-catenin negative feedback loop suggests that RNF43 inactivation may result in deregulated Wnt activity in GC, thus contributing to GC initiation and progression.

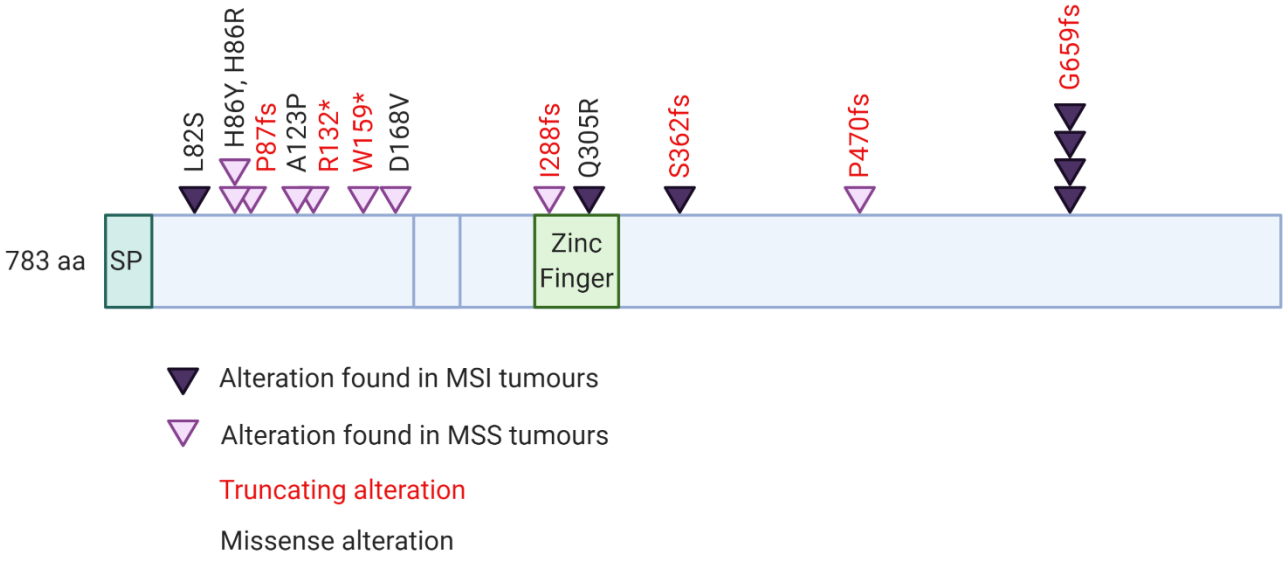


Figure 5.2 Distribution of protein alterations encoded in *RNF43* in GC. Generated from TCGA dataset on cBioportal. Conserved domain mapping is from UniProt. SP, signal peptide; MSI, microsatellite instability; MSS, microsatellite stable. Multiple arrows indicate high frequency of mutation in gastric tumours.

The expression level of RNF43 is significantly correlated with the stage of tumour: Low RNF43 expression is associated with low histological differentiation, bigger tumour size, deeper invasion, advanced TNM stage, and poorer prognosis for the patient (Niu et al., 2015). The poor prognosis may be linked to the fact that RNF43 has been shown to inhibit chemotherapy resistance *in vitro*, and this protective mechanism is eliminated by the loss of RNF43 (Gao et al., 2017b). Furthermore, the protecting effect of RNF43 by inhibiting the self-renewability of GC stem cells, could be partially reversed by adding R-spondin and Wnt5a *in vitro* (Gao et al., 2017b). This further supports the concept of Wnt pathway overexpression leading to GC initiation and progression.

Gastric tumours harboring RNF43 mutations become hypersensitive to Wnt due to the increased cell surface expression of FZD receptor, making FZD receptors an attractive target for treatment of tumours harbouring RNF43/ZNRF3 mutations. Furthermore RNF43 mutational status could potentially be used as a biomarker to identify Wnt-dependent tumours which would respond to treatment (Hao et al., 2016). Therefore, inhibition of Wnt secretion, via a PORCN inhibitor, is a potential therapeutic strategy for counteracting the overexpression of FZD receptors on the cell surface due to LOF mutations to RNF43/ZNRF3. However, the functional significance of RNF43/ZNRF3 first needs to be fully understood in the context of GC.

There have been very few functional investigations into RNF43's role in GC. One study has reported, through both gain- and loss-function assays that RNF43 could suppress cell proliferation and was negatively correlated with Lgr5 (Niu et al., 2015). This is supported by a more recent study which demonstrated that loss of endogenous RNF43 function enhances proliferation and tumour growth in GC (Neumeyer et al., 2019). Initially endogenous expression of RNF43 was depleted *in vitro* via transfection of GC cells with RNF43 targeted shRNA. Loss of endogenous RNF43 increased the proliferative and invasive capacity of GC cells. The effect of RNF43 loss in GC cells was also investigated *in vivo* via

xenograft models. Mice injected with RNF43-deficient GC cells developed larger tumours than those derived from respective sh-control cells. This confirmed that loss of RNF43 enhanced tumour growth. Interestingly, loss of RNF43 function *in vivo* lead to gastric hyperproliferation, and gastric organoids derived from the stomach of RNF43 mutant mice grew bigger than organoids from gastric tissue of WT-mice, suggesting that RNF43 loss leads to a hyperproliferative phenotype in the stomach. However, the authors only generated a robust mouse model using the Cre-loxP system for the intestine by introducing two *loxP* sites flanking the exon VIII that encode for the RING domain (Koo et al., 2012) and crossing with mice specifically expressing Cre in the intestinal epithelium. For the *in vivo* experiments examining RNF43 loss in the stomach, mutations were introduced via targeted mutagenesis; the first mutation was a 57 bp deletion in exon 8 of RNF43 and the second was two point mutations in the RING domain. While these mutations have been demonstrated to transactive Wnt signalling *in vitro* (Loregger et al., 2015) this has not been confirmed *in vivo* and is not confirmed to cause LOF to RNF43. Importantly, it has still not been functionally demonstrated that the phenotypes associated with conditional deletion of RNF43 is due to deregulation of FZD and whether the phenotypes can be rescued with co-deletion of RNF43 and a FZD gene. This would allow further characterization of GC due to loss of RNF43 and help identify which other members of the Wnt signalosome may be working together to lead to GC.

Therefore, this chapter begins to examine this by investigating the functional significance of RNF43/ZNFR3 mutations in gastric cancer development by characterization of tumours from a novel mouse model in which RNF43/ZNFR3 are conditionally deleted in the gastric epithelium.

5.2 Results

5.2.1 *RNF43/ZNFR3* knockout generated gastric epithelium phenotype.

To investigate the function of *RNF43* and *ZNFR3* in the gastric epithelium, we crossed floxed *RNF43/ZNFR3* mice with Claudin18CreER^{T2} mice to enable deletion of *RNF43*, *ZNRF3* or both *RNF43* and *ZNRF3* specifically in the gastric epithelium (Figure 5.3). Claudin18 is a highly specific tight junction component of the stomach and is specifically expressed within the gastric mucosa (Coati et al., 2019). The *RNF43* gene has a loxP site inserted upstream of the exons encoding the RING finger domain and the *ZNRF3* gene has a loxP site inserted between the exons encoding the RING domain as well as a loxP site downstream of these exons. This were inserted via homologous recombination. There is no effect on *RNF43* or *ZNRF3* gene function until the locus is recombined and the mice can be bred to homozygosity.

The addition of a dTOM cassette was inserted after a STOP cassette to allow observation of recombination. Upon induction recombination was observed almost exclusively in the gastric epithelium, in both the corpus and the antrum regions (Figure 5.4). A few cells were observed in the Brunner glands; these are located in the duodenum and provide a protective function against the acidic content from the stomach by the production of mucous (Krause, 2000).

Mice were bred to generate the following cohorts: Claudin18CreERT2; *Rnf43*^{flox}; *Znrf3*^{WT}; *dTOM*^{LSL} , Claudin18CreERT2; *Rnf43*^{WT}; *Znrf3*^{Flox}; *dTOM*^{LSL} mice, and Claudin18CreERT2; *Rnf43*^{flox}; *Znrf3*^{Flox}; *dTOM*^{LSL} mice.

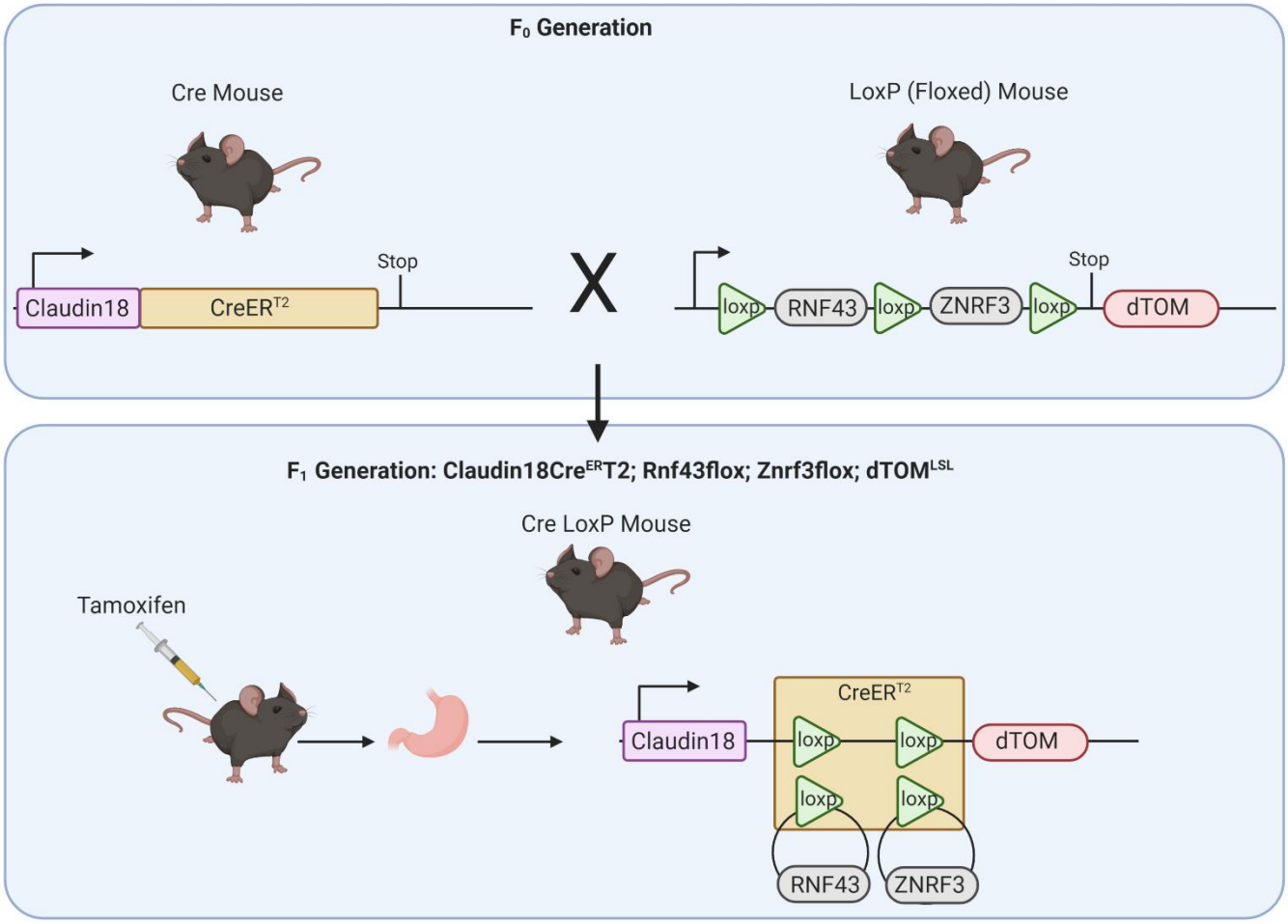


Figure 5.3 Breeding strategy for Claudin18CreERT2; Rnf43^{flox}; Znrf3^{flox}; dTOM^{LSL} mice. In the Cre mouse, the expression of Cre is under the control of a Claudin18 promoter that is specific to the cells of the gastric epithelium. The floxed target gene mouse contains LoxP sites flanking RNF43 and ZNRF3 and a dTOM reporter downstream of a STOP cassette. When the two mouse lines are bred and induced with tamoxifen the Cre enzyme recognizes the LoxP sites and deletes RNF43 and ZNRF3 only in the gastric epithelium. The target gene remains floxed and theoretically functional, in all other tissues. Cells where recombination has occurred can be tracked by the dTOM reporter.

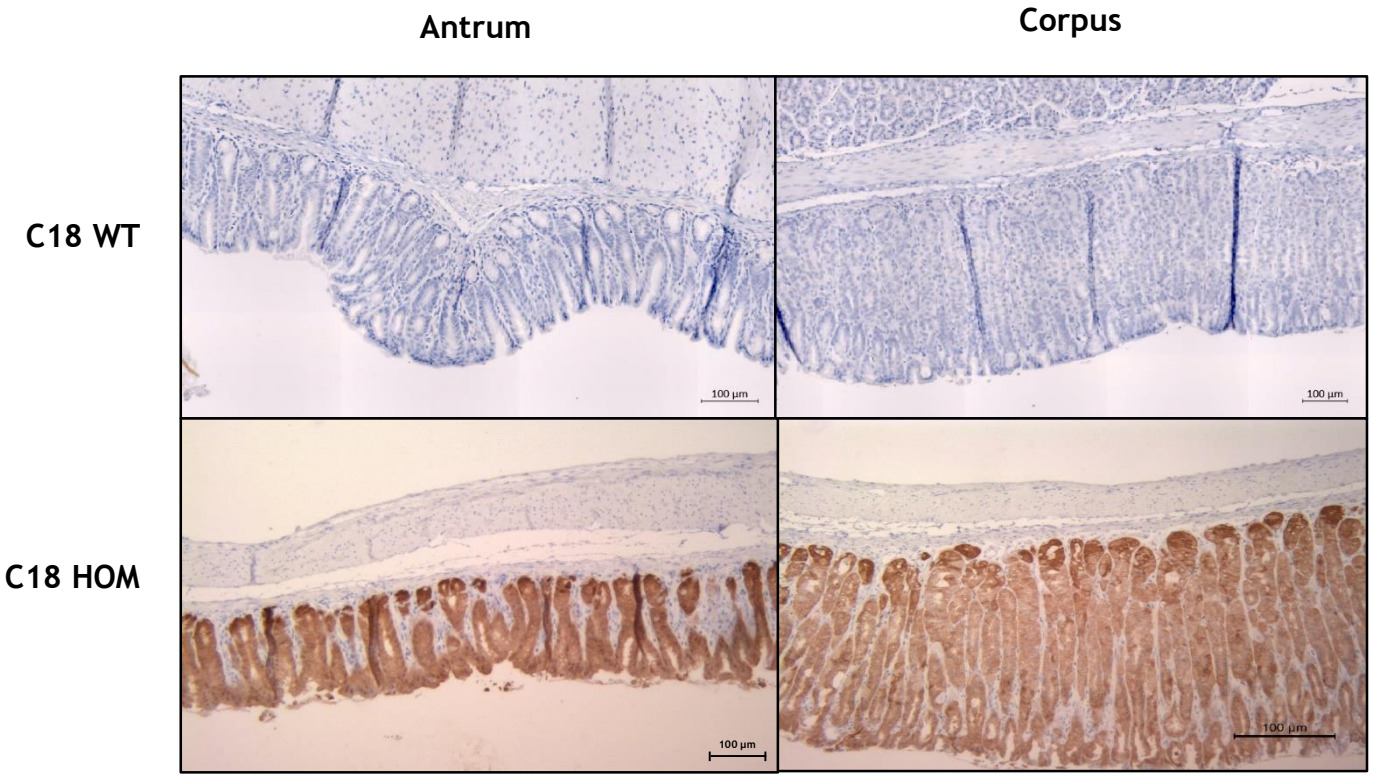


Figure 5.4 Recombination is exclusive to the Cre^{+} gastric epithelium cells. Immunohistological staining for red florescent protein in the gastric epithelium following short-term induction with tamoxifen. No staining was observed in gastric epithelium of mice who were negative for Cre. Full recombination was observed in both the antral and corpal glands in Cre^{+} mice. Scale bar= 100 µm

Mice were induced with tamoxifen 1x daily for 4 days. 140 days after induction, *Claudin18*CreER^{T2}; Rnf43flox; Znrf3flox; dTOM^{LSL} mice homozygous for *RNF43* and *ZNRF3* began to show signs of sickness, such as: cold feet, rough coat, hunched posture and weight loss. Mice from all cohorts were sacrificed and dissected at this 140-day time point. The gastric epithelium from *RNF43*$^{-/-}$/ *ZNRF3*$^{-/-}$ mice displayed a phenotype unique from WT mice. The gastric epithelium of *RNF43*$^{-/-}$/ *ZNRF3*$^{-/-}$ mice showed signs of uncontrolled proliferation as the surface was significantly raised with ridges and lumps indicative of gastric tumours (Figure 5.5). Mice homozygous for a single E3 ligase (RNF43 or ZNRF3) showed a less severe phenotype with the presence of metaplasia, with the biggest change compared to WT mice observed in the corpus region (Figure 5.5). Stomach weight relative to total body weight were calculated per mouse. The *RNF43*$^{-/-}$/ *ZNRF3*$^{-/-}$ cohort had significantly heavier stomachs compared to the WT cohort. Both the *RNF43*$^{-/-}$ and *ZNRF3*$^{-/-}$ cohorts did not have a significant difference in stomach weight compared to the WT control cohort (Figure 5.6).

Hematoxylin and eosin (H&E) staining of the gastric epithelium of the *RNF43*$^{-/-}$ / *ZNRF3*$^{-/-}$ cohort displayed a substantial lack of differentiated cells in the corpus region and large growths compared to the WT cohort (Fig 5.7-9) Additionally the antrum region also displayed signs of uncontrolled proliferation and lack of differentiation within the gastric glands (Figure 5.7-9). H&E staining did not reveal any gross differences between the epithelium of the WT mice and the single *RNF43*$^{-/-}$ or *ZNRF3*$^{-/-}$ cohorts (Figure 5.7). However, tumourigenic lesions were observed in *RNF43*$^{-/-}$ (Figure 5.8-9) suggestive of early neoplasms.

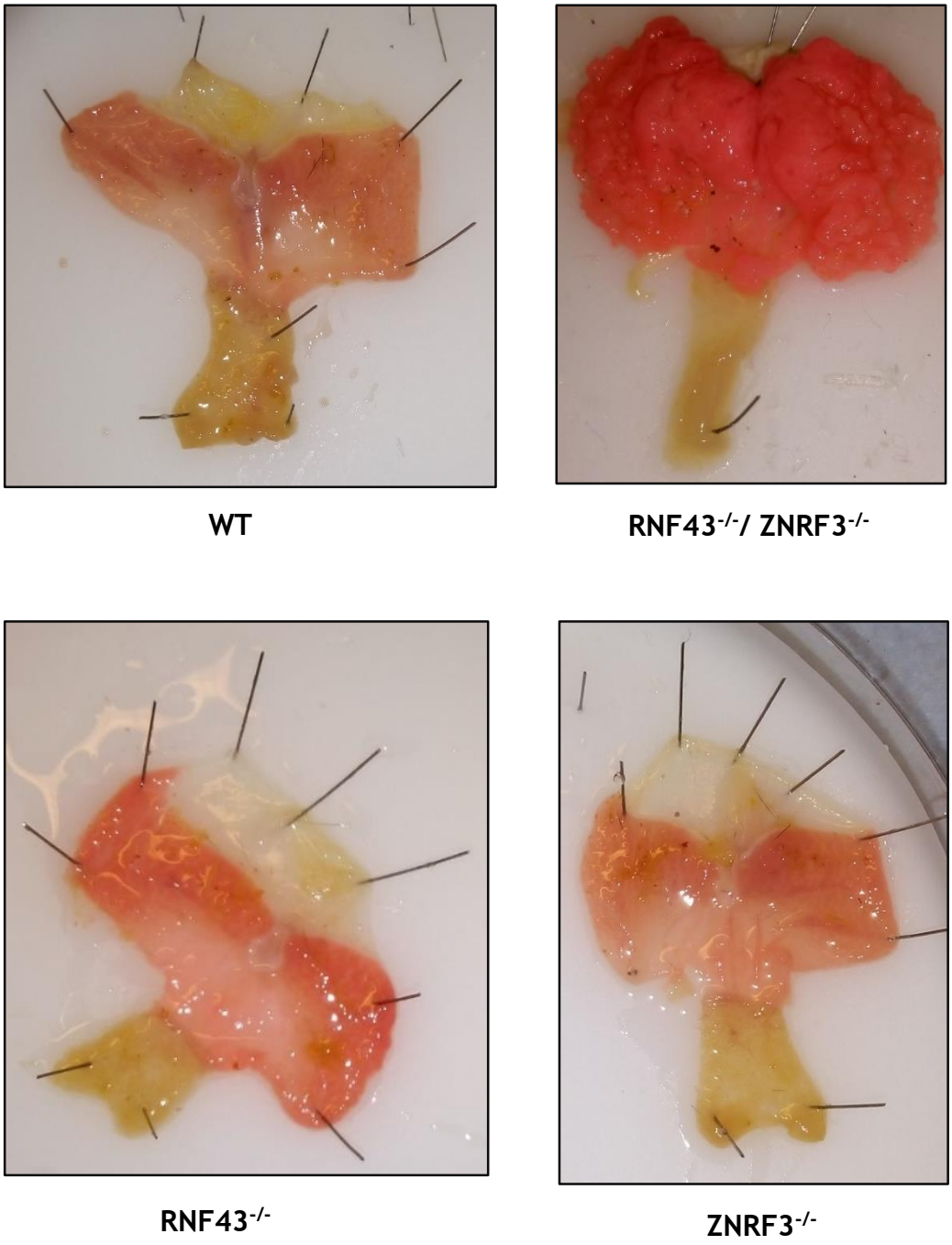


Figure 5.5 Stomach dissections from 140 post-induction mice. 140 days following induction with tamoxifen mice were sacrificed and their stomachs harvested and dissected. Stomachs from RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice had evidence of gastric tumours and a thick layer of mucous. Stomachs from the single knockout mice displayed evidence of mild metaplasia.

