The role of Frizzled-7 in the normal intestinal epithelium and colorectal cancer

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Abstract

The Wnt signalling pathway is pivotal for many biological processes, it controls cell fate during embryonic development, is a key regulator of homeostasis in adult self-renewing tissues, is necessary for regeneration and deregulation of the pathway leads to malignant transformation. The intestinal epithelium, which is composed of a single layer of intestinal epithelial cells that line the gastrointestinal tract, is the best-understood example for the roles of Wnt signalling during homeostasis and tumorigenesis. The Wnt signalling pathway was originally linked to the genesis of colorectal cancer (CRC) through linking the genes that are mutated in human CRC to the regulation of Wnt pathway mediated transcription. Through a great deal of attention directed at understanding the link between mutated intracellular components of the pathway and the initiation of CRC, it was shown to have a fundamental role in the regulation of intestinal stem cells and in malignancies of the intestinal epithelium as well as play an important role in intestinal homeostasis. Although our knowledge about the Wnt signalling pathway has increased immensely since its discovery, there are still numerous questions regarding Wnt signalling to explore. Thus, this thesis attempts to expand our knowledge of its role in the intestine and CRC, more specifically the role of the transmembrane receptors responsible for transducing Wnt signals, the Frizzled receptors, particularly Frizzled-7, one of ten seven-transmembrane receptors of the Frizzled family.

One of the aims was to determine which Wnt(s) interact with Frizzled-7 (Fzd7), as a role for Fzd7 in CRC morphogenesis was previously shown. Thus in Chapter 2 I demonstrate that Wnt secretion is necessary for the phenotype transitions that are associated with the dissemination of the cells from the primary tumour site and that Wnt2b and Wnt3a can cooperate with Fzd7 in this process. Furthermore, I confirm the interaction between Fzd7 and Wnt3 and demonstrate an interaction between Fzd7 and Wnt2b via co-immunoprecipitation.

In chapter 3, the aim was to investigate the role of Fzd7 in the homeostasis of the intestinal epithelium. I demonstrate that conditional deletion of Fzd7 in vitro in 3D “mini-gut” organoid cultures in LGR5⁺ CBC stem cells is detrimental to the organoids, which verified our previous in vivo findings and suggests that Fzd7 is required for
intestinal homeostasis and has a critical role in transmitting essential Wnt signals in stem cells of the intestinal epithelium.

In CRC over-expression of Fzds, specifically Fzd7, has been well documented as well as its involvement in the survival, invasion and metastatic capabilities of CRC cell lines. So, in Chapter 4 I explore the potential therapeutic benefit of Fzd7 inhibition in 3d adenoma organoid cultures, by employing antibodies that target Fzd7. Blocking the Fzd7 receptors is sufficient to reduce tumour growth and limit proliferation. Furthermore, conditional deletion of Fzd7 in vitro in the cancer stem cells reveals a strong drive to restore Fzd7+ cells.

Collectively, the findings from this thesis demonstrate a role for Frizzled-7 in maintaining intestinal stem cells in homeostasis and potentially in cancer. Moreover a role for the upstream components of the Wnt signalling pathway, i.e. Wnts and Fzd7, in cancer was shown.
Declaration

This is to certify that

i) The investigations conducted in this thesis are entirely my own work, unless stated otherwise.

ii) Materials and work from other sources and publications have been appropriately acknowledged.

iii) This thesis is fewer than 100,000 words in length, excluding figures, tables, maps, bibliographies and appendices.

Renate H. M. Schwab
Acknowledgements

“The master has failed more times than the beginner has even tried”
- Stephen McCranie

A PhD is something that is not accomplished in isolation and as such I would like to thank and acknowledge the following people in helping me achieve this goal.