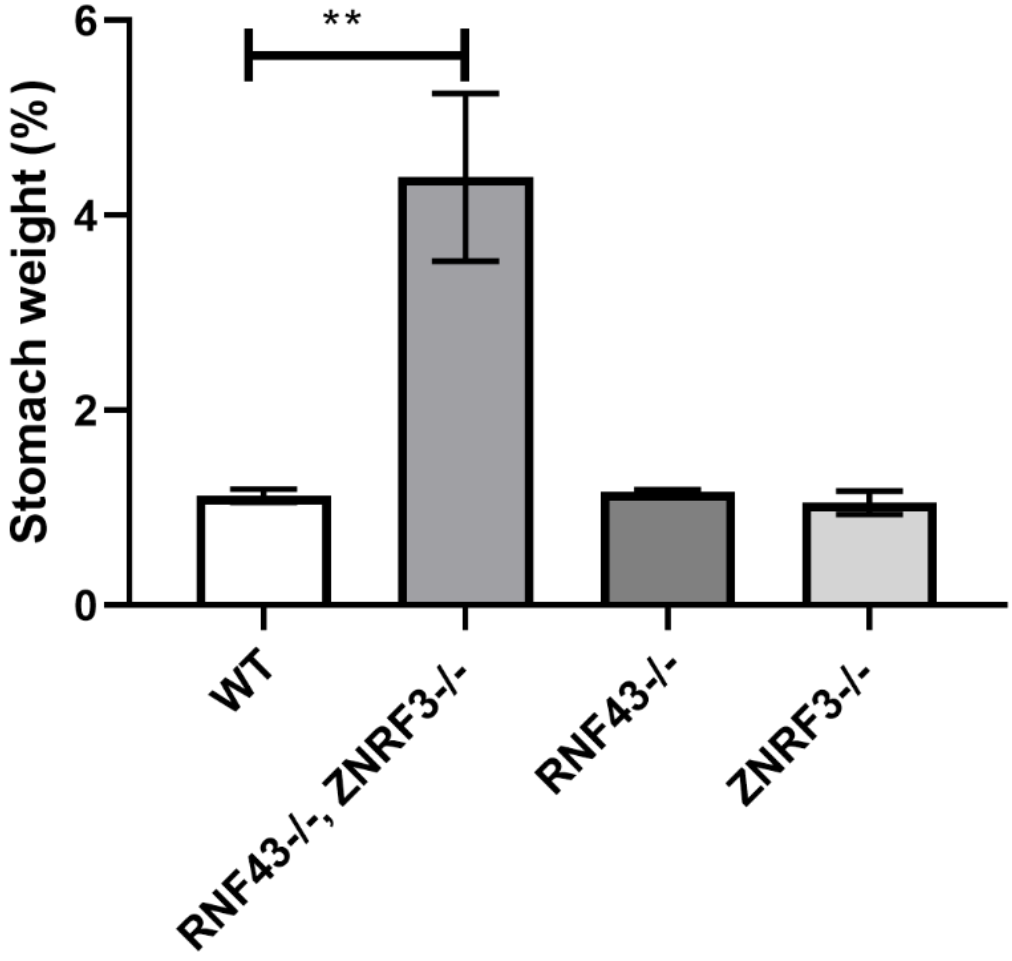


Figure 5.6 Average stomach weight. Mice were weighed before being sacrificed and their final body weight was recorded. Harvested stomachs were weighted and the stomach weight as a percentage of their body weight was calculated. RNF43$^{-/-}$ / ZNRF3$^{-/-}$ stomachs had significantly larger stomachs compared to WT control. (**= $p \leq 0.01$, mean ± SEM, t-test, n=6 mice per cohort).

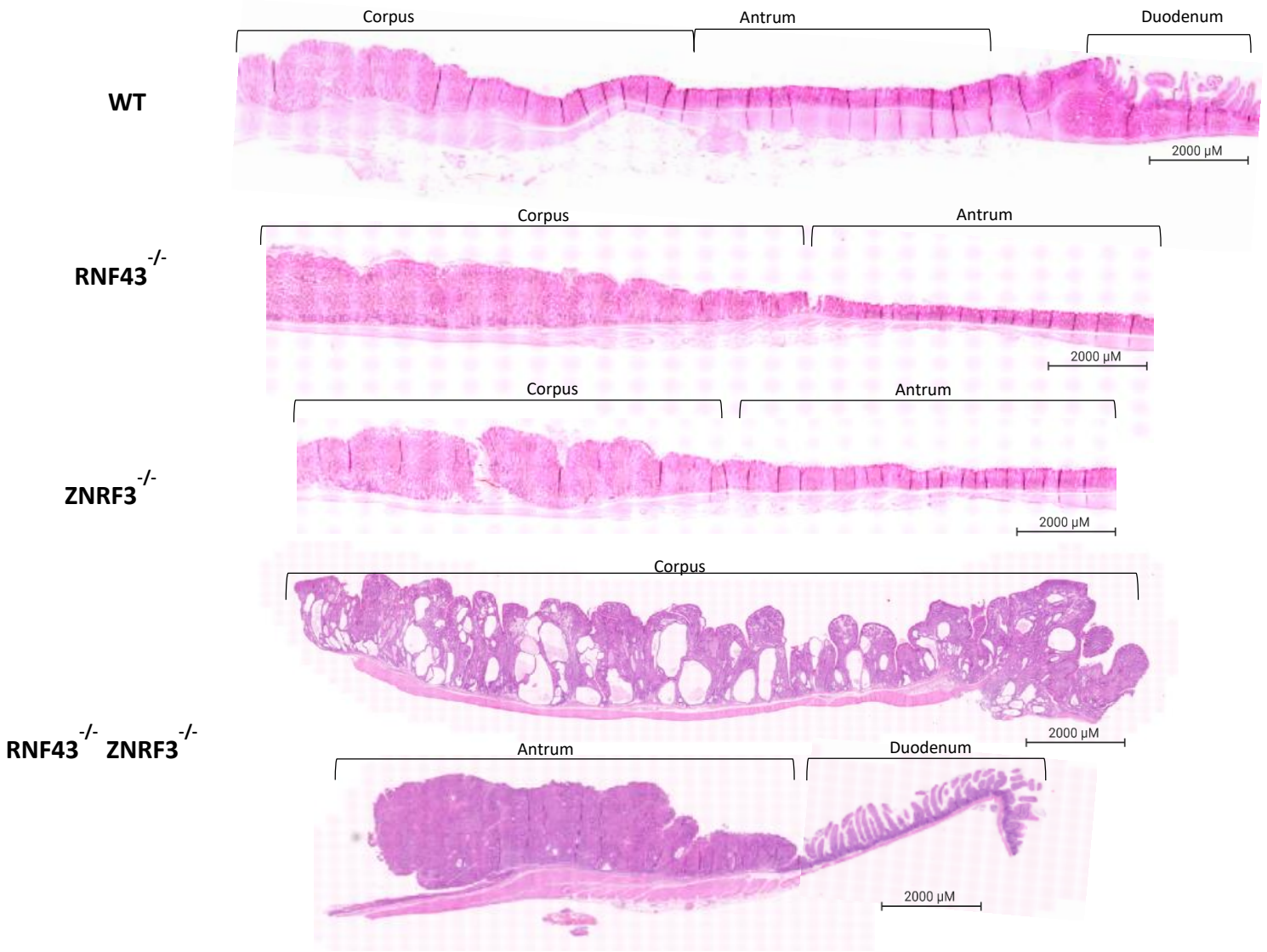


Figure 5.7 Tumours present in the gastric epithelium of RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice. H&E staining of the gastric epithelium from WT, RNF43$^{-/-}$, ZNRF3$^{-/-}$ and RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice. Presence of tumours can be seen in the corpus & antrum region of the RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice, Scale bar= 100 µm.

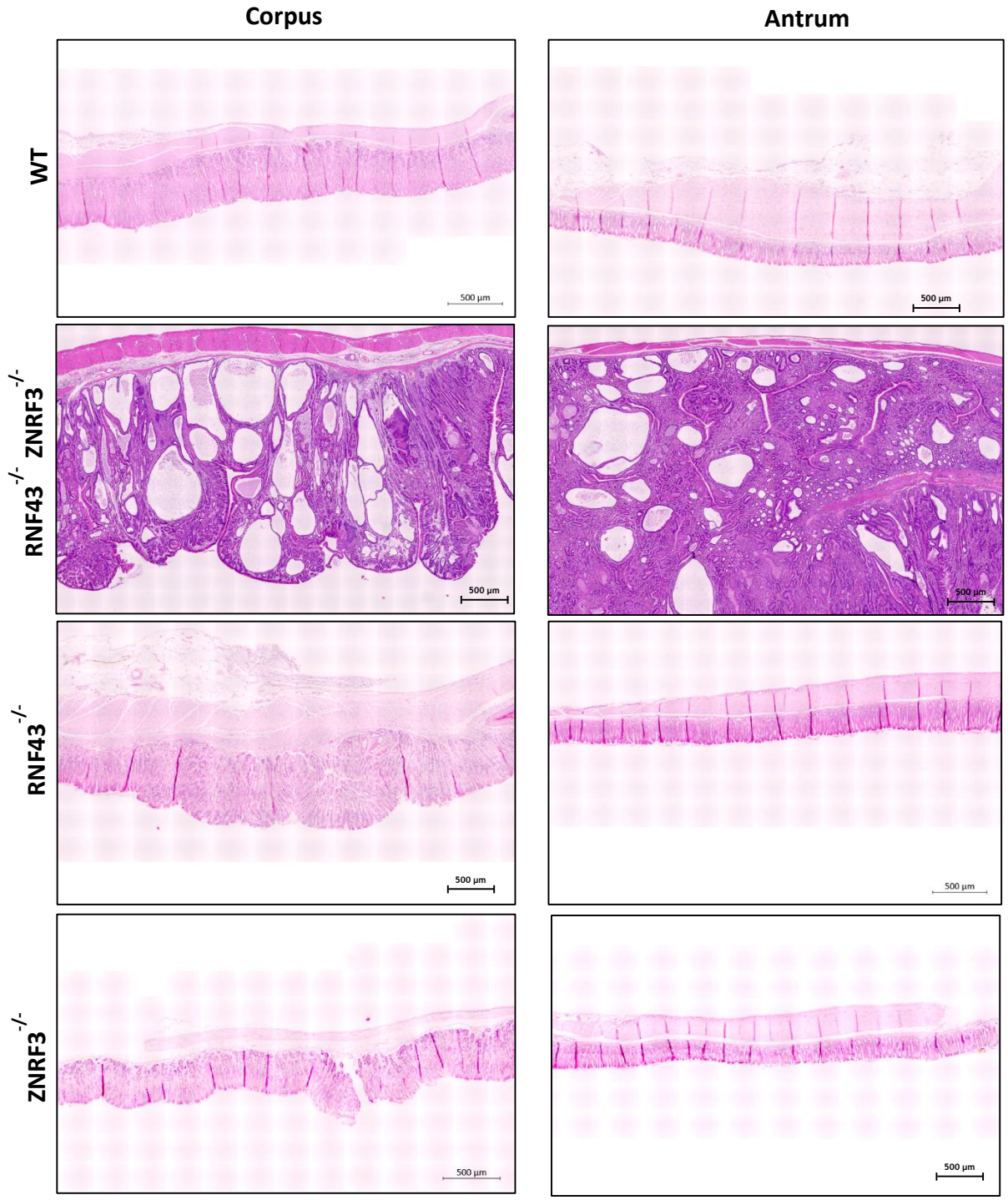


Figure 5.8 Tumours present in the gastric epithelium of RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice. Additional images of H&E staining of the gastric epithelium from RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice. Presence of tumours can be seen in the corpus region of the and RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice and absence of tumours in the antrum region. Scale bar= 500 µm.

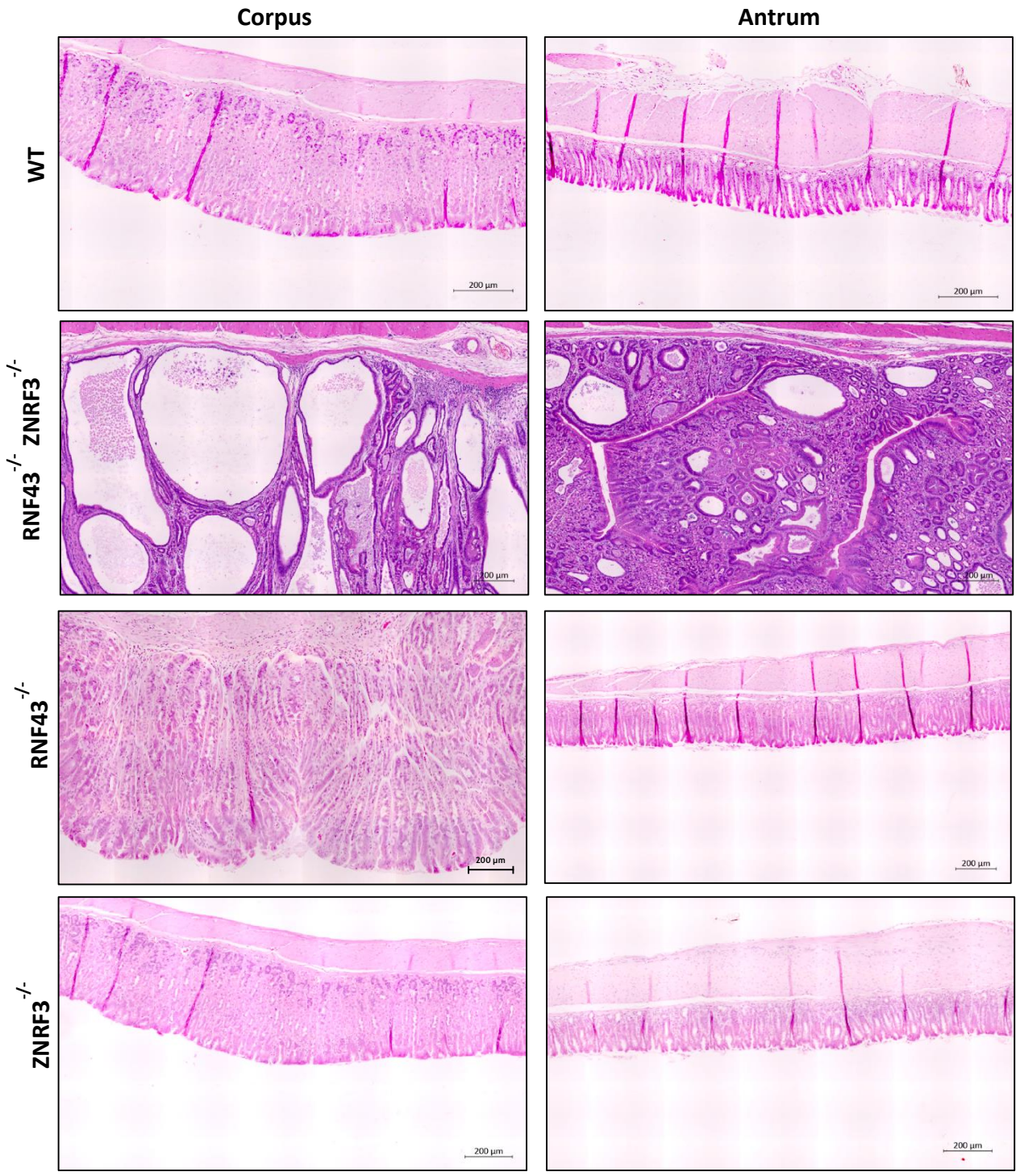


Figure 5.9 Tumours present in the gastric epithelium of RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice. Additional images of H&E staining of the gastric epithelium from RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice. Presence of tumours can be seen in the corpus region of the and RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice and absence of tumours in the antrum region. Scale bar= 200 µm.

5.2.2 Characterization of *RNF43*$^{-/-}$/ *ZNRF3*$^{-/-}$ gastric tumours.

The observation that the gastric epithelium in which *RNF43* and *ZNRF3* had been conditionally deleted displayed signs of increased proliferation and loss of differentiation indicated the possibility that gastric epithelial homeostasis is acutely disrupted and the initiation of GC has taken place. To further test this possibility sections from tamoxifen induced *Claudin1*8CreER^{T2} ; *Rnf43*$^{fl/fl}$, *Claudin1*8CreER^{T2}; *Znrf3*$^{fl/fl}$, and *Claudin1*8CreER^{T2}; *Rnf43*$^{fl/fl}$; *Znrf3*$^{fl/fl}$ mice were immunohistochemically labelled for makers of proliferation (PCNA), differentiation (H^+/K^+ ATPase), apoptosis (cleaved-caspase 3) and active Wnt signalling (ß-catenin). Sections from tamoxifen induced *Claudin1*8CreER^{T2}; *Rnf43*$^{fl/fl}$; *Znrf3*$^{fl/fl}$ mice stained for PCNA had increased expression throughout the whole gastric gland compared to tamoxifen induced Cre-negative control (Figure 5.10 & 5.12). This was quantified and demonstrated that *Rnf43*$^{fl/fl}$; *Znrf3*$^{fl/fl}$ mice had 80% PCNA-positive cells compared to 20% PCNA-positive in WT mice (Figure 5.11). PCNA expression was also increased in induced *Rnf43*$^{fl/fl}$ mice outside of the proliferation zones (Figure 5.12) and a significant increase in PCNA-positive cells compared to WT (Figure 5.11*);* this mislocalisation of proliferating cells is suggestive of a pro-neoplastic environment in the epithelium. No change in PCNA expression was observed in *Znrf3*$^{fl/fl}$ mice (Figure 5.10 & 5.12). This was reflected in quantification of PCNA-positive stained cells (Figure 5.11).

H^+/K^+ ATPase is a proton pump that is specific to the parietal cells, and therefore the corpal epithelium of the stomach. Additionally, parietal cell loss is a precursor to SPEM, a step in the pathogenesis of GC. As expected, sections from WT mice stained positively for H^+/K^+ ATPase expression in the corpus and negatively for H^+/K^+ ATPase in antrum (Figure 5.13). Sections from *Rnf43*$^{fl/fl}$ mice displayed a decrease in expression of H^+/K^+ ATPase on the luminal half of the corpal epithelium (Figure 5.10 & 5.14) confirmed by quantification showing an approximately 50% decrease of parietal cells compared to WT (Figure 5.11). No significant changes were observed in the expression of H^+/K^+ ATPase in the

Znrf3$^{fl/fl}$ mice (Figure 5.10 & 5.11). Sections from *Rnf43*$^{fl/fl}$; *Znrf3*$^{fl/fl}$ mice showed an almost complete loss of H^+/K^+ ATPase expression in the corpal tumours, illustrating loss of parietal cells (Figure 5.10 & 5.14). The percentage of positively-stained H^+/K^+ ATPase reduced from an average of 93% in the WT mice to an average of 5% in the *Rnf43*$^{fl/fl}$; *Znrf3*$^{fl/fl}$ mice (Figure 5.11).

Only sections from *Claudin18*CreER^{T2}; *Rnf43*$^{fl/fl}$; *Znrf3*$^{fl/fl}$ mice showed the presence of rare caspase-3 ('apoptotic') positive cells (Figure 5.10 and 5.11).

Staining for ß-catenin was similar between WT mice and *Znrf3*$^{fl/fl}$ mice, with mostly membrane staining (Figure 5.15). A significant increase in ß-catenin was observed in *Rnf43*$^{fl/fl}$ and *Rnf43*$^{fl/fl}$; *Znrf3*$^{fl/fl}$ mice which demonstrates active Wnt signalling (Figure 5.10 & 5.15). The largest change in ß-catenin was in the *Rnf43*$^{fl/fl}$; *Znrf3*$^{fl/fl}$ mice with 90% positive cells in (Figure 5.11). 30% of cells stained positivity for ß-catenin in the *Rnf43*$^{fl/fl}$ mice (Figure 5.11).

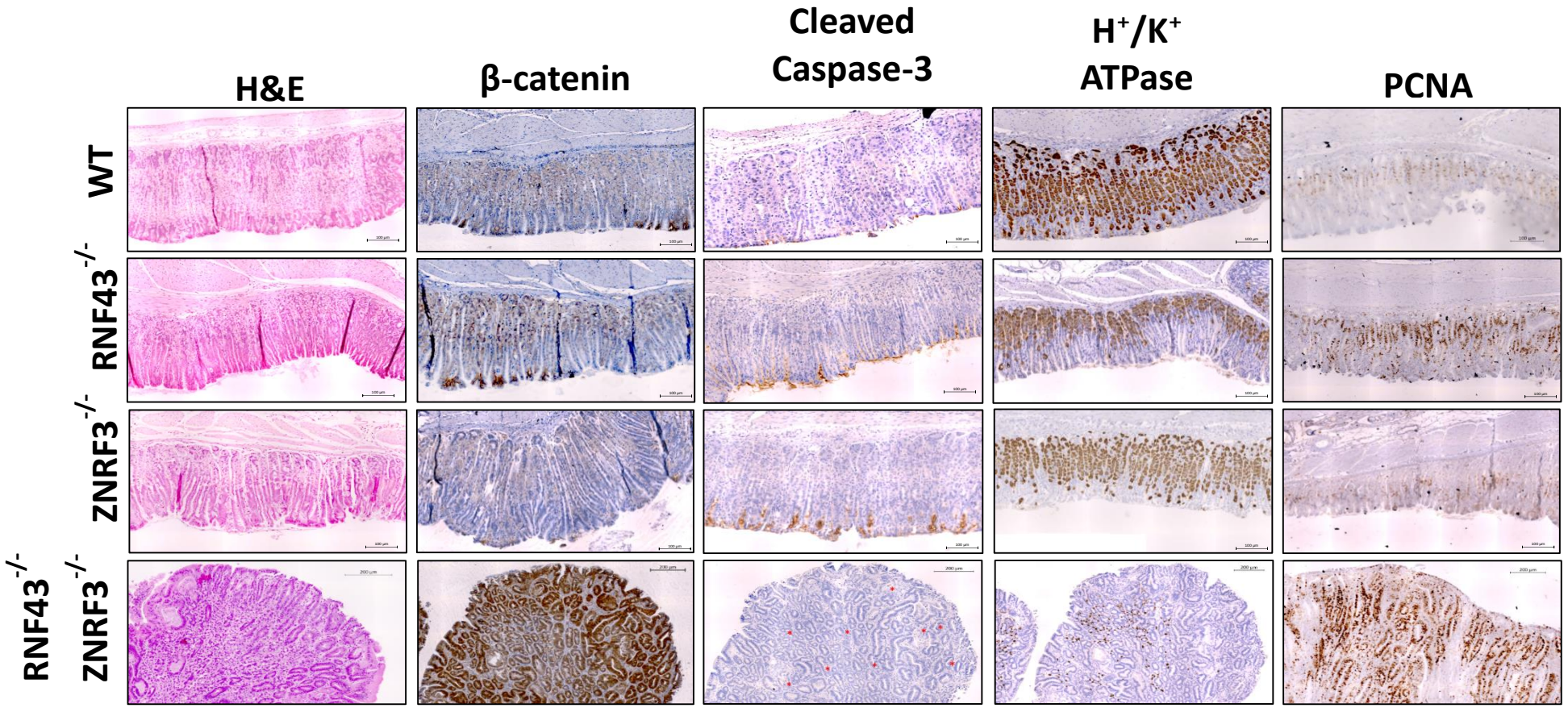


Figure 5.10 RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice display a GC phenotype. RNF43$^{-/-}$/ ZNRF3$^{-/-}$ mice showed the biggest change in expression of various markers compared to WT and single knockout cohorts. RNF43$^{-/-}$/ ZNRF3$^{-/-}$ tumours stained strongly for β-catenin and PCNA, showed evidence of rare caspase-3 positive cells and showed a decrease in expression of H^+/K^+ ATPase compared to WT mice, indicating parietal cell loss. Scale bar= 100/200 μm.

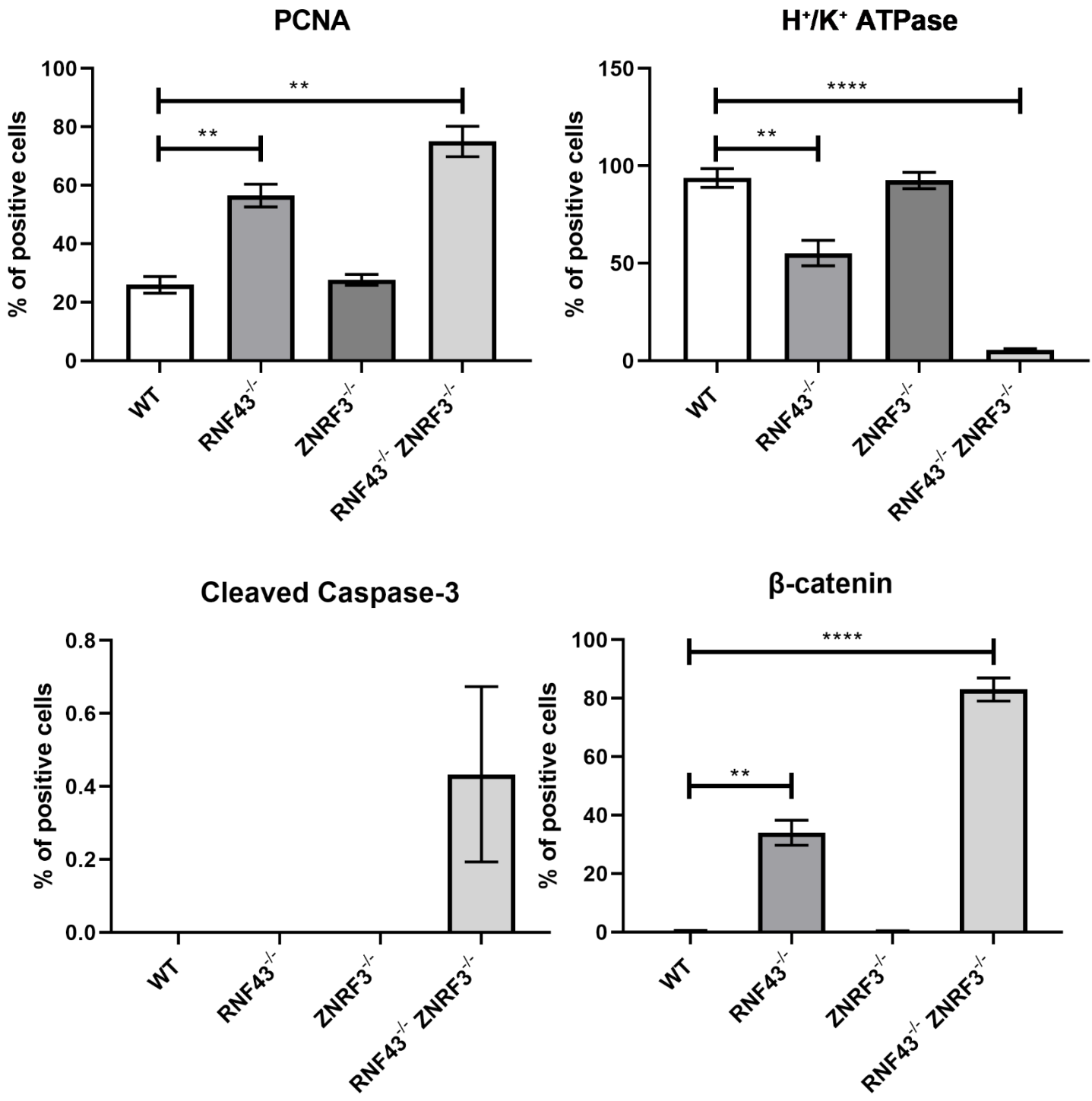


Figure 5.11 Loss of RNF43 leads to GC phenotype. Quantification of IHC stains from Figure 4.10. Cells were counted in three fields of view (1000 cells counted per view) and the percentage of positive cells calculated. (**=p≤0.01, ****= p ≤0.0001, mean ± SEM, t-test, n=3)

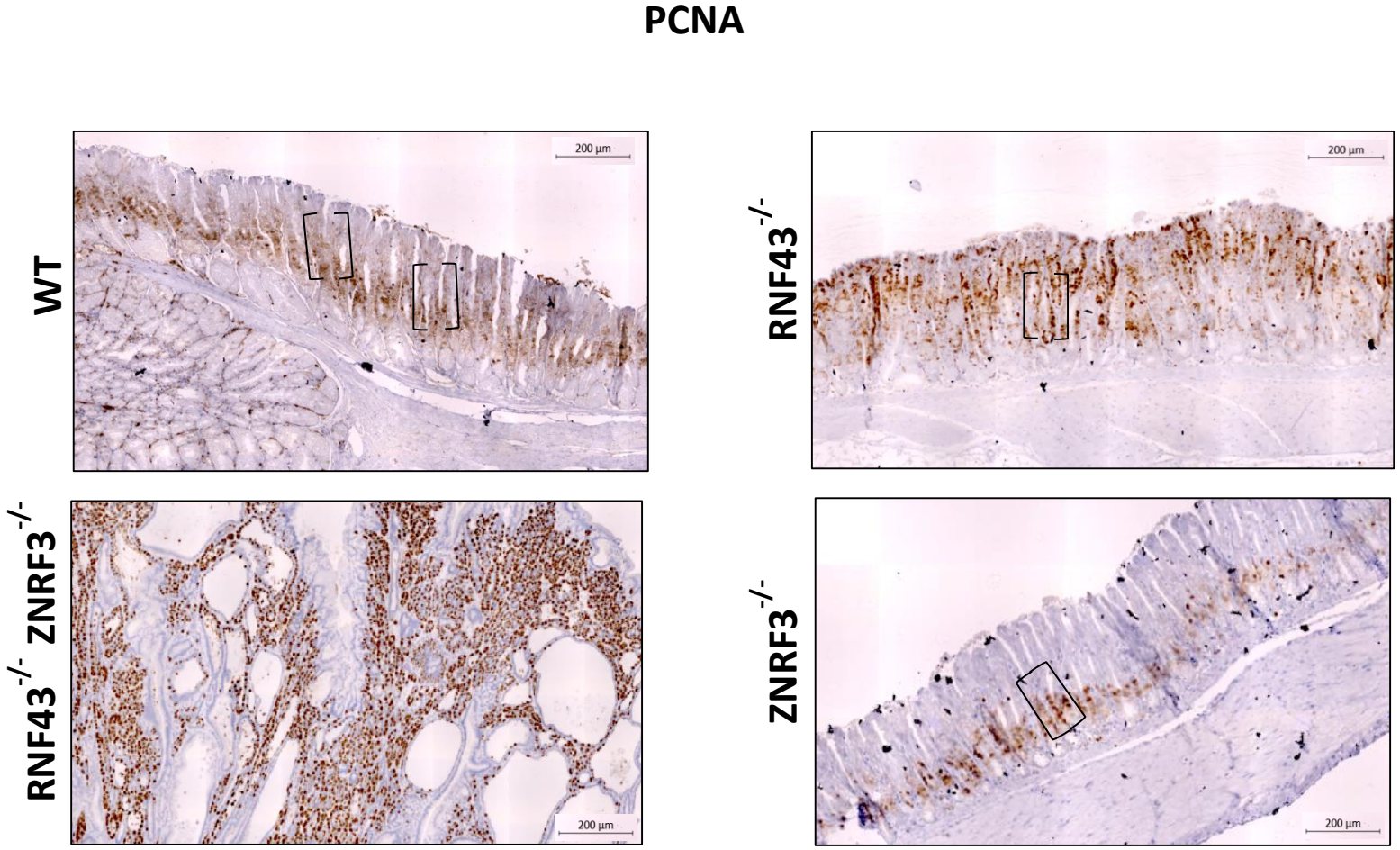


Figure 5.12 PCNA staining in *RNF43/ZNRF3*-deficient mice. Staining for PCNA, a marker of proliferating cells, in the gastric epithelium of WT, RNF43$^{-/-}$, ZNRF3$^{-/-}$, and RNF43$^{-/-}$ ZNRF3$^{-/-}$ mice. Abundant PCNA staining was observed in mice deficient in both RNF43 and ZNRF3. RNF43$^{-/-}$ mice displayed mislocalisation of proliferating cells outside the gastric proliferative zone. Brackets indicate gastric proliferative zone. Scale bar= 200 µm.

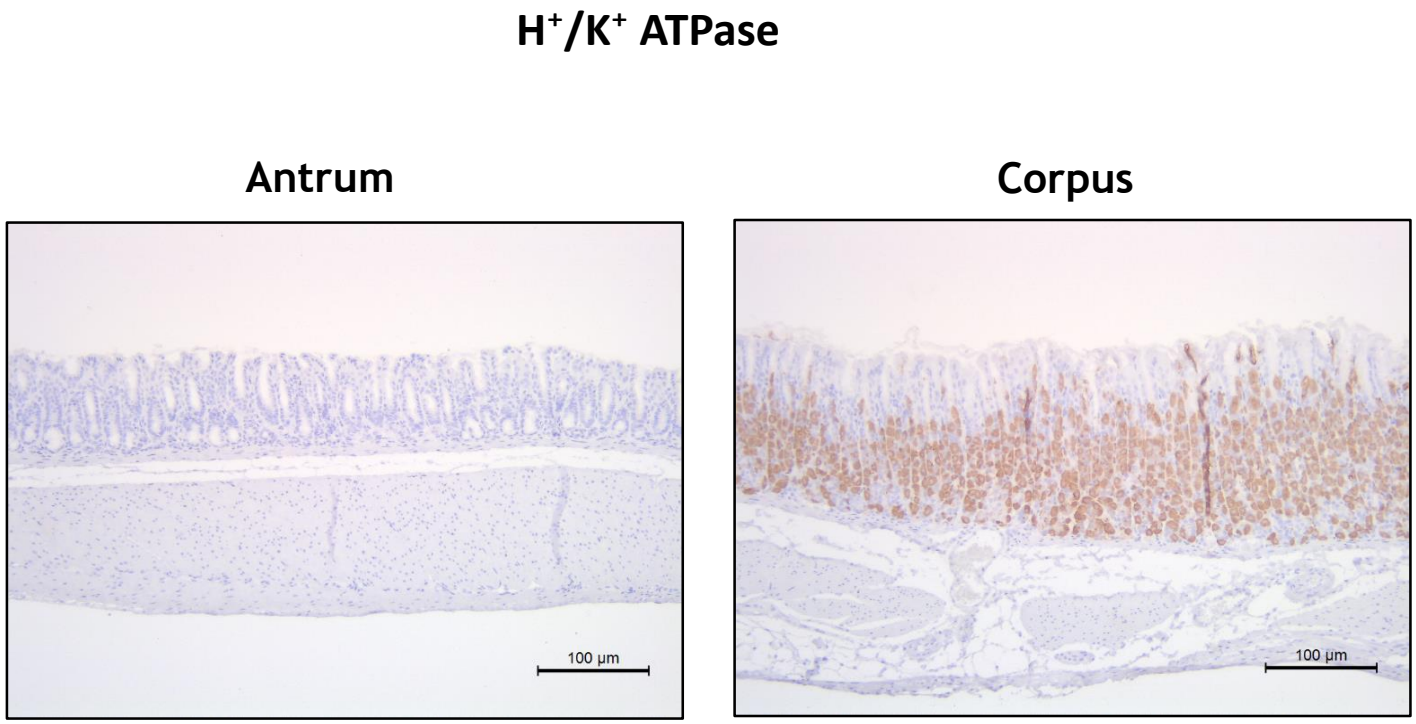


Figure 5.13 H^+/K^+ ATPase expression only present in the corpus region of gastric epithelium. Staining for H^+/K^+ ATPase in the gastric epithelium of WT mice. Staining only observed in the corpus region confirming the specificity of the antibody and validity of the ICH. Scale bar= 100 µm.

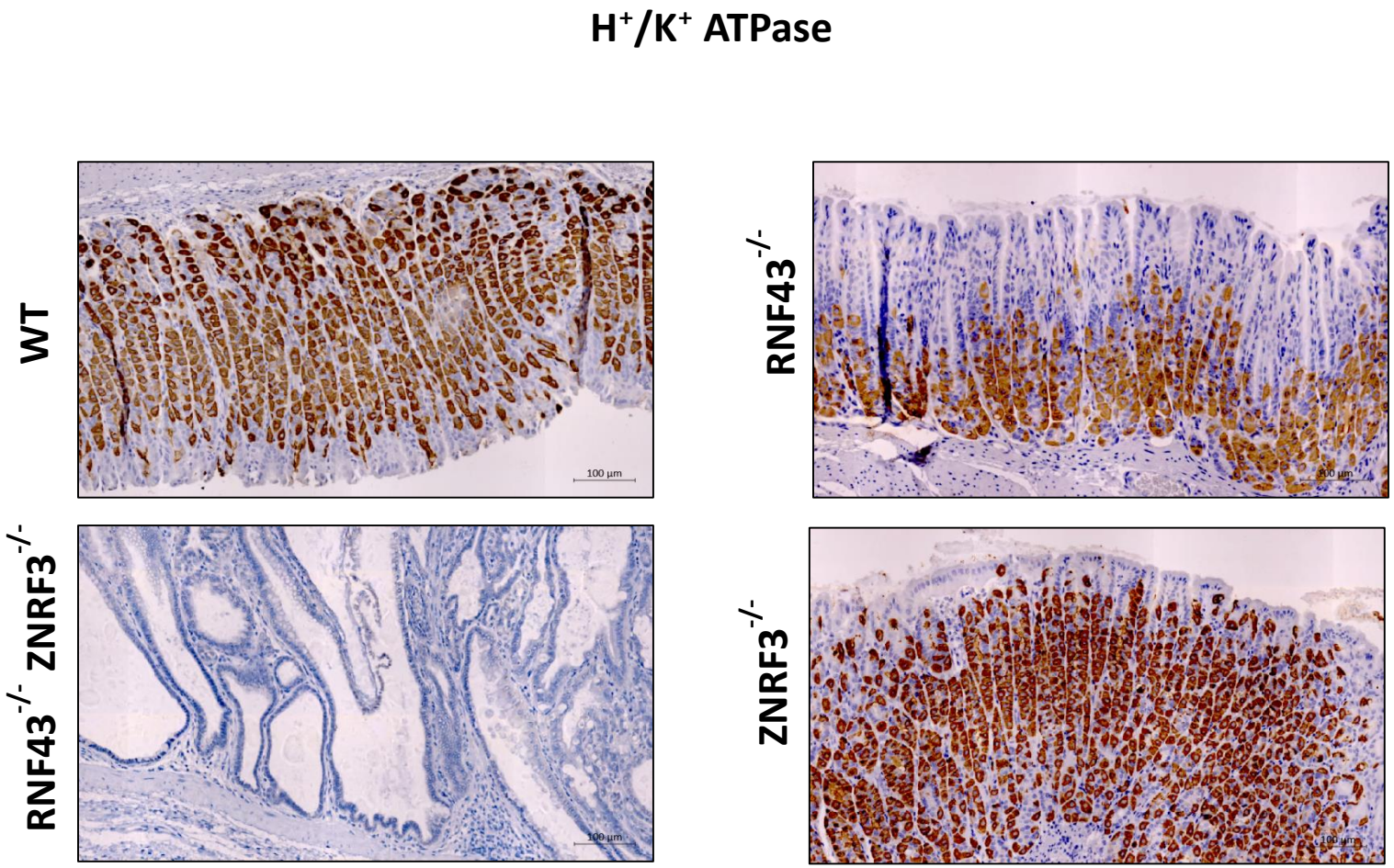


Figure 5.14 H$^+$/K$^+$ ATPase staining in *RNF43*/*ZNRF3*-deficient mice. Staining for H$^+$/K$^+$ ATPase, a marker of parietal, in the corpal epithelium of WT, RNF43$^{-/-}$, ZNRF3$^{-/-}$, and RNF43$^{-/-}$ ZNRF3$^{-/-}$ mice. RNF43$^{-/-}$ mice had loss of parietal cells in approximately 50% of the gland. RNF43$^{-/-}$ ZNRF3$^{-/-}$ mice displayed total loss of parietal cells. Scale bar= 100 µm.

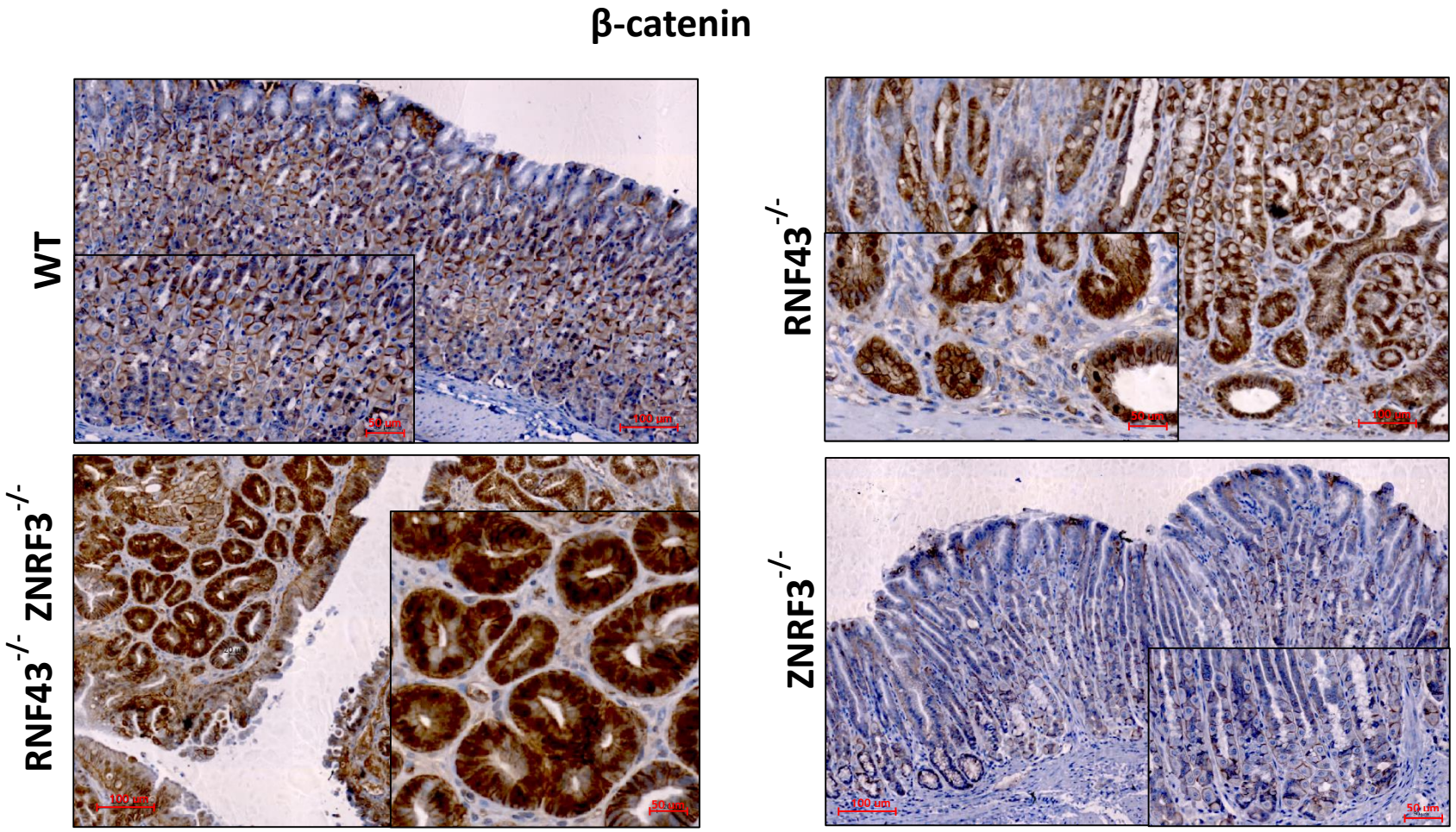


Figure 5.15 ß-catenin staining in RNF43-deficient mice. Staining for ß-catenin in the gastric epithelium of WT, RNF43$^{-/-}$, ZNRF3$^{-/-}$, and RNF43$^{-/-}$ ZNRF3$^{-/-}$ mice. Strong ß-catenin was observed in mice deficient in RNF43. Scale bar= 100/50 µm.