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<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>ADF</td>
<td>Advanced DMEM-F12 medium</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>Ascl2</td>
<td>Ascete-like-2</td>
</tr>
<tr>
<td>CBC</td>
<td>Crypt base columnar</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
</tr>
<tr>
<td>CCND2</td>
<td>Cyclin D2</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-rich domain</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>Dkk</td>
<td>Dickkoff</td>
</tr>
<tr>
<td>dp-PCR</td>
<td>Deleted-product polymerase chain reaction</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>Gsk3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>ISC</td>
<td>Intestinal stem cell</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>Lgr5</td>
<td>Leucine-rich G-coupled protein receptor-5</td>
</tr>
<tr>
<td>M-MLV RT</td>
<td>Moloney Murine Leukemia Virus Reverse Transcriptase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered-saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase-C</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>R-Spo</td>
<td>R-Spondin</td>
</tr>
<tr>
<td>SDS</td>
<td>Soidum dilauryl Sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sFRP</td>
<td>Secreted Frizzled related protein</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short-hairpin RNA</td>
</tr>
<tr>
<td>TA</td>
<td>Transit amplifying</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered-saline-tween</td>
</tr>
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Chapter 1

Literature review
1.1 Introduction

The gastrointestinal tract (GIT) is a large, muscular tube that extends from the mouth and ends at the anus. Food and drink enter through the mouth then travel through the pharynx, oesophagus, stomach, small and large intestines, where with the help of hormones and enzymes, the food is digested and the nutrients (e.g. sugars, amino acids, fatty acids, etc.) absorbed and utilised by the cells of the body. The waste material is then expelled from the body through the rectum and finally the anus. These processes take place primarily in the stomach and small intestine respectively, whereas the large intestine absorbs water, from the remaining indigestible food matter, and any remaining nutrients and electrolytes. Along with selectively absorbing nutrients, the intestinal epithelium that lines the organs of the GIT, is also tasked with simultaneously excluding luminal pathogens and pro-inflammatory molecules from host tissues. The intestinal epithelium, composed of a single layer of intelligent intestinal epithelial cells (IECs), lines the luminal surface of the GIT, functioning as a physical barrier against luminal insults (e.g. harmful microorganisms, antigens and toxins), secreting factors necessary for digestion (e.g. mucus, gastric acids, hormones and proteases) as well as protective factors (e.g. immunoglobulins and antimicrobial peptides). It also controls gastric motility and nutrient and water uptake. The absorptive and protective functions of the GIT are dependent on an intact and functional intestinal epithelium, which is under constant exposure to chemical, biological and mechanical stresses. Thus the intestinal epithelium is constantly renewed to preserve homeostasis, which is maintained by continuous and rapid replacement of differentiated cells, at a turnover rate of approximately 3-5 days. This turnover is governed by the rate of cell proliferation and cell shedding. Dedicated pools of intestinal stem cells (ISCs), residing in defined niches, drive the epithelial renewal and regeneration of the GIT. Research using mouse models has shown that the ISCs can give rise to all cell types of the intestinal epithelium throughout the lifetime of the animal. Indeed, research efforts into better understanding and characterizing factors which control and influence the ISCs and their contribution to homeostasis and tumourigenesis has been immense. Several molecular pathways
regulate the activity of the ISCs; however the Wnt signalling pathway is the most prominent.

The Wnt signalling pathway plays a critical role in development of the GIT and homeostasis, and is also very important in regeneration of the intestinal epithelium following injury. Wnt signals are extremely pleiotropic in their activity, with consequences ranging from mitogenic effects to control and regulate cell proliferation, to differentiation, changes in polarity and differential cell adhesion. Therefore, the Wnt pathway must be tightly controlled to minimise aberrant Wnt activity, which has been implicated in a number of diseases, including cancer. Recently, research in colorectal cancer (CRC) has shown that the level of Wnt signalling is important and also additional regulation from each step of the pathway further regulates Wnt signalling. Thus, the focus of this review is on the role of Wnt signalling, especially the Wnt/β-catenin pathway, in the intestine and how Wnt signalling regulates homeostasis and the consequences of abnormal Wnt pathway activation. Special attention is paid to the contribution of Frizzled receptors, particularly Frizzled-7, as it is conserved in the gut through evolution from hydra to man and is prominent in stem cell function and cancer.
1.2 Anatomy of the mouse and human gastrointestinal tract