To confirm if aberrant Wnt signalling was contributing to the development of gastric tumours following conditional deletion of *RNF43* and *ZNRF3* in *Claudin1*8CreER^{T2} mice, the expression of several Wnt /ß-catenin target genes were analyzed. Total RNA prepared from extracted corpal epithelial cells from tamoxifen induced *Claudin1*8CreER^{T2}; Rnf43flox, *Claudin1*8CreER^{T2}; Znrf3flox, *Claudin1*8CreER^{T2}; Rnf43flox; Znrf3flox, and WT mice was analyzed by qRT-PCR. No significant changes were observed across all Wnt target genes (*Myc*, *AXIN2*, *CD44*, *LGR5*, and *CCND1*) in the *RNF43$^{fl/fl}$* or *ZNRF3$^{fl/fl}$* mice compared to tamoxifen induced WT control, suggesting that conditional deletion of just one E3 ligase is not sufficient to induce Wnt signalling (Figure 5.16). There was a significant increase in expression of all Wnt target genes (*Myc*, *Axin2*, *CD44*, *LGR5*, and *CCND1*) in the *RNF43$^{fl/fl}$; ZNRF3$^{fl/fl}$* mice compared to WT control (Figure 5.16). This suggests that conditional deletion of both *RNF43* and *ZNRF3* is sufficient to initiate aberrant Wnt signalling in the corpal epithelium, which may drive tumorigenesis.

As aberrant Wnt signaling often induces feedback mechanisms to inhibit the pathway, the expression of the FZD receptor family genes (FZD1-10) was analyzed by qRT-PCR to assess if conditional deletion of *RNF43* and/or *ZNRF3* changed the transcript level expression of FZD receptors. Interestingly, only the expression of *Fzd7* was upregulated in *RNF43$^{fl/fl}$*, *ZNRF3$^{fl/fl}$*, and *RNF43$^{fl/fl}$*; *ZNRF3$^{fl/fl}$* compared to WT control. The most significant increase in *FZD7* mRNA expression was seen in the *RNF43$^{fl/fl}$; ZNRF3$^{fl/fl}$* cohort (Figure 5.17). This could be due to FZD7s role as a Wnt target gene.

Additional analysis to investigate the expression of markers of proliferation, apoptosis and differentiation were about to be performed to support the IHC in Figure 5.10 but were prevented due to the COVID19 lockdown. This would have further characterized the gastric tumour and aberrant gastric epithelium to yield a more complete understanding of the changes caused by RNF43/ZNRF3 knockout.

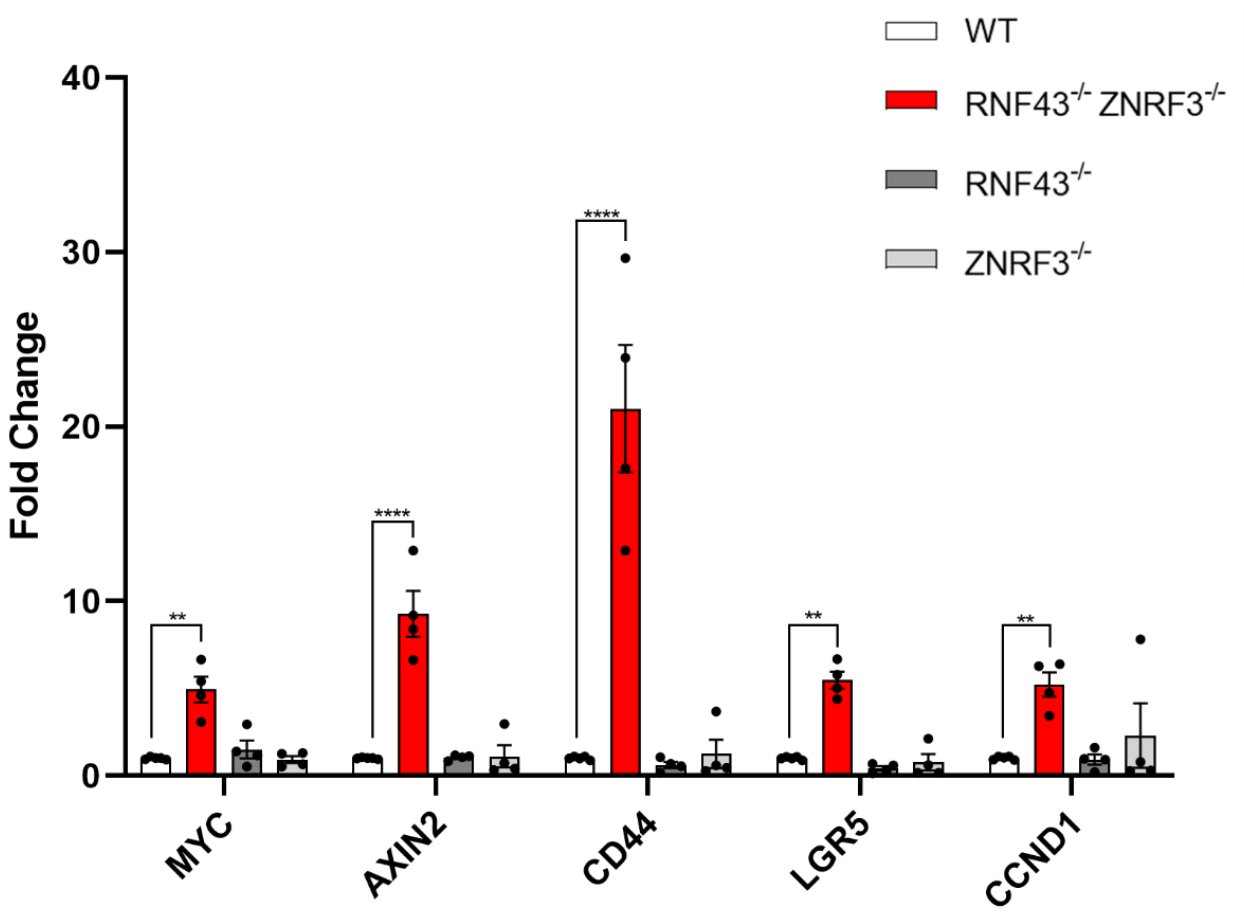


Figure 5.16 RNF43/ZNRF3 knockout increases Wnt target genes. qRT-PCR analysis for the expression of Wnt target genes on RNA extracted from the epithelial cells of the corpus. RNA from the RNF43$^{-/-}$/znrf3$^{-/-}$ cohort had significantly increased expression of all Wnt target genes compared WT control cohorts. No significant change in expression was observed in the single knockout cohorts. Normalized to GAPDH. (**=p≤0.01, ****= p ≤0.0001, mean ± SEM, t-test, n=6 mice)

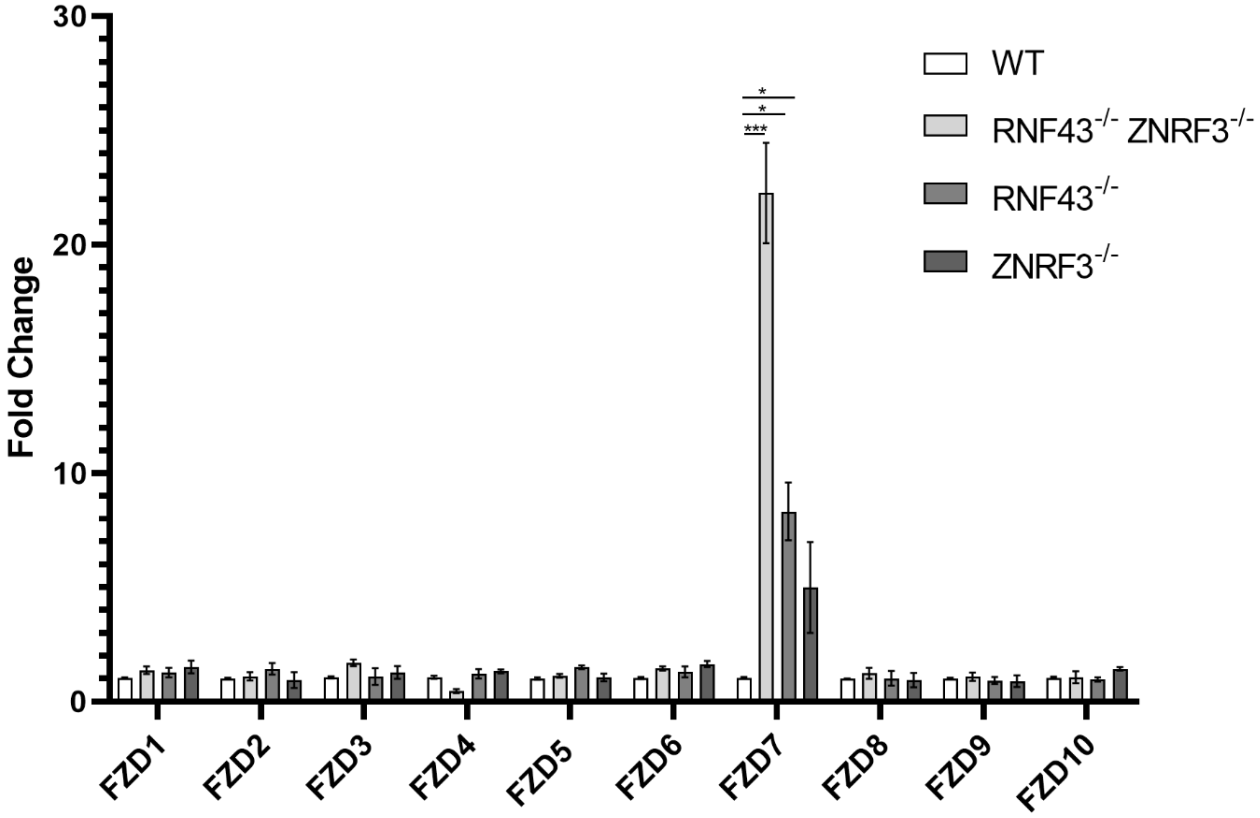


Figure 5.17 FZD7 expression increased in RNF43 mutant mice. qRT-PCR analysis for the expression of FZD genes on RNA extracted from the epithelial cells of the corpus. RNA from the RNF43$^{-/-}$/znrf3$^{-/-}$ cohort had significantly increased expression FZD7 compared WT control cohorts. A significant increase in FZD7 expression was also observed in the single knockout cohorts. Normalized to GAPDH. (**=p≤0.01, ****= p ≤0.0001, mean ± SEM, t-test, n=6 mice)

5.2.3 Co-deletion of *RNF43* and *FZD7* may rescue *RNF43*$^{fl/fl}$ phenotype.

To investigate if the phenotypes associated with conditional deletion of *RNF43* are due to deregulation of *FZD7* and therefore if the phenotype can be rescued with co-deletion of *RNF43* and *FZD7*; *Claudin18*CreER^{T2}; Rnf43flox; Znrf3flox; dTOM^{LSL} mice have been crossed with floxed *FZD7* mice (Figure 5.18). This could functionally confirm if the tumourigenesis observed when *Rnf43* and *Znrf3* are deleted in the stomach is specifically due to deregulated Fzd7, which we have previously shown is important for transmitting oncogenic Wnt signalling in the stomach.

The *Claudin18*CreER^{T2}; Rnf43flox; Znrf3flox; *Fzd7*flox; dTOM^{LSL} have been bred but induction was not carried out as planned due to the COVID19 pandemic lockdown. This set of experiments would have confirmed the relationship between RNF43 and FZD7 and provided mechanistic insights into the molecular pathogenesis of GC.

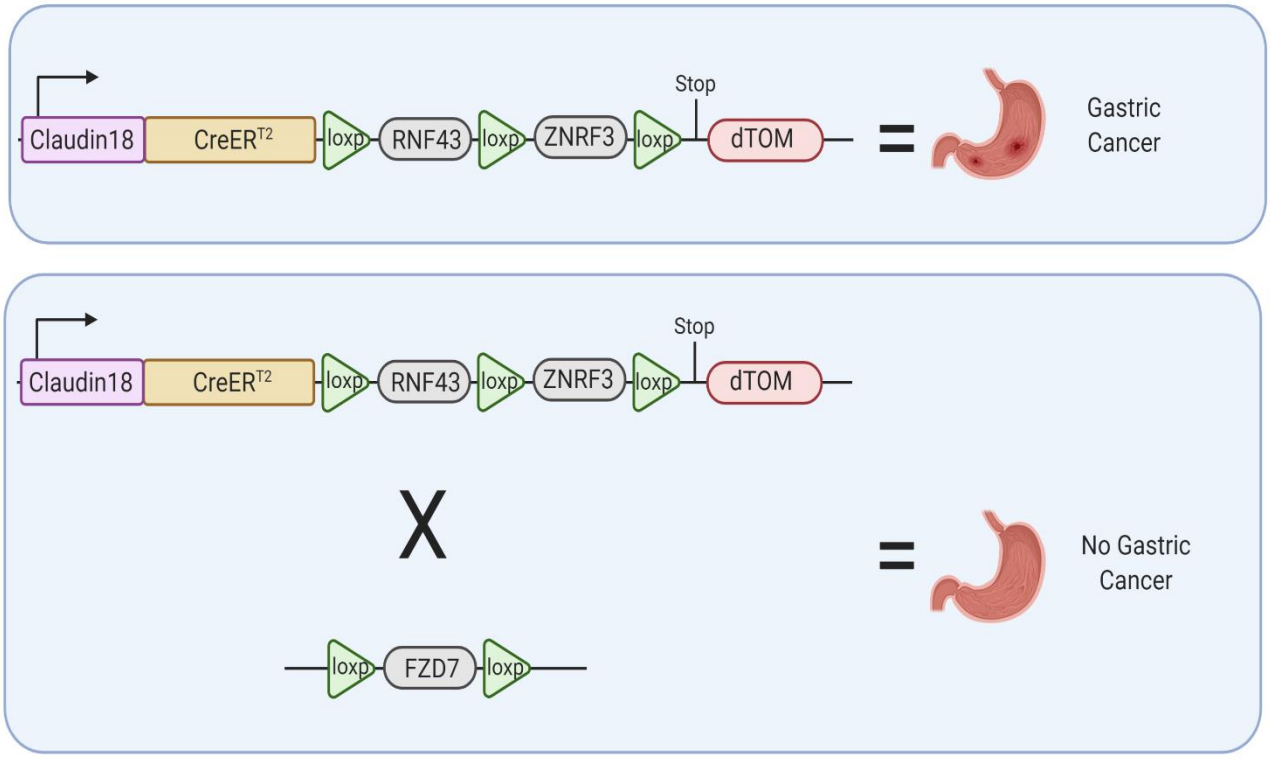


Figure 5.18. FZD7 deletion may rescue RNF43-mutant GC phenotype. Breeding strategy for *Claudin18*CreER^{T2}; Rnf43flox; Znrf3flox; *Fzd7*flox; dTOM^{LSL} cohort. The additional conditional deletion of *FZD7* should rescue the phenotype we have demonstrated following conditional deletion of *RNF43* and *ZNRF3*.

5.3 Discussion

RNF43, and its homologue ZNRF3, are integral E3 ubiquitin ligases located in the transmembrane region. They are responsible for ubiquitylating cytoplasmic sites on FZD receptors which drives FZD lysosomal degradation and negatively regulates its abundance at the cell surface (Hao et al., 2012; Koo et al., 2012). The activity of RNF43 and ZNRF3 at the cell surface is regulated by R-Spondin ligands and the co-receptors LGR5/6 (Carmon et al., 2011), with the heterotrimeric complex of LGR-RSPO-RNF43/ZNRF3 inhibiting the ubiquitylation of FZD and increasing the FZD cell surface abundance and cellular sensitivity to Wnts. This is clinically relevant since LOF mutations to *RNF43* are found in multiple Wnt-addicted cancer types (Madan et al., 2016; Wang et al., 2014b)

A key goal of precision medicine is to identify actionable mutations that will allow patient stratification and improved clinical outcome. LOF RNF43 mutation may be a potential actionable mutation for many cancers since LOF RNF43 mutations drive progression by increasing cellular sensitivity to Wnt ligands. Therefore, these cancers are uniquely sensitive to inhibitors that block Wnt secretion, such as PORCNi. LGK-974 (a PORCNi) has been demonstrated to have an anti-tumour response in *in vivo* breast, pancreas, and head and neck cancers (Blagodatski et al., 2014). Importantly, all LGK974-sensitive pancreatic cell lines carried a LOF mutation in the *RNF43* gene (Liu et al., 2013). Therefore, as PORCNi and other upstream inhibitors, such as OMP-18R5, enter and progress through clinical trials it is important to identify the right patients to treat with these upstream Wnt inhibitors. A phase I evaluation of LGK-974 is currently underway (NCT01351103) recruiting patients with melanoma, breast, and pancreatic cancer. Hence a comprehensive understanding of RNF43s role in GC is required.

This treatment stagey has potential to be a viable option in Wnt-addicted GCs. Through whole-genome sequencing analysis, RNF43 has been found to be frequently mutated in GC: RNF43 was mutated in 4.8% of MSS tumours and

54.6% of MSI tumours, 62.5% of which were truncating mutations (Wang et al., 2014b). However, RNA-SEQ analysis from the TCGA-STAD (stomach adenocarcinoma, cancer.gov/tcga) dataset reported that expression of RNF43 was slightly up-regulated compared to normal tissues. However this dataset contained 375 tumour samples and only 32 normal samples, additionally "normal" samples were obtained from adjacent mucosa, which may have their own unique transcriptome resulting from a crosstalk between tumor and adjacent tissue (Russi et al., 2019).

Conversely, in line with the whole-genome sequencing analysis study by Wang *et al*, a small study looking at GC tissue with matched tissue reported, through qRT-PCR and western blotting, that both RNF43 mRNA and protein was downregulated in GC tissue and GC cell lines compared to normal gastric tissue (Niu et al., 2015). Furthermore, RNF43 expression was found to be absent in 48.39% (15/31) of the stage II GC tissues and 96.67% (29/30) of the stage III GC tissues (Niu et al., 2015) .This was further confirmed by another study reporting that RNF43 expression is decreased in GC tissue samples compared to normal gastric tissue and that loss of RNF43 lead to increased stemness of GC stem-like cells through the Wnt/β-catenin pathway (Gao et al., 2017b). The human protein atlas (Uhlén et al., 2015) reported an increase in RNF43 protein expression in GC samples, however this was only based off 12 samples (https://www.proteinatlas.org/ENSG00000108375-RNF43). Additionally, RNF43 expression was localized to the nucleus and the nuclear membrane and may be reflective of the novel alternative mechanism of RNF43-mediated Wnt inhibition, through TEF4 tethering (Loregger et al., 2015).

Evidently, a clear and comprehensive understanding of RNF43 function in GC has still not emerged. Therefore, to establish the requirement of RNF43 in GC we conditionally deleted it *in vivo*. Our results support a role for both RNF43 and ZNRF3 in the initiation of GC as co-deletion of both genes resulted in strong aggressive GC phenotype. This is in line with reported findings that loss of RNF43 in GC patients was significantly associated with poor prognosis and an

aggressive phenotype (Gao et al., 2017b). Additionally, we have reported here that co-deletion of RNF43 and ZNRF3 resulted in the loss of parietal cells from the corpal epithelium. Loss of parietal cells is considered a key step in the pathogenesis of GC and is a pre-curser to SPEM and the transformation of metaplastic cells into neoplasia (Nam et al., 2010). We observed a thick layer of mucous in the double knockout mice which is consistent with the parietal cells being replaced by proliferating cells that secrete mucins which is frequently seen in pre-malignant SPEM lesions (Nozaki et al., 2008)

Furthermore, SPEM arises from a subpopulation of cells that express the Wnt regulated stem cell marker, Lgr5 (Barker et al., 2007). Consistent with this, we observed a significant increase in Lgr5 mRNA expression in the corpal epithelium from the RNF43/ZNRF3 knockout mice. This suggests that loss of RNF43/ZNRF3 leads to an increase in Lgr5+ cells that promote SPEM that persists into a cancerous lesion. The negative correlation between RNF43/ZNRF43 and Lgr5 that we have overserved is further supported by a study which also observed a negative correlation between the two (Niu et al., 2015). They knocked-down RNF43 in GC cells via targeted shRNA and demonstrated that the protein level of Lgr5 was significantly increased compared to negative control. Additionally, they saw the same increase in Lgr5 expression in normal gastric cell lines transfected with shRNF43 (Niu et al., 2015). Taken together, these data suggest that RNF43 might be inversely related to the potential of the cancer stem cell marker Lgr5 in GC. However, the exact mechanism still needs to be elucidated.

In line with the above, we saw a significant increase in all Wnt target genes following conditional deletion of RNF43/ZNRF3. This illustrates that loss of RNF43/ZNRF3 leads to strong activation of the Wnt pathway which could be driving GC initiation through the recruitment of Lgr5+ cells back into the cell division cycle, thus, promoting GC (Leushacke, 2017). This is further supported by our observation of increased PCNA staining and subsequent increased proliferation within the corpal epithelium of double-knockout mice. Loss of

RNF43/ZNRF3 is likely leading to increased expression of FZD receptors on the cell surface and propagating aberrant Wnt/ß-catenin and the transcription of Wnt target genes. Therefore, RNF43-mutant GC will likely respond to PORCN inhibitors.

Despite our data and others clearing implicating LOF RNF43/ZNRF3 mutations with the development of GC and a worse prognosis for GC patients, two very recent studies have been published (after the initiation of this project) that argue the most common RNF43 mutation in many cancers, including GC, remains fully functional and does not compromise RNF43 activity (Li et al., 2020; Tu et al., 2019). The most frequent RNF43 mutation in GC is G659Vfs, present in 65% of RNF43 mutant tumours according to the data on cBioportal. This mutation results in a deletion of a G-C base pair in a 7-G repeat near the 3' end of its open reading frame (Figure 5.2). This frameshift has been thought to truncate the enzyme at Gly659 leading to an inactive enzyme and increased Wnt signalling that drive tumourigenesis (Tate et al., 2018). However, both studies argue that the majority of tumours with RNF43- G659Vfs also have low expression of MLH1, a key player in DNA mismatch repair, therefore, RNF43-G659Vfs is the result of error-prone replication of a 7-G repeat and is simply a bystander mutation (Li et al., 2020; Tu et al., 2019). Both studies utilized *in vitro* assays and transient transfections. Li et al., used CRISP-Cas9-medicated knockout of G659FS RNF43 expression in KM12 (CRC) cells and generated comparable mutations in HEK293T cells and assessed Wnt signalling activity via TOPflash assays. They reported that G659fs mutants demonstrated the same inhibitory effect on Wnt signalling as RNF43 WT cells (Li et al., 2020). They also assessed the ability of RNF43 truncation mutants on promoting turnover of FZD receptors through immunoblotting and co-immunoprecipitation assays. Collectively, they concluded that C-terminal truncation mutants retaining the RING domain were still effective in FZD receptor turnover when overexpressed (Li et al., 2020). Tu et al., utilized the same techniques but additionally looked at correlation of BRAF and RNF43 mutations (Tu et al., 2019). Both studies

have not elicited an underlying mechanism for how RNF43-G659Vfs mutants retain functionally.

There are several limitations to the transient transfections experiments used in the above studies such as the highly simplified promoter used the *in vitro* assay, the nature and type of the cells used, and simply the fact that the assays were performed *in vitro* while mutant tumours grow in a far more complex environment *in vivo*. For example, in the study by Li et al., they used a MSI colorectal cell line, KM12, with *APC* and *AXIN1* mutations which activate Wnt signalling downstream of FZD.

Conversely, another recent study argues in favour that frequent G659fs RNF43 mutation still leads to loss of function and increased Wnt signalling (Yu et al., 2020). Importantly unlike the previous studies, Yu et al., used in depth *in vivo* analysis of various C-terminal truncating mutations including G659fs and demonstrated them to be loss of function and potentially actionable mutations for PORCN inhibitor treatment. This study systematically examined a spectrum of 135 patient-derived RNF43 mutations from a variety of cancers and they found that all truncation or frameshift mutants are loss of function, as well as nearly all missense mutants in the RING domain being loss of function also. They also demonstrated that C-terminal truncating mutations in RNF43 lead to increased cell surface FZD expression, increased Wnt/β-catenin signalling and are responsive to therapeutic doses of PORCN inhibitors *in vivo* through patient-derived xenograft models (Yu et al., 2020). This provides clear and robust evidence of RNF43 mutation leading to loss of function of the protein and is consistent with our results.

Furthermore, novel and emerging inhibitors targeting Wnt secretion or Wnt receptors, such as the new Lrp5/6 anti-body (Fenderico et al., 2019) use RNF43 mutational status to stratify patients that will likely respond. Additionally, Anti-Lrp5/6 blocked Rnf43/Znrf3-mutant intestinal organoid growth (Fenderico et al., 2019). Together this strengthens the support that RNF43 mutations in

cancer, including GC, do in fact lead to LOF and can be exploited for therapeutic gain.

Our findings did not show any increase in Wnt target gene expression or evidence of uncontrolled proliferation in the gastric epithelium of the *ZNRF3*$^{fl/fl}$ mice. This is likely due to the Rnf43/Znrf3 module harboring a strong level of functional redundancy and therefore being able to compensate for one another when one is mutated or lost. This is validated by a study in which knockout of each gene separately had no noticeable effect on mouse intestinal homeostasis whereas double knockout resulted in strong proliferation and Wnt/ß-catenin activation (Koo et al., 2012). Therefore, loss of a single E3 ligase may not be sufficient to reach the threshold of active Wnt signalling required for neoplastic transformation in the gastric epithelium. Alternatively, the single mutants may need longer to develop the GC phenotype and so longer-term single knock-out experiments should be set up to investigate this. This hypothesis is supported by our data showing that single RNF43 mutant mice had significantly increased expression of ß-catenin positive cells compared to WT mice. This result was not reflected in the qPCR analysis of Wnt target genes, with no significant changes in expression reported between WT and RNF43$^{fl/fl}$ mice. This may be due to the Wnt threshold not being met and so despite active Wnt signalling, it is not sufficient to lead to the transcription of Wnt target genes. This would also explain why despite upregulation of FZD7 in RNF43$^{fl/fl}$ mice there was no change in the expression levels of other Wnt target genes and Wnt activity. Loss of RNF43 is likely leading to a direct increase in FZD7, of which it regulates, however this is still not sufficient to cross of the threshold for gastric-specific Wnt signalling. This is a possibility as the RNF43$^{fl/fl}$ mice had approximately 20% ß-catenin positive cells compared almost 90% in the double knockout mice. This is supported by a study by Buchert *et al.*, in which they described tissue-specific response levels for the Wnt/ß-catenin signalling pathway (Buchert et al., 2010). Thus further building on the "just-right" model (Albuquerque et al., 2002) where distinct dosages of Wnt activation are required to perturb the self-

renewal of stem cell populations and lead to neoplastic transformation. Furthermore, the RNF43$^{fl/fl}$ mice an increase in proliferating cells outside of the gastric proliferative zones. This mislocalisation of proliferating cells is strongly suggestive of a pro-neoplastic environment in the epithelium. This paired with the active Wnt signalling not meeting the required threshold for transformation may explain why gastric tumours were not observed in the RNF43$^{fl/fl}$ mice despite an increase in active Wnt signalling and proliferating cells.

Evidence strongly indicates that RNF43 mutations are loss of function and lead to the overexpression of FZD receptors on the surface. As we have demonstrated here the conditional deletion of RNF43/ZNRF3 in the gastric epithelium leads to the development of gastric tumours, therefore, it is worth investigating which FZD receptor is overexpressed and transmitting aberrant Wnt signaling in RNF43-mutant tumours. FZD7 has been shown to be the predominant FZD receptor transmitting Wnt signalling to regulate stem cell function in the gastric epithelium (Flanagan et al., 2017a) and we have shown in the previous chapters that FZD7 is likely the predominant FZD receptor transmitting oncogenic Wnt signalling in the context of GC (Flanagan et al., 2019a). This may explain why we observed increased levels of *FZD7* expression across all our RNF43/ZNRF3 mutant cohorts. Therefore, it is likely that loss of RNF43 function in GC is leading to overexpression of FZD7 specifically on the cell surface and development of GC. Consequently, we plan to cross our *Claudin18*CreER^{T2}; Rnf43flox; Znrf3flox; dTOM^{LSL} mice with floxed *FZD7* mice with the hypothesis it rescues the phenotype. This would functionally demonstrate that deletions in RNF43/ZNRF3 induce tumourigenesis by deregulating FZD receptors and has the potential to open up a new therapeutic avenue for stratifying patients with RNF43 mutations who would respond to treatment with OMP-18R5 (Anti-FZD Ab).

5.4 Conclusions

Despite conflicting evidence for the role of RNF43 in gastric tumourigenesis, we have shown that conditional deletion of *RNF43* and *ZNRF3*

in vivo leads to uncontrolled proliferation in the gastric epithelium and the development of gastric tumours; these tumours displayed significantly increased expression of Wnt target genes and loss of parietal cells. This provide evidence that RNF43 has a significant functional role in the development of GC. Therefore, a large proportion of GC patients are likely to be responsive to Wnt inhibition. Additionally, if shown that FZD7 can rescue the Rnf43$^{fl/fl}$; Znrf3$^{fl/fl}$ phenotype further therapeutic options will open up for GC patients and provide further evidence and guidelines for patient stratification based on RNF43 mutational status for the emerging upstream Wnt inhibitors and potentially those specifically targeting Fzd7.

Chapter 6: Generation of Tools to Better Investigate the Molecular Mechanisms Underlying GC

6.1 Introduction

The previous chapters presented in this thesis, along with other confounding evidence, has shown that the exact molecular mechanisms underlying aberrant Wnt signalling in GC is complex and remains largely poorly understood. We have demonstrated Wnt pathway activation can be modulated at the level of the receptor/ligand, even in cells with mutations to the cytoplasmic Wnt regulator, *APC* (Flanagan et al., 2019a). Therefore, investigations into the molecular mechanism of Wnt signalling in GC, and how mutant APC modulates the response of GC cells to FZD7 inhibition is vital in order to clarify the molecular mechanisms regulating GC and develop more effective therapies for GC patients. A hurdle in understanding these molecular mechanisms is a lack of tools that accurately recapitulate tumour biology, from clinical mutations to the microenvironment.

A huge advancement in the field of developmental biology and cancer biology was the development of a three-dimensional (3D) culture system termed "organoids" which have opened up new opportunities in preclinical personalized therapy testing. Organoids recapitulate many, but not all, of the aspects of the tissue they are derived from such as the differentiation capacity to the tissue-specific lineages and stem cell self-renewal (Merker et al., 2016). Due to successful culturing of normal tissue, organoids have also been developed for several human cancers (Gao et al., 2014; Sato et al., 2011a). While being more technically challenging than traditional 2D *in vitro* culture, 3D organoids yield numerous advantages by bringing more faithfulness to the *in vivo* environment. One such advantage is a lower stiffness environment that closer resembles that of tissues; stiffness directly affects cell adhesion, spreading, migration and differentiation (Bayir et al., 2019). Furthermore, organoid cultures offer some advantages compared to *in vivo* xenograft models due to the shorter establishment time frame and the ease of manipulation (Andersson-Rolf et al., 2017). However, organoids do not contain every type of

the tissue they are derived from or cells from the surrounding microenvironment, such as immune cells.

Initially developed based on the growth requirements of small intestinal stem cells, organoids have now been developed for several organs, including the liver and the brain (Akbari et al., 2019; Cakir et al., 2019). Gastric and GC organoids are slowly emerging, with a handful of groups publishing different methods of their culture with various levels of success (Nanki et al., 2018; Yan et al., 2018). Gastric organoids present an exciting platform to further advance GC research, especially as despite clear views of the genetic diversity across human GCs, the mechanistic link between their genotypes and phenotypes remains unclear, owing to the lack of functionally controllable platforms. To date, numerous genetically engineered mice have been generated to model spontaneous gastric tumourigenesis *in vivo* (Hayakawa et al., 2013; Poh et al., 2016). While these models have provided valuable insights into GC pathogenesis, their genetic backgrounds are mostly irrelevant to human GC genetics. Furthermore, tumourigenesis in these genetic mouse models requires long latency, which is suggestive of multiple genetic alternations being essential for the development of full-blown cancers. Alternatively, GC cell lines have served as accessible pre-clinical models for GC drug development. However, their biological distinction from clinical cancers can often lead to biases in the interpretation of their phenotypes. Patient-derived xenograft models offer a way to mitigate these challenges, but several other obstacles, such as the low establishment efficiency, low throughout, and genetic intractability, remain as roadblocks (Wang et al., 2017b). Therefore, organoids provide an exciting compromise for culturing patient-derived GCs.

A bottleneck in GC organoids can be the availability of GC tissue due to the lower incidence in the Western world (Sitarz et al., 2018) and the high percentage of patients that have received chemotherapy which may alter the molecular landscape of the GC. Therefore, it is vital to develop tools that will allow the introduction of clinically relevant mutations in normal gastric

organoids, thus, transforming them into GC organoids allowing the consistent investigation in GC molecular mechanisms. This can be achieved by genome editing technologies.

The initial wave of modern day genome editors was zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). However, their use has been limited due to the need for difficult and laborious engineering of a new version of the editing protein for each new target in the genome and the fact that designing the nucleases to a induce double-stranded break (DSB) in a specific loci relies on predicting protein-DNA interactions (Gaj et al., 2013; González-Romero et al., 2019). By contrast, the recent breakthrough of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology, which is based on nucleic acid interactions, has enabled specific genome editing in a versatile and uncomplicated manner (Sternberg and Doudna, 2015).

The Cas9 nuclease is guided by small RNAs through Watson-Crick base pairing with target DNA (Figure 6.1) and promotes genome editing by stimulating a DSB at a target genomic locus (Garneau et al., 2010). Upon cleavage by Cas9, the target locus undergoes two major pathways of DNA repair (Figure 6.2): the error-prone non-homologous end joining (NHEJ) or the high-fidelity homology-directed repair (HDR) pathway, both of which can be used to achieved the desired editing outcome (Ran et al., 2013). In the absence of a repair template, DSBs are re-ligated through the NHEJ pathway, which leaves insertion/deletion (indel) mutations; this pathway is utilized to mediate gene knockouts, as indels within coding exons can lead to frameshift mutations and premature STOP codons (Perez et al., 2008). The HDR pathway can be leveraged to generate precise defined modifications at the target locus in the presence of an exogenously introduced repair template. However, this pathway typically occurs at lower and substantially more variable frequencies than the NHEJ pathway. Additionally, it is only active in dividing cells, and its efficiency can vary greatly depending on the cell type (Saleh-Gohari and Helleday, 2004).

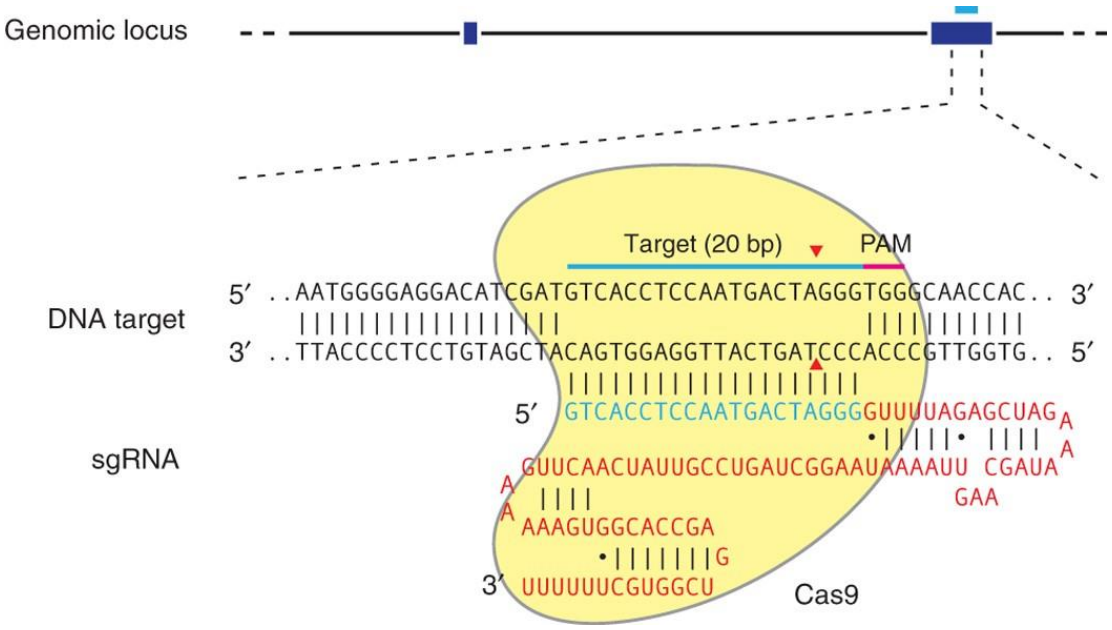


Figure 6.1 Schematic of the RNA-guided Cas9 nuclease. The Cas9 nuclease from *S.pyogenes* (yellow) is targeted to genomic DNA by a single-guide RNA (sgRNA) consisting of 20 nucleotide guide sequence (Blue) and a scaffold (red). The guide sequence pairs with the DNA target (blue bar on top strand), directly upstream of a requisite 5'-NGG adjacent motif (PAM; pink). Cas9 mediates a DSB ~3 bp upstream of the PAM (red triangle). Reproduced from (Ran et al., 2013)

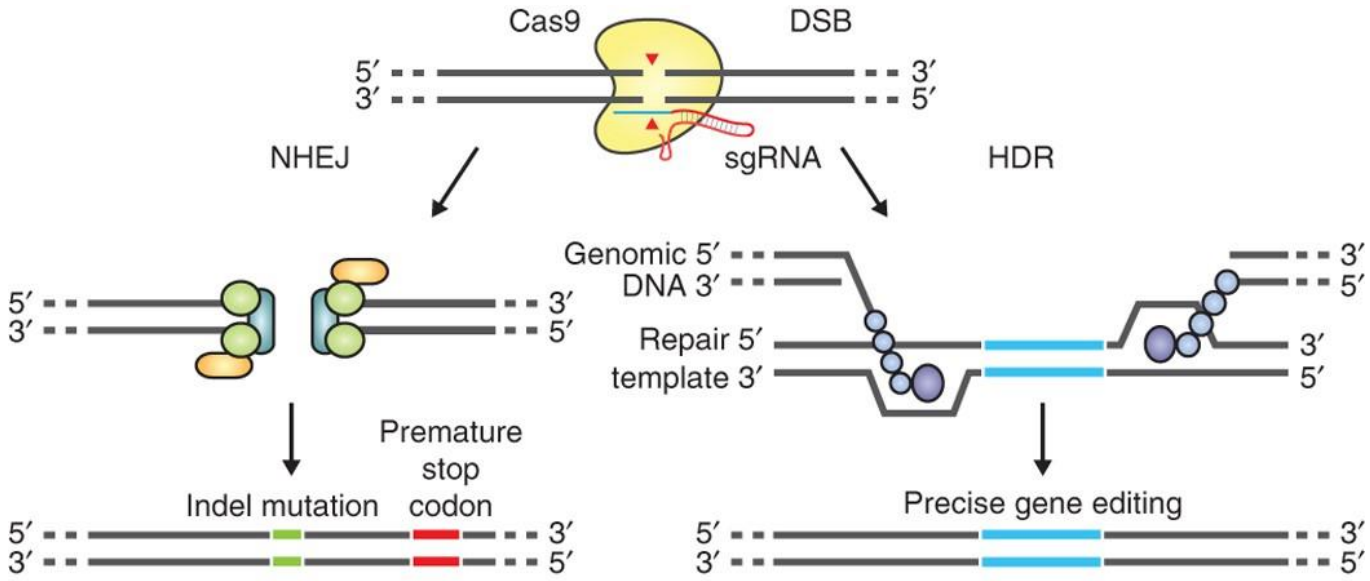


Figure 6.2 DSB promotes gene editing. DBSs induced by Cas9 can be repaired via one of two pathways. In the NHEJ pathway, the ends of a DSB are processed by endogenous DNA repair machinery and rejoined, which can result in random indel mutations. If these occur within the coding region of a gene, frameshifts and the creation of a premature stop codon can result leading to a gene knockout. In the HDR pathway, a repair template in the form of a plasmid can be supplied to leverage the HDR pathway, which allows high fidelity and precise editing. Reproduced from (Ran et al., 2013).

This new technology can be harnessed to investigate precise molecular mechanisms underlying in GC. The data presented in this thesis has demonstrated that inhibition of Fzd7 is able to prevent the growth of gastric cancer cells with mutant Apc. Therefore, generation of tools using CRISPR-Cas9 technology will help to begin to reveal the molecular mechanism regulating Wnt signalling in GC.

In this chapter we demonstrate how we have established a human gastric organoid platform, through refinement of the culture protocol, to allow for future investigations into the molecular mechanisms of GC and treatment responses. Additionally, we have generated CRISPR-Cas9 constructs that can be used as tools in combination with the newly established organoid platform to investigate the precise requirement of APC in GC.

6.2 Results

6.2.1 Generation of Human gastric cancer organoids.

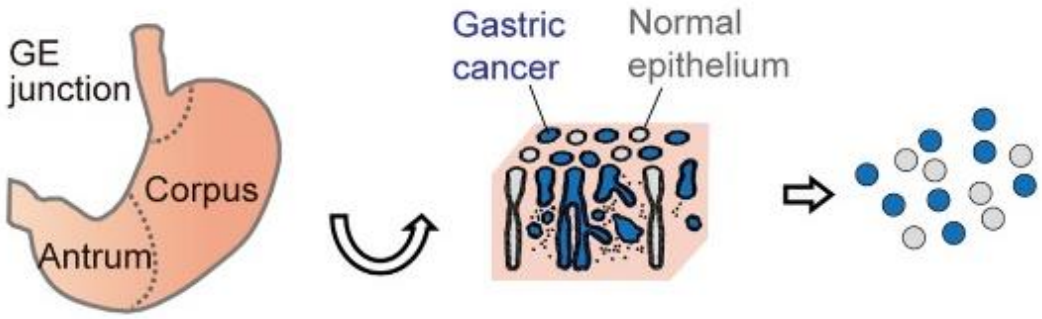
Tissue with histologically diagnosed gastric adenocarcinoma was obtained from surgical resection specimens at University Hospital Wales. The establishment of GC organoid cultures was complicated by a variety of factors. Firstly, due to the nature of harvesting the sample, GC samples could often be contaminated with normal gastric glands (Figure 6.3). An unexpected effect of this was once in culture the normal organoids out competed the tumour organoids within the mixed population, which has been reported previously and also observed in other cancer organoid cultures such as prostate (Nanki et al., 2018; Wang et al., 2017d). To increase successful initiation of GC organoid cultures and minimize the growth of normal gastric glands, the tissue digestion method and time was optimized. Instead of using a standard EDTA chelating buffer, a more aggressive digestion solution was used. Washed tissue samples were digested at 37°C with constant agitation using Liberase TH (0.28 WÃ¼nsch units/mL)(Roche)) which contains highly purified collagenase I, collagenase II, and thermolysin (non-clostridial neutral protease). The use of Liberase TH also allowed greater experimental reproducibility between GC samples due to precise blended ratio of the two collagenase isoforms; this allows higher lot-to-lot consistency.

Secondly, the GC samples often had a stromal composition resulting in a stiff, fibrous, and sometimes calcified sample. This effected digestion of the samples and made releasing tumour cells from the bulk of the tissue difficult. Therefore, a range of digestion times was utilized, from 1 hour to overnight, depending on tissue stiffness. Tissue debris was removed by passing the mixture through a 100 µm cell strainer.

Finally, the cultured GC organoids often had signs of red blood cell (RBC) contamination, likely due to the increase in neoangiogenesis commonly observed in tumours. The centrifuged pellet of GC organoids prior to resuspension in matrigel was colored red and when plated hundreds of small

cells could be seen, this made clear imaging difficult. To overcome this, the final pellet of GC organoids was bathed in an ammonium chloride solution (1:10 dilution in PBS) to lyse the red blood cells. We did not observe a difference in cultures that received this treatment prior to plating compared to those that did not (Figure 6.3B).

A



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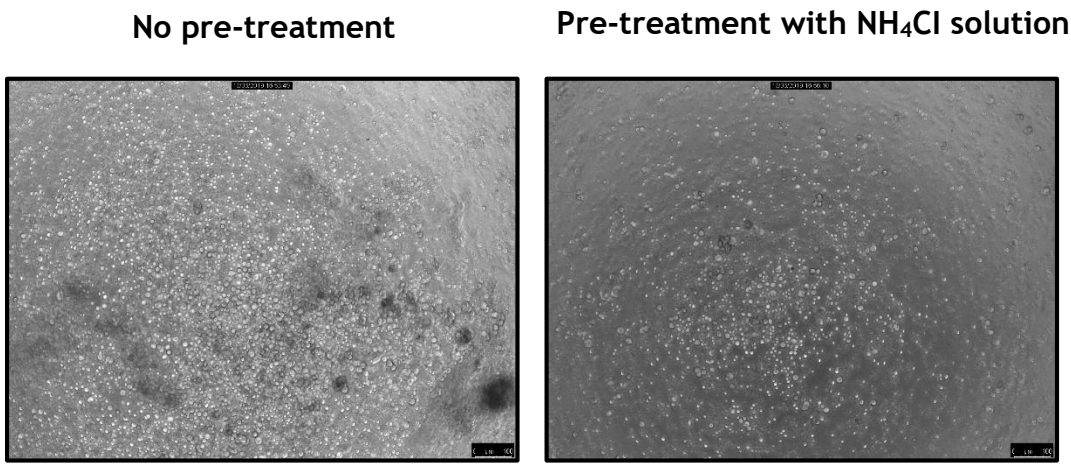


Figure 6.3 Human GC organoid challenges (**A**) Human GC samples were frequently contaminated with normal gastric glands creating a mixed population of organoids in culture. (**B**) Human GC samples containing RBC contamination in culture. No significant was observed between NH4Cl-treated organoids and non-treated organoids. RBS; Red blood cells. Scale bar = 100 μM

Despite these challenges, we were able to successfully culture human GC organoids (Figure 6.4). Interestingly, we observed that the growth of the Human GC organoids was extremely slow and GC organoids were approximately half the size of normal gastric organoids after 15 days (Figure 6.5). Some successful Human GC organoids still suffered from normal gastric organoid contamination which hampered the slow-growing GC organoids. Therefore, we developed a strategy to enrich for mutant GC organoids (Figure 6.6). This strategy harnessed recurrently dysregulated signals in human GCs: the TP53, RHO, TGF-B, and RAS-PI3K pathways (Wang et al., 2014a). Firstly, Nutlin-3, an MDM2 inhibitor would be added to the culture media to select for TP53 mutant organoids (Matano et al., 2015); Nutlin-3 resistant organoids would most likely contain TP53 mutations. Secondly, as ROCK inhibition is essential for the recovery of individualized organoid cells, we planned to remove the ROCK inhibitor from the media to enrich for RHO-deregulated GCs. Following this, organoids would be treated with TGF-B in the absence of A83-01 (TGFß kinase/activin receptor-like kinase inhibitor) to select for organoids insensitive to TGF-B stimulation. Finally, we used a media free from the growth factors EGF and FGF10 to select for organoids with ligand-independent receptor tyrosine kinase signal activation. These positive selections were chosen to yield a range of Human GC organoids subtypes, lesions and histologic types. Unfortunately, we suffered with fungal contamination within the tissue culture room around the time of these experiments and therefore we were not successful in growing GC organoids long-term and implement our selection strategy.

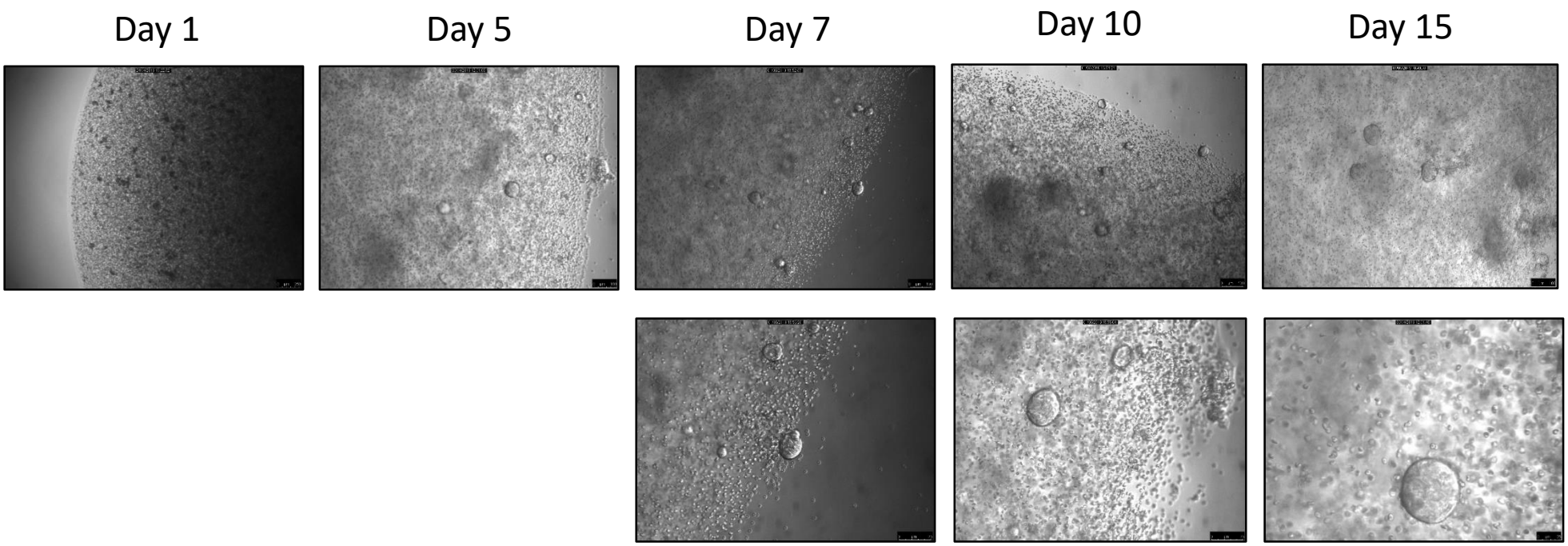
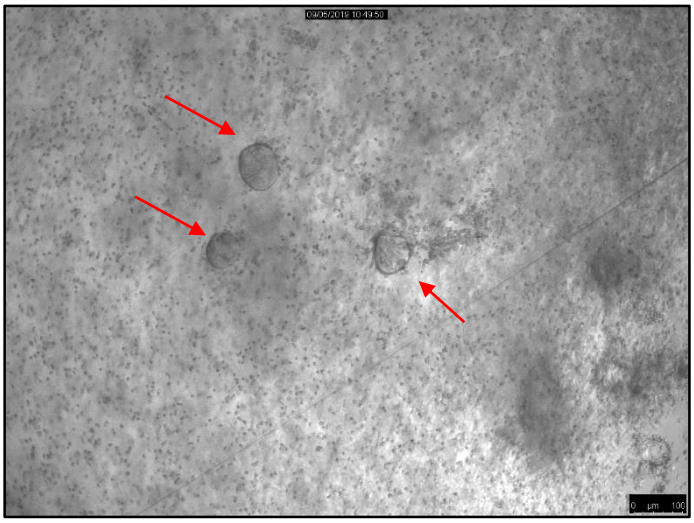


Figure 6.4 Human GC organoids. Representative images of Human GC organoids from day 1 of culture following 15 days of growth. Scale bars = 100/25 μM

GC organoid



Normal gastric organoid

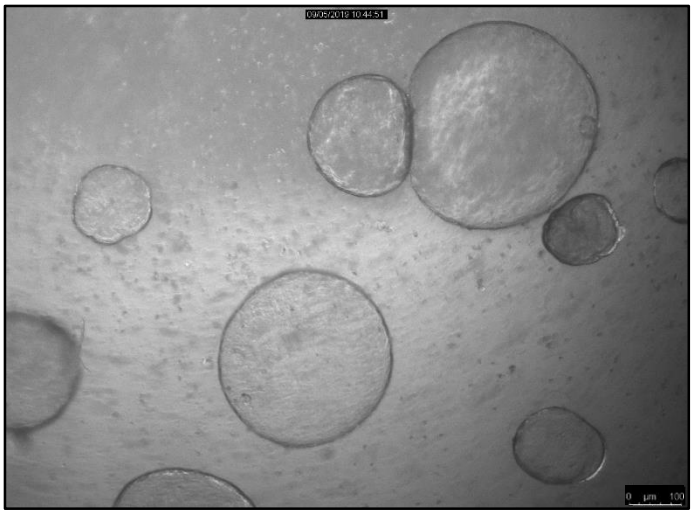


Figure 6.5 Normal gastric organoids grew faster. Representative images of GC and normal gastric organoids 15 days after first culture. Normal gastric organoids grew exceptionally faster than GC organoids. Scale bars = 100 μM

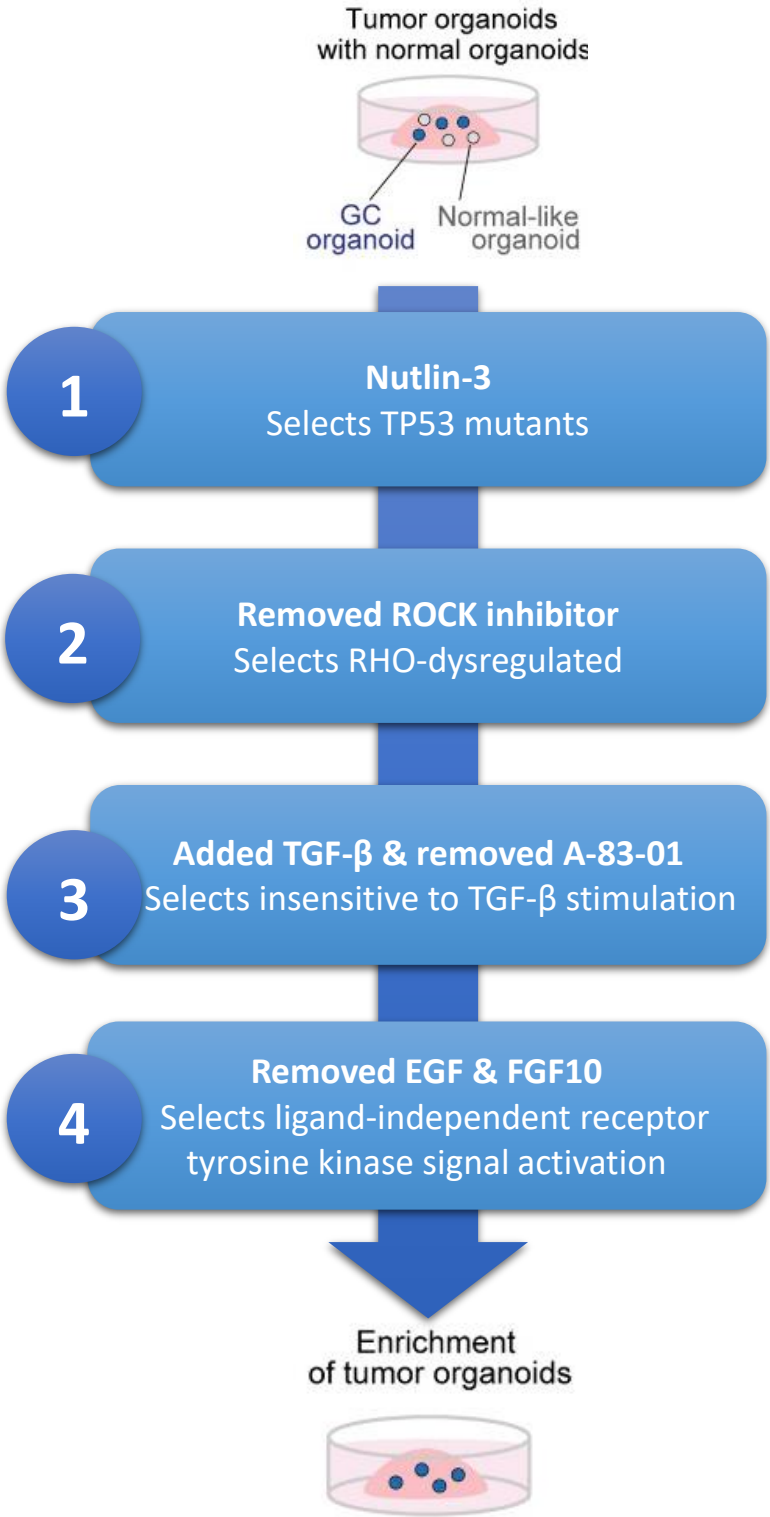


Figure 6.6 Establishment of GC organoids. Strategy for the enrichment of GC organoids by niche factor-based selections

6.2.2 Generation of normal Human gastric organoids.

Normal gastric organoids were less challenging to culture than the human GC organoids. Matched normal gastric organoids were derived from the same region (corpus or antrum) of the stomach as the location of the gastric adenocarcinoma but far enough away to prevent cross-contamination. After 24 hours' single glands had developed into round cystic-like organoids, as is typical for a gastric organoid (Figure 6.7). They continued to grow steadily for 15 days after which time they were passaged. Organoids were passaged by breaking up the matrigel by pipetting and centrifuging. The resulting supernatent contained a pellet of glands at the bottom of the tube topped with a cloudy layer containing the matrigel debris and single cells. The cloudy layer was carefully removed and the organoid pellet broken up with a small p200 tip before being resuspend. Organoids remained viable after first passage and continued to grow normally (Figure 6.8). At time of first passage, some organoids were frozen and stored to form a Biobank of human gastric organoids.

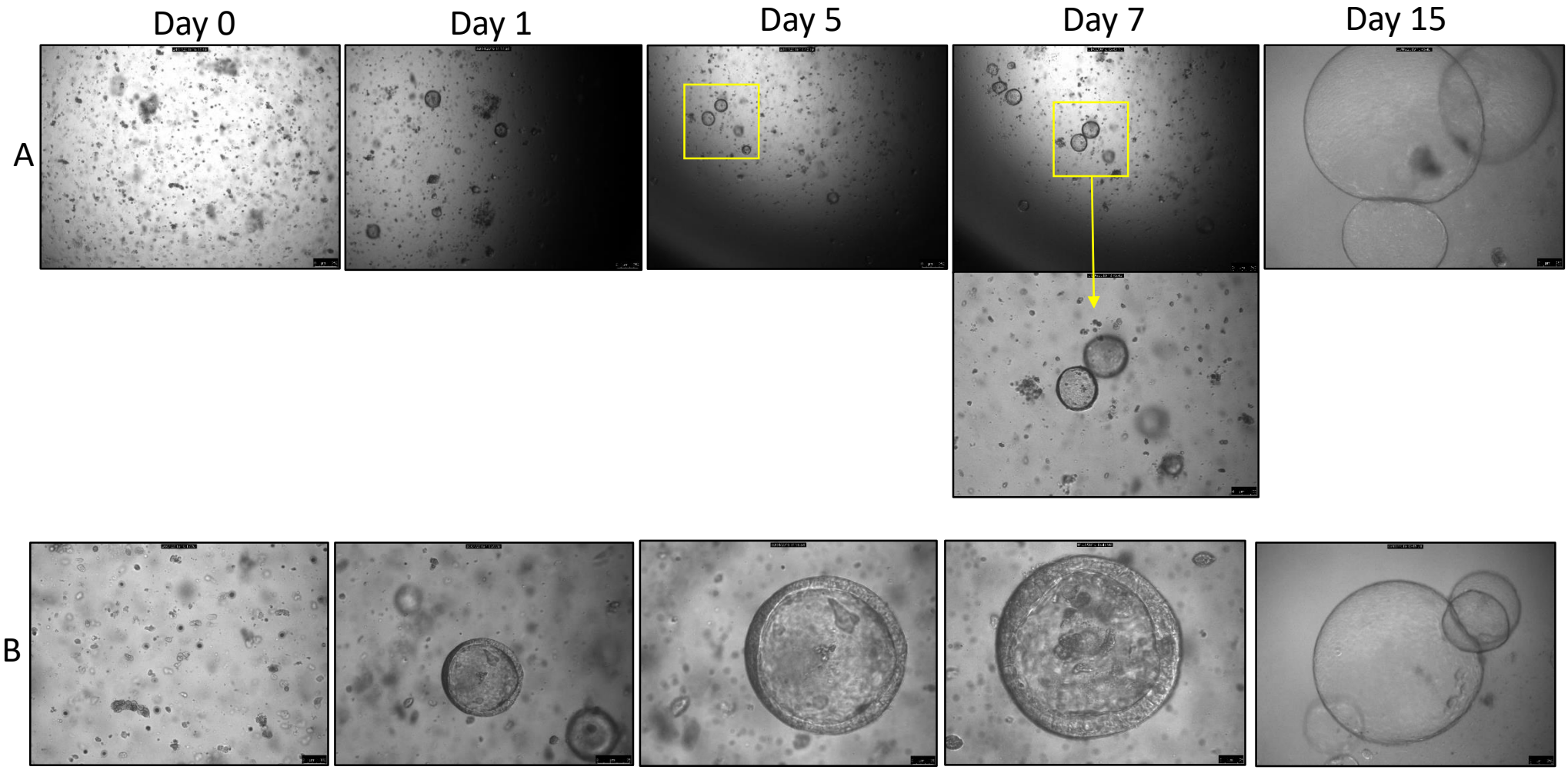


Figure 6.7 Normal Human gastric organoids. Representative images of the growth of two organoid samples (A & B) from different patients over 15 days

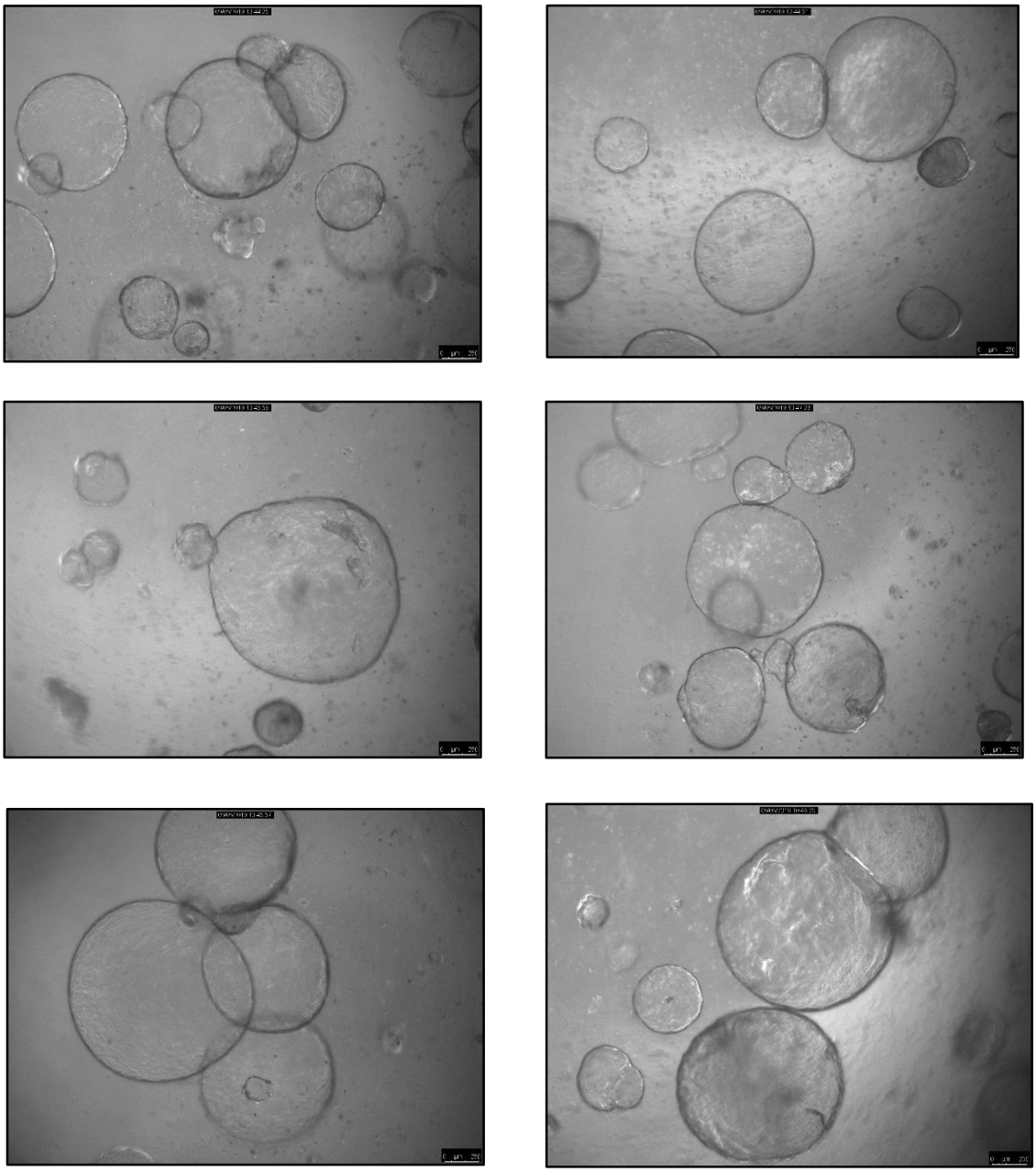


Figure 6.8 Normal Human gastric organoids following passage. Representative images of normal Human gastric organoids 7 days after passage. No changes to growth patterns or viability were observed following passage.

6.2.3 Design of FZD7-knockout and APC-mutant constructs

Inhibition of Fzd7 is able to prevent the growth of gastric cancer cells with mutant APC (Flanagan et al., 2019a). We hypothesize that this is due to the mutant APC still being transcribed and translated into a semi-functional protein which still allows regulation of the pathway from the level of the receptor. This is consistent with the 'just right' model of Wnt signalling in which too little Wnt does not transform cells, but too much is cytotoxic (Albuquerque et al., 2002) (Meniel et al., 2013). In order to more accurately assess the requirement of APC to allow gastric cancer cells to respond to inhibition of Fzd7, CRISPR-Cas9 genome editing technology will be employed to created precise truncated APC protein products as well as complete knockout as well as a construct to knockout *FZD7* and *FZD5* (as a control*)*. These will eventually be transfected into human GC cell lines and gastric organoids. The use of sgRNA and Cas9 protein will induce DSB at specific targets on the APC genome, these will be repaired by the NHEJ repair pathway. This will lead to frameshift mutations and premature STOP codons at the required sites therefore leading to precise truncated protein products or complete gene knockout.

We designed sgRNA to generated 6 different length APC proteins at known functional sites (Figure 6.9) including the serine alanine methionine proline (SAMP) repeat at 1615aa (axin binding), the mutation cluster region at 1375 aa, the 15 amino acid repeats at 1225aa, the Armadillo repeat at 809aa and downstream of the oligomerisation domain at 407aa. This was more complex than the FZD7 knockout as we required cleavage between specific functional domains. These were often restrictive in size, for example between the armadillo domain and the 15 amino acid repeats there are only 867 bp. This limited the availability of PAM sites downstream of target sequences without off-target activity.

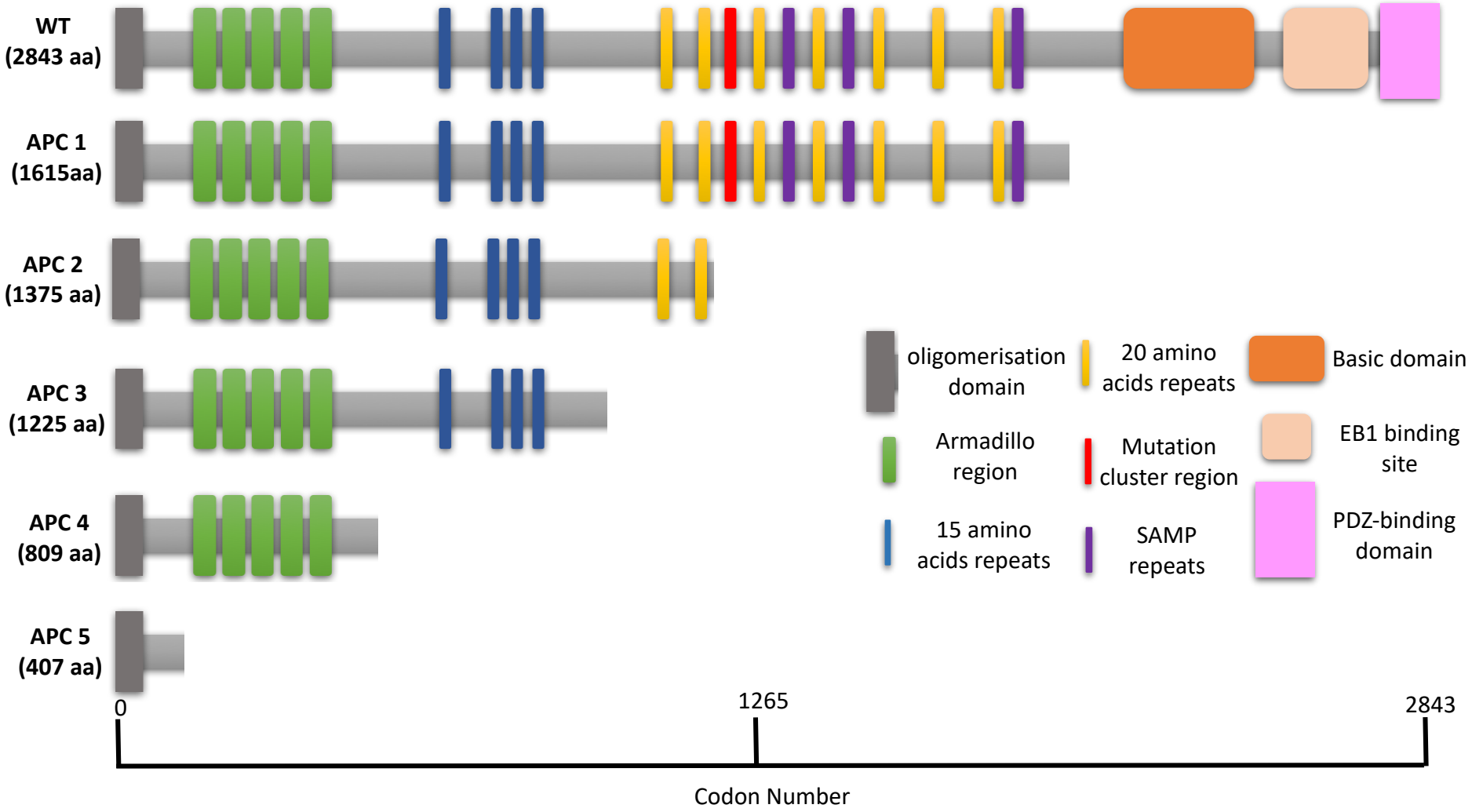
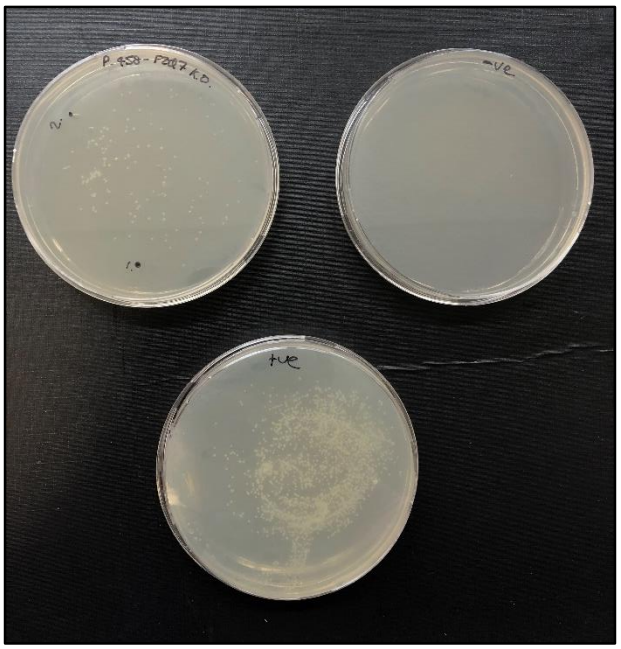


Figure 6.9 Schematic representations of human WT APC protein and the corresponding truncating mutants generated by the CRISPR-Cas9 technique.

It was decided that the sgRNA would be delivered as a sgRNA-expressing plasmid. The carefully designed sgRNA were annealed and ligated into the px458 plasmid which contained both Cas9 and the remainder of the sgRNA as an invariant scaffold immediately following the oligo cloning site. Additionally, the px458 plasmid contained 2A-GFP to allow for selection of transfection cells downstream. Following ligation, plasmids were transformed into competent *E.coli* and plated onto ampicillin-LB agar plates. Colonies were observed for all constructs, numerous colonies were seen on the positive control plate (uncut px458) and zero colonies were seen on the negative control plate (Figure 6.10). This is highly suggestive of successful insertion of sgRNA into px458. To confirm, bacterial cultures were grown from colonies and the plasmid DNA purified and sent for sequencing. All results confirmed the successful insertion of sgRNA insert into the px458 vector.

Plasmids were transfected into the GC cell line, MKN45 (WT for APC). Transfection was deemed successful due the expression of GFP in MKN45 under a fluorescent microscope (Figure 6.10). Next, the isolation of clonal cell lines was required to generate a monoclonal population. This was attempted by isolating single cells through FACs using GFP as the marker followed by an expansion period to establish a new clonal cell line. Unfortunately, our cell lines did not survive as single cells and an alternative method needs to be develop such as the gentler serial dilution protocol. Nevertheless, we have generated a panel of successfully cloned plasmids that are ready for functional testing to investigate the mechanism of how Apc mutant cells respond to inhibition of Fzd7.

A



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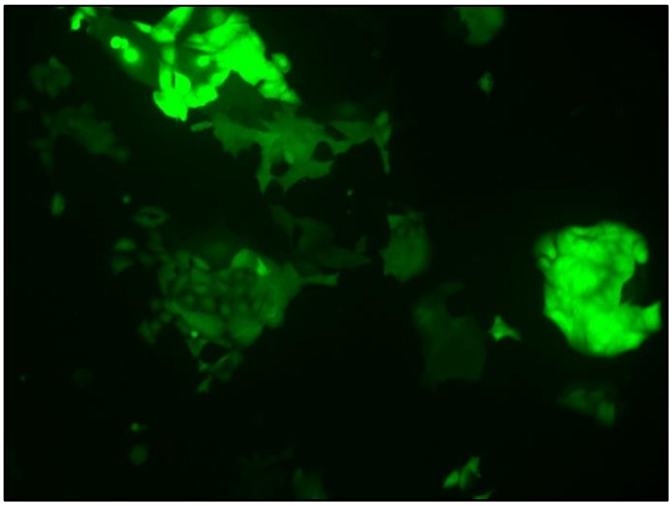

Figure 6.10 Positive selection of clones (A) Representative image of bacterial colonies includes example of FZD7-knockout plasmid, positive control and negative control. **(B)** Representative image of MKN45 cells transfected with FZD7-knockout plasmid containing GFP reported. Imaged under fluorescent microscope.

6.3 Discussion

The *APC* truncating mutation is the hallmark of the vast majority of human CRCs and is also frequently present in GCs. Its loss is thought to contribute to the initiation of GC through the constitutive activation of the Wnt pathway (Flanagan et al., 2017b). Most *APC* somatic mutations occur in the "mutation cluster region" (MCR) between codons 1,286 and 1,513 (Vogelstein and Kinzler, 2004) with region-specific APC mutations being associated with distinct β-catenin transcriptional activity and tumour susceptibility (Gaspar and Fodde, 2004). Different functional domains have been described in the central region of the APC protein, including the β-catenin-binding 15- and 20-amino acid repeats and the Axin-binding Ser-Ala-Met-Pro motif (SAMP) repeats that are vital for regulating β-catenin level. We have previously shown that Wnt receptor FZD7 plays an essential role in gastric tumourigenesis and that inhibition of this upstream receptor can modulate Wnt signalling irrespective of APC mutation status (Flanagan et al., 2019a). Importantly, data from our lab has shown that tamoxifen induced *TffCre; Apcfl/fl* mice, in which exon 14 of *Apc* is deleted and consequently truncated from codon 580 onwards (Phesse et al., 2014) develop gastric tumours with 100% penetrance**.** Additionally, recent data from our lab demonstrated that biallelic deletion of *Fzd7* was able to block gastric tumourigenesis in *TffCre; Apcfl/fl* mice. Furthermore, the results presented in chapter 2 used MKN28 GC cells which contain an APC truncating mutation (p.R1450*) at codon 1450, inside the MCR. We demonstrated that MKN28 GC cells responded to inhibition of FZD receptors in vitro and in vivo despite the mutation to APC. Together these data suggest that there was sufficient translation of the Apc protein to allow upstream factors to modulate Wnt pathway activity as the 'just right' model predicts (Albuquerque et al., 2002). However, the molecular mechanism of how GC cells with APC mutations can respond to inhibition of FZD receptors and which regions of APC are functionally and clinically significant remain unknown. For example, would complete deletion of Apc result in cells activating Wnt signaling that are

unresponsive to modulation of Fzd7 or upstream signaling events? Here, we have designed sgRNA to target specific loci between the known functional domains of APC, successfully cloned then into a Cas9 expressing vector and validated them through sequencing. We have also generated and validated a construct that will lead to complete knockout of *FZD7*. The constructs are ready be used for functional assays to help develop the understanding of the molecular mechanism of Wnt signalling in GC, and how mutant APC modulates the response of GC cells to FZD7 inhibition. This will deepen and strengthen the results from previous chapters of this thesis by providing insights into the potential mechanism that allows disruption of the FZD receptor to have an effect on GC cells with APC mutations.

These generated tools can be used in combination with our newly established human gastric organoid biobank platform. The truncated APC constructs can be transfected into normal human gastric organoids and the truncated APC mutations will transform the organoids to tumouroids as recently demonstrated in CRC (Novellasdemunt et al., 2017). These organoids can be treated with OMP-18R5 or co-transfected with the construct to knock-out *FZD7* to investigate which APC protein lengths respond to genetic knock-out of *FZD7* or pharmacological inhibition of the receptor. The combination of organoids and CRISPR-Cas9 will help determine the mechanism by which APC mutant GC cells are able to respond to deletion of FZD7, and thus modulation of the receptor upstream of intracellular mutations. These experiments will build on the findings in this thesis by investigating modulation of the FZD receptors across a broad-range of clinical types of GC and shed light on the mechanism of action underlying the results observed in the previous chapters. This will ultimately help striate which GC patients will be most likely to respond to therapy with anti-FZD drugs based on the APC mutation they harbor.

GC has extremely high molecular and cellular heterogeneity which is exhibited both within tumours and between patients (Carrasco-Garcia et al., 2018). This heterogeneity can make the choice of therapy difficult and so addressing this

molecular heterogeneity is critical for achieving an optimal therapeutic approach against GC. GC heterogeneity is manifested in marked differences in disease aggressiveness and treatment outcome, however, in current clinical practice the choice of therapeutic strategy against GC does not consider this molecular heterogeneity and is mostly based on tumour stage (Carrasco-Garcia et al., 2018). This highlights the need for novel and relevant biomarkers for patient stratification to direct targeted therapies to the right patients, with an overall goal of personalized medicine.

The use of organoids opens up new opportunities in preclinical personalized therapy testing. Our newly established platform will allow the generation of a large organoid collection over time that will function as a living human biobank. However, a weakness of this current study is the lack of validation of the normal gastric organoids. These must be confirmed as normal and not a fast-growing tumour organoid by analysis of biomarkers. The usefulness of organoid biobanks has already been demonstrated, for example, a CRC biobank has been shown to be amenable to drug screens to individualize patient treatment and screen for novel therapeutics (van de Wetering et al., 2015). Additionally, the establishment of GI organoids from metastatic lesions has been shown to be feasible and treatment of these organoids recapitulates the clinical response of the corresponding patients (Vlachogiannis et al., 2018). Our expanding collection of human GC organoids can be classified according to their molecular profile, assessed for their chemotherapeutic response, and given targeted treatments according to their specific druggable mutations. However, it is important to note that organoids consist only of the epithelial layer with no surrounding mesenchyme, blood vessels or immune cells, therefore drugs that target the microenvironment cannot be evaluated.

Recently, two groups independently established human gastric organoid biobanks of 37 GC samples (Nanki et al., 2018) and 46 GC samples (Yan et al., 2018). Both groups performed detailed whole-exome and transcriptome analysis to characterize the tumours and performed drug screening that could

potentially guide patient drug selection. Interestingly however, the groups did not observe the same genetic alterations between different GC subtypes and produced conflicting results, for example Nanki *et al.*, did not report a correlation between KRAS mutations and the MSI GC subtype, whereas Yan et al., reported a strong correlation between this aberration and the MSI subtype. Additionally, genetic alterations observed between did not agree with the datasets generated by the TCGA (Wang et al., 2014b). For example, *Nanki et al.*, reported a low mutation date of PIK3CA whereas the TCGA reports it as high. Overall, both new studies showed a lack of consistency with the TCGA report and did not demonstrate a robust genetic distinction amongst subtypes. This may be due to the small size of their datasets not allowing accurate patterns to emerge and highlights the heterogeneity of GC and the need to increase the numbers of organoids collected from different patients to fully elucidate the landscape of the molecular and genetic alterations of human GC.

Nevertheless, these investigations validate the viability of establishing human gastric biobanks and their use to identify novel druggable targets as well as highlighting the importance of organoids for the next advancements of GC research.

COVID19 lockdown prevented us from attempting to use CRISPR-Cas9 to manipulate Fzd7. We had planned to transfect organoids with the previously described construct to knockout Fzd7. We hypothesized that we would observe organoid death following transfection due to the requirement of Fzd7 in the gastric epithelium. As the human organoids were slow to establish, we planned to utilized mouse organoids which are less technically difficult to culture. A previous study investigated the role of Fzd7 in the gastric epithelium and found it to be a requirement for the culture of gastric organoids (Flanagan et al., 2017a). However, the Cre used (*Tff1Cre*) only recombined in the antral glands (Flanagan et al., 2017a) and the role of FZD7 in the corpus remains unknown. Therefore, we planned to culture organoids from the corpus region to

investigate the requirement of FZD7 through knockout using our CRISPR-Cas9 constructs.

6.4 Conclusions

We have begun to establish a human gastric organoid biobank which will help to identify the mechanistic connection between GC genotype and phenotype. These GC organoids might serve as living biomarker to predict therapy response and resistance in GCs containing specific mutations, thereby helping to guide personalized therapy approaches. Additionally, the protocol for establishing normal human gastric organoids has been refined. The treatment of patient-derived organoids alongside patients from whom cultures were derived will ultimately test their usefulness to predict individual therapy response and patient outcome. These organoids can begin to be used in combination with our newly generated CRISPR-Cas9 constructs to unravel the mechanism of how APC mutant GC cells respond to inhibition at the level of the receptor.

Chapter 7:
General Discussion & Future Directions

7.1 General Discussion & Future Directions

GC remains a major contributor to cancer-related mortality worldwide and is still the fourth most common cancer. The 5-year survival rate is less than 5% in advanced unresectable or metastatic disease (Ferlay et al., 2015), a stage which is observed in approximately 80% of patients at diagnosis (Correa, 2013). This poor survival rate is reflected in the limited treatment options for GC patients; these options are largely stratified as palliative or curative. The preferred treatment option is partial or complete resection of the stomach, however, as most patients present with advanced-stage disease a majority of patients lose the opportunity to undergo surgical resection (Song et al., 2017). In this instance, the goal of comprehensive treatment is to prolong survival and improve quality of life. This includes neoadjuvant and adjuvant chemotherapy, radiotherapy, or a combination of the two (Wagner et al., 2006) although >95% of non-operable GCs develop chemoresistance (J.J.G. Marin, 2016). After this the only remaining treatment option is Trastuzumab, a monoclonal antibody targeted against HER2. This is the only approved molecular-targeted therapy for GC, however only around 20% of GC are classed as HER2-positive (Kim et al., 2014). Clearly there is an urgent and unmet need to develop a wider range of effective targeted treatments for GC. This thesis aimed to enhance the understanding of the molecular pathways linked to GC in order to identify novel therapeutic targets for GC.

The lack of GC treatment option stems from an incomplete understanding of the molecular pathways underlying this highly heterogeneous disease. The Wnt signalling pathway is central to gastric homeostasis and therefore its aberrant activation is linked to GC development, how the precise molecular mechanisms of aberrant Wnt signalling in GC have remained unknown in the field. The studies in this thesis have been the first investigations to examine the role of the Wnt receptor FZD7 in the context of GC. We have demonstrated that inhibition of FZD7 leads to a reduction in GC initiation, even in APC mutant cells. In addition, we have shown for the first time the functional significance

of the Wnt pathway negative regulator, RNF43/ZNRF3, in GC through its conditional deletion *in vivo*. Collectively all result chapters presented in this thesis highlight for the first time how modulation of Wnt signalling at the level of the receptor may be a potential and novel therapeutic strategy for the treatment of GC irrespective of downstream mutations.

Aberrant activation of Wnt/ β-catenin signalling is a necessary initiating event in the formation of many cancers of epithelial origin, including GC (Clevers and Nusse, 2012a). Many GC patients harbor activating mutations to key intracellular components of the Wnt/β-catenin pathway, such as *APC* (Wang et al., 2014a) however ever-growing evidence is suggestive that additional modulation of Wnt/β-catenin signalling can help attenuate cancer progression (Caldwell et al., 2004; Suzuki et al., 2004) however this had not been confirmed in GC until the generation of this thesis. As such, it has been shown that FZD7 is upregulated in many cancers, including GC, with constitutive Wnt activity, which robustly increases already elevated levels of Wnt/β-catenin signalling and subsequent target gene expression (Zhao et al., 2014). This is presumably due to the fact that *FZD7* is a downstream target of Wnt/β-catenin signalling (Vincan et al., 2010), which may serve as a feed-forward mechanism to fuel Wnt/β-catenin signalling, thus facilitating cancer progression. Importantly, implementing targeted strategies to reduce the availability of FZD7 has been shown to reduce cell viability, cell migration, and cell invasion within various cancer types both *in vitro* and *in vivo* (Ueno et al., 2009; Vincan et al., 2007a; Wei et al., 2011; Yang et al., 2011) in line with the results we have presented in the context of GC. However, targeting a component of a crucial adult tissue homeostasis signalling pathway has many associated caveats, such as the possible disruption to tissue proliferation and homeostasis of surrounding non-tumorigenic tissue. Thankfully, recent studies support our hypothesis that targeting of FZD7 within the gastric epithelium is a novel, viable and attractive target for the treatment of GC without severe unwanted side effects. Published work from our lab in conjunction with this project has demonstrated that

conditional deletion of FZD7 from gastric adenomas of Tff1Cre$^{ERT2/+}$;gp130$^{F/F}$;Fzd7$^{fl/fl}$ mice significantly reduced tumour burden with associated decreases in cell growth, proliferation and angiogenesis (Flanagan et al., 2019a). Additionally, conditional deletion of FZD7 from the gastric epithelium is well tolerated, as demonstrated by the fact that FZD7-deficient cells are repopulated and the integrity of the epithelium is restored soon after (Flanagan et al., 2017a). Furthermore, Fzd7-deficient mice were reported to be viable, healthy and fertile. Additional support comes from a study where Fzd7 was deleted from the intestinal epithelium, triggering rapid epithelial repopulation (Flanagan et al., 2015b) which is suggestive of a survival mechanism to eradicate any cells that have undergone critical genetic ablation. Furthermore, given the pivotal role of Lgr5 gastric stem cells play in gastric homeostasis (Barker et al., 2010b) and the recent study demonstrating that the function of Lgr5+ cells in the gastric epithelium do not require FZD7 to maintain this homeostasis (Flanagan et al., 2019b) is highly suggestive that therapeutic FZD7 inhibition within the context of GC treatment is unlikely to affect this population of cells.

However, as with the majority of cancer therapies, mono-therapies show limited promise in long-term cancer treatment due to the heterogeneity of the disease and the development of drug-resistant cells (Arkenau, 2009). As such, it is now almost standard in clinical trials to treat all malignancies in combination with other agents as this often shows synergistic effects (Le et al., 2015). As highlighted in chapter 2 and 3 of this thesis, the use of OMP-18R5 as a single agent was successful in inhibiting GC cell growth both *in vitro* and *in vivo*, and at inhibiting the ability of GC cells to migrate. However, in a clinical setting it would be advisable to be implemented in combination with other routine chemotherapy or radiotherapies. Importantly, due to the systemic pharmacological action of OMP-18R5, careful pre-testing and validation should be performed to gauge the appropriate human therapeutic dose and identify any potential unwanted toxicity to Wnt-sensitive tissues. A first-in-human

phase I dose-escalating clinical trial (NCT01345201) using OMP-18R5 in solid cancers found that the best tolerated dose was up to 2.5 mg/kg once every 3 weeks. The most commonly reported side effect was reduction in bone density, however, this was successfully elevated by co-treatment with zoledronic acid (Smith et al., 2013); a common approach when using Wnt inhibitors in a clinical trial setting.

We have elucidated that FZD7 is the predominant Wnt receptor transmitting cell-intrinsic Wnt signals in human GC cells and therefore may be an attractive target for GC treatment. Our *in vitro* studies demonstrated reduced GC cell growth and progression following FZD inhibition or FZD7 knockdown which is in line with several *in vitro* studies that have shown targeted inhibition of FZD receptors is sufficient to block growth of GC cells (Li et al., 2018; Tomizawa et al., 2015). A weakness of the study presented in this thesis was that transient transfections were used to investigate the effect of FZD7 knockdown. This may have led to different levels of FZD7 knockdown between experimental repeats and various experiments. A more robust approach would be to generate stable FZD7-knockdown GC cell lines. This would ensure consistency and reproducibility between experiments and minimize human error.

However, it is well documented that *in vitro* studies do not fully recapitulate the complex cellular and molecular interactions that are present in tumours (Hanahan and Weinberg, 2011). Our results in chapter 2 of this thesis demonstrated that pharmacological inhibition of FZD receptors, via OMP-18R5 treatment, resulted in significantly smaller gastric tumour xenografts when MKN28 and MKN45 GC cells were injected *in vivo*; illustrating that inhibition of FZD receptors slows the growth of GC cells. This is supported by a parallel experiment from our laboratory in which it was shown that gastric adenomas required Fzd7 for optimal growth using genetic and pharmacological strategies in two independent mouse models (Flanagan et al., 2019a). Furthermore, these data are confirmed by earlier work that demonstrated that the targeting of multiple FZD receptors blocked the growth of several different cancers (Gurney

et al., 2012), which we can now extend to include GC. Future work to validate the effectiveness of targeting FZD7 in GC could be through the treatment of Human GC organoids/PDX models to accurately represent the variety of the molecular and genetic landscape of GCs seen in the clinic; this would also address a weakness of this current study, whereby, the newly classified molecular GC subtypes are not represented by our cell lines.

As mentioned above, metastasis is the key contributor to the high mortality rate observed in GC patients as well as being a major challenge in the development of novel treatments. We have demonstrated that FZD7 plays a predominant role in the invasion and migration capabilities of GC cells *in vitro*, suggesting it is also important for metastasis of GC cells *in vivo*. This is consistent with evidence that over-expression of FZD7 is associated with GC metastasis, advanced clinical stages and poor patient prognosis (Li et al., 2018). These findings are further supported by a similar investigation in the context of CRC. They reported that FZD7 is involved in the progression of CRC through the enhancement of survival, invasion and metastatic capabilities of CRC cells through transfection of CRC cells with FZD7 siRNA (Ueno et al., 2009). Interestingly, they reported that FZD7 was transmitting signals via both the canonical and non-canonical signalling pathways. Accumulating evidence suggests that non-canonical Wnt signalling is important in regulating cellular polarity and movement (Veeman et al., 2003) and so it is likely to play a role with the context of cancer metastasis. Wnt5a, a ligand that activates the non-canonical branch of the Wnt pathway, can play a role as a tumour suppressor or by promoting cancer invasion and migration, although the molecular mechanisms explaining these roles have not been fully elucidated (Astudillo, 2020). Overexpression of Wnt5a has been implemented in promoting GC progression (Kurayoshi et al., 2006) with one study reporting that Wnt5a-targeted knockdown in GC cell lines reduced cell migration through inhibition of Rac1, a driver of GC invasion. Additionally, suppression of Wnt5a with the use of an anti-Wnt5a antibody blocked clathrin-mediated internalization of the

FZD2 and Ror2 receptors (Hanaki et al., 2012). However, the function of this ligand has not been investigated in the context of GC *in vivo*, although accumulating evidence does point to a role for Wnt5a in GC metastasis. Therefore, as FZD7 is able to transmit signals through both the canonical and non-canonical pathways (Phesse et al., 2016) future experiments investigating if FZD7 is playing a role in the non-canonical pathway in the context of GC metastasis is vital to further support the findings of this thesis. Future experiments should inhibit FZD7, via FZD7-targeted shRNA and OMP-18R5 treatment, and look at the expression of c-Jun, the phosphorylation of JNK and c-Jun, and activation of RhoA. It has been reported that Wnt5a failed to induce tumour initiation. An inducible *Wnt5a* transgenic mouse model was generated by crossing TetO-Wnt5a mice with hnRNP-rtTA mice which drive ubiquitous expression of the doxycycline inducible rtTA2S-M2 Tet-trans-activator (Katsantoni et al., 2007). Induced Wnt5a expression was well-tolerated in adult mice for multiple durations, including 1 day, 1 week and 3-5 months (Bakker et al., 2012). This is suggestive that Wnt5a alone is not sufficient for GC establishment and may be working in concert with other pathways. Nevertheless, this thesis has clearly demonstrated that FZD7 is transmitting oncogenic signals via the canonical Wnt/ß-catenin pathway, which are promoting the invasion and migration capabilities of GC cells. We demonstrated that inhibition of FZD7 reduced the expression of Wnt target genes associated with the canonical ß-catenin pathway including *MYC*, *AXIN2*, *CCND1* and *LGR5*. Furthermore, inhibition of FZD7 down-regulated EMT and reduced GC cells colony forming ability, indicative of reduced CSC activity, which is associated with attenuated canonical Wnt/ß-catenin signalling. Additionally, gastric organoids required Wnt3a in the culture medium in addition to the Wnt agonist R-spondin, demonstrating that canonical Wnt ligands are required for GC growth.

EMT enables cancer cells to obtain migratory capacity, infiltrate surrounding tissue and metastasize to distant sites. Wnt signalling has been reported to

promote EMT through the up-regulation of transcription factors slug, snail, vimentin, and twist1 (DiMeo et al., 2009). Similarly, FZD receptors are thought to be critical factors for the EMT processes. Recent investigations have shown that down-regulation of FZD7 expression significantly inhibited cell invasion and migration, accompanied with a decreased expression of vimentin and snail, and increased expression of E-cadherin, in cervical and ovarian cancers (Asad et al., 2014; Deng et al., 2015). Wnt3/FZD7 signalling is up-regulated in hepatocellular carcinoma and leads to an attenuation of E-cadherin expression (Kim et al., 2008b) and over-expression of FZD7 was been reported to promote cell mobility, metastasis and EMT in esophageal cancer (Cao et al., 2017). The results presented in this thesis have demonstrated that inhibition of FZD receptors caused an increase in E-cadherin expression along with decreases of N-cadherin, vimentin, snail, slug, and twist1. However, a potential weakness of this study is that only a selection of EMT markers were evaluated. The expression profile of cells undergoing EMT is not validated and can vary between tissue-types, therefore, a larger and more expansive panel of epithelial and mesenchymal markers should be utilized to fully evaluate the EMT status in GC. Nevertheless, this work provides novel evidence that one of the 5 FZD receptors inhibited by OMP-18R5 (FZD1, 2, 5, 7, and 8) could promote EMT, enhancing the metastatic potential of GC cells. Of the 5 inhibited receptors, FZD7 is likely the key receptor promoting EMT due to the gene expression analysis performed in chapter 2 showing that FZD7 is abundantly expressed in the GC cell lines used for our experiments. Furthermore, the expression of FZD1, 2, 5 and 8 was not observed to be highly expressed in our cell lines. Further experiments to confirm that FZD7 is the predominant receptor in promoting EMT are required. The EMT experiments outlined in chapter 3 should be repeated with the use of FZD7-targeted shRNA in place of the OMP-18R5 treatment; this would have taken place if not for lab restrictions due to COVID19 lockdown. This would have confirmed FZD7 is the predominant receptor regulating EMT in GC.

The role of FZD7 in GC metastasis has not be investigated *in vivo*. Therefore, this remains a key area to be investigated in this field to build on the novel findings from this thesis. This project has the scope to examine this through the use of the CRISPR-Cas9-sgFZD7 construct generated in chapter 6. This can be cloned into a DOX-inducible Cas9 vector which will be stably transfected into GC cell lines which can be used in our *in vivo* abdominal metastasis model to elucidate the functional role of FZD7 in GC metastasis. The injected GC cells can be left to develop into palpable tumours, after which stage, the mice will be injected with doxycycline to induce Cas9 expression and the subsequent knock-out of FZD7. The weakness remains of not being able to explore the function of FZD7 in GC in a setting more representative of the clinic, for example with immune cells. To complement this, *in vitro* 3D co-culture experiments of GC cells with CAFS or tumour-associated macrophages, could be established to investigate the relationship of GC cells with the TME.

We have demonstrated that inhibition of FZD receptors or Wnt secretion leads to a reduction in migration, invasion, EMT and stemness, illustrating that Wnt modulates these aspects of the metastatic cascade in GC. However, our *in vivo* data indicated that Wnt is not required in our model of GC, which was representative of the later stages of the metastatic cascade when cells are surviving in the metastatic niche and establishing secondary sites. Therefore, the requirement for Wnt in the process of MET and regaining an epithelial phenotype should be investigated further in GC to further strengthen the insights gained from this project.

As we have demonstrated, modulation of the FZD-Wnt complex, either by inhibition of FZD receptors or inhibition of Wnt secretion, leads to a reduction in GC initiation and metastatic potential. Another key player involved in close concert with the FZD-Wnt complex is RNF43. Loss-of-function mutations to RNF43 have been thought to drive the development of many cancers due to the upregulation of FZD receptors on the cell surface (Giannakis et al., 2014; Jiang et al., 2013). Currently there are conflicting reports of the significance of

RNF43 loss in cancer, including within the setting of GC. Recent publications argue that the most frequently observed RNF43 mutation, G659fs, retains functionality in the inhibition of Wnt signalling and is unlikely to contribute to tumorigenesis (Li et al., 2020; Tu et al., 2019). Whereas, an opposing publication reports through *in vivo* studies that C-terminal truncation RNF43 mutants, including G659fs, are in fact loss of function and likely to contribute to tumourigenesis caused by active Wnt signalling (Yu et al., 2020). We have demonstrated the functional significance of RNF43 in GC by robust conditional deletion *in vivo*. Genetic knockout of RNF43, and its homologue ZNRF3, resulted in the development of gastric adenomas with increased levels of Wnt target gene expression. This supports the idea that LOF mutations to RNF43 are significant in GC development and is additionally supported by a study in which knockdown of RNF43 enhanced the tumourigenic potential of gastric cell lines (Neumeyer et al., 2019). Clinically this is an important finding as it supports the idea what these Wnt-addicted RNF43 mutant tumours will be responsive to Wnt inhibitors, such as LGK-974 (Liu et al., 2013; Madan et al., 2016; Yu et al., 2020). Our data contributes to the increasing evidence in support of the significance of RNF43 LOF mutants in cancer which will facilitate the selection of patients who may benefit from upstream Wnt pathway inhibitors. Additionally, the findings presented in this thesis show for the first-time the functional significance of RNF43 in the context of the gastric epithelium.

Furthermore, we observed metaplasia and lesions indicative of early tumour development within the single RNF43 knockout mice. This is suggestive that loss of a single E3 ligase will eventually lead to the development of less gastric tumours. An important next step for this project will be to age the single knockout mice for longer and observe if gastric tumours develop, as a weakness of this study was the short time-point, which is not necessarily reflective of the long latency of GC seen in the clinic. As this thesis has demonstrated, FZD7 plays a predominant role in GC initiation and progression, therefore, another important next step for this project will be to assess if the phenotype observed

in the double knockout can be rescued by co-deletion of *FZD7*. Furthermore, our double mutant mice could be treated with OMP-18R5 and their tumour burden quantified, as this is more clinically relevant. This study was the first investigation into the functional significance of RNF43 in the gastric epithelium using a robust genetic *in vivo* model, however, the data generated is in the early stages and further characterization of the resulting tumours and aberrant gastric epithelium needs to be undertaken to support and strengthen the findings. Additionally, this study looked at full genetic knock-out of RNF43, whereas in human GC, truncation of RNF43 is more commonly observed.

It is important to note that non-mutational drivers, such as microRNAs (miRNA), are able to drive Wnt activity. For example, miR-103/107 has been demonstrated to prolong Wnt/β-catenin signalling and CRC stemness by targeting Axin2 (Chen et al., 2019). miRNAs are also capable of targeting Wnt at the level of the Wnt receptors. For example, miR-100 has been demonstrated to inhibit Wnt/β-catenin signalling by targeting FZD8 leading to the suppression of the migration and invasion of breast cancer cells (Jiang et al., 2016). Mir-188-5p has been reported to activate the Wnt/β-catenin pathway in GC and its expression is positively correlated with GC metastasis (Li et al., 2019c). Therefore, miRNAs should be an avenue in future GC research.

Collectively, our data has shown that targeted inhibition of Wnt receptors, specifically FZD7, is rate-limiting for the growth of GC cells with and without APC mutations. Therefore, we hypothesis that a possible mechanism to allow APC mutant cells to respond to FZD7 inhibition is due to the continued transcription and translation of the mutant APC gene itself (Figure 7.1) as opposed to complete deletion as previously assumed in GC. This would allow the restoration of the destruction complex. This hypothesis is supported by the "just right" model of Wnt signalling in which it has been demonstrated that different APC mutations result in different levels of Wnt pathway activation (Albuquerque et al., 2002). Despite our evidence that demonstrates Wnt signalling can be further regulated at the level of the receptor in APC mutant

cells, other studies have indicated that this phenomenon is context dependent and may not be observed in all cancers with APC mutations (Flanagan et al., 2019; Huels et al., 2018).

There is no evidence in if disruption of the FZD receptors has the same effect in GC cell lines with β-catenin mutations, although this is less clinically relevant as mutations to β-catenin is rare in GC. Of interest, a study in *CTNNB1* mutant CRC cells did not observe the same response as seen with APC mutant cells, suggesting a specific role for mutant APC (Saito-Diaz et al., 2018). However, this may be cancer-specific and not translate to GC.

Therefore, a key next step for this project will be to examine the molecular mechanism by which APC mutant GC cells are responding to inhibition of FZD7. The CRISPR-Cas9 constructs generated in chapter 6 can be employed to examine which exact regions of the APC protein are required to permit GC cells to respond to FZD7 inhibition. These constructs will generate different mutant APC proteins of specific lengths and be used in combination with the human gastric organoid culture platform to ensure it is clinically relevant. The normal human gastric organoids will be transfected with the Cas9-APC-mutant constructs and treated with FZD inhibitors. The viability of the organoids assessed and characterized to expand the fields understanding of Wnt signalling in mutant GC cells.

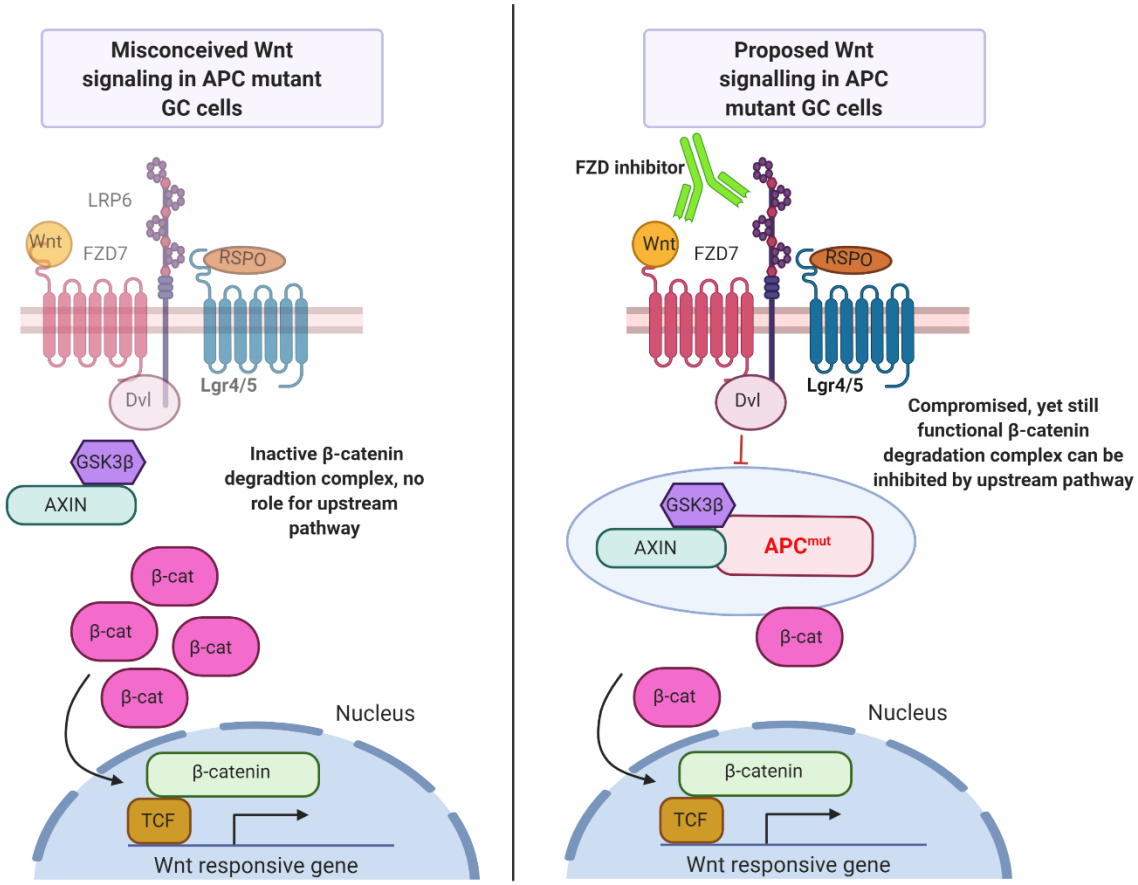

Figure 7.1 Proposed Wnt signalling in mutant APC GC cells. In APC mutant cells, it is often misconceived that APC is completely deleted and therefore the degradation complex is non-functional and Wnt signalling cannot be regulated upstream of the degradation complex at the level of the receptor (Illustration faded out, left panel). However, mutant APC is transcribed and translated resulting in a compromised, yet function, ß-catenin degradation complex. This explains how upstream inhibitors, FZD inhibitor, can still modulate Wnt signal activity (right hand panel)

7.2 Conclusions

In this thesis we have demonstrated a novel and predominant role for the Wnt receptor FZD7 in the initiation, invasion and metastatic capabilities of GC cells. For the first time, we have functionally shown that the loss of RNF43/ZNRF3 promotes the development of GC through overexpression of FZD receptors on the cell surface. Importantly, we have confirmed that modulation of the Wnt pathway at the level of the receptor/ligand is sufficient to block GC initiation, migration, and invasion irrespective of the downstream APC mutational status. These findings provide novel insights into the molecular understanding of GC. These can be translated into an attractive and novel therapeutic strategy for the treatment of GC and facilitate a new framework for patient stratification based on RNF43 mutational status.

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