In physiology and anatomical structure, the mouse and human are quite similar; hence the widespread use of mouse models in biomedical studies. Particularly, the gastrointestinal tracts (GIT) are composed of organs that are anatomically similar in both species (Nguyen et al., 2015), albeit prominent differences exist as well (Table 1.1). Morphological differences in mouse small intestine compared to human, is that the mouse villi are much taller, which increases the surface area and has been suggested to be a compensation mechanism for the lack of mucosal folds. Additionally the mouse colon is rather smooth with no divisions, compared to the human large intestine, which is sub-compartmentalized into pouches. At the cellular level, notable differences between humans and mice are in the distribution of goblet and Paneth cells in the large intestine. In mice, the mucin-producing goblet cells are abundant along the crypts of the proximal colon and in the distal colon and rectum they decrease in number at the base of the crypt. However, in humans, goblet cells are abundant from the cecum to the rectum. Paneth cells on the other hand are rare but present in the cecum and ascending colon of humans, but absent in the mouse colon and uniquely found in the cecum (Nguyen et al., 2015). Overall, the mammalian GIT is strongly conserved and the differences between species are likely due to variations in diet.
Table 1.1: Similarities and differences in the anatomy of the mouse and human GIT

<table>
<thead>
<tr>
<th>Features</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall anatomy: gastrointestinal tract is composed of mouth, esphagus,</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>stomach, small intestine, large intestine and anus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Composition of sectional tissue of small intestine: mucosa, lamina propria,</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>muscularis mucosae, submucosa, muscular tunics, nervous plexi, serosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of cells in small intestine: absorptive enterocytes, goblet cells,</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>enteroendocrine cells, Paneth cells, microfold (M) cells, caveolated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(chief) cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Composition of sectional tissue of the large intestine: mucosa, lamina pro</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>pria, muscularis mucosae, submucosa, muscular tunics, serosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of cells in the colon: absorptive colonocytes, goblet cells,</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>enteroendocrine cells, microfold (M) cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>Divided into non-glandular/fore-stomach and glandular stomach, the</td>
<td>Lack fore-stomach, no limiting ridge</td>
</tr>
<tr>
<td></td>
<td>two parts separated by limiting ridge</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>Taller villi with no mucosal folds</td>
<td>Shorter than mouse villi, presence of mucosal folds</td>
</tr>
<tr>
<td>Cecum</td>
<td>Large, fermentation happens here</td>
<td>Small, no fermentation</td>
</tr>
<tr>
<td>Appendix</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Colon</td>
<td>Rather smooth and no division</td>
<td>Clearly divided into different sections: ascending, transverse and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>descending colon</td>
</tr>
<tr>
<td>Bowel of colon</td>
<td>Thin muscularis mucosae</td>
<td>Variable thickness</td>
</tr>
<tr>
<td>Presence of haustrum and taenia coli in the colon</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Distribution of Paneth cells</td>
<td>Present only in the small intestine</td>
<td>In the cecum and ascending colon</td>
</tr>
<tr>
<td>Distribution of goblet cells</td>
<td>Abundant in proximal colon, number decrease at the base of the crypt</td>
<td>Abundant from cecum to rectum</td>
</tr>
<tr>
<td></td>
<td>in distal colon and rectum</td>
<td></td>
</tr>
<tr>
<td>Distribution of transverse folds</td>
<td>Restricted to the cecum and proximal colon</td>
<td>Along the length of the colonic mucosa</td>
</tr>
</tbody>
</table>

Adapted from Nguyen et al., 2015
1.3 Structure and function of the small intestinal epithelium in the mouse

Small intestinal architecture

Anatomically, the small intestine is divided into three parts, the duodenum, jejunum and ileum and is approximately 35cm from the stomach to the cecum. The intestine has four layers: an inner mucosa layer; the submucosa; the muscularis layer, and the serosa, which covers the outer intestine (Shackelford, 1999). The mucosal layer facing the intestinal lumen is where the important absorptive functions occur, and is further divided into the epithelium (innerlayer), lamina propria and muscular mucosae. The epithelium consists of continuous villi and crypts, with the villi decreasing in length progressively from the duodenum to the ileum (Fox et al., 2006). The villi, are finger-like projections that extend into the intestinal lumen to maximise nutrient exchange and are encircled by at least six crypts. The villi are populated by differentiated epithelial cells that constitute the functional compartment of the epithelium. The differentiated cells are no longer capable of dividing and have the features of mature epithelial cells. They are categorized based on their function: enterocytes that function to absorb nutrients, goblet cells that secrete a protective mucus barrier, enteroendocrine cells that release gastrointestinal hormones and secretory tuft cells (Gerbe et al., 2011). On the other hand, the Paneth cells, which secrete antibacterial peptides, reside at the base of the proliferative compartment in the mucosal invaginations called the crypts of Lieberkühn. The crypts are populated by the undifferentiated and rapidly cycling cells that represent the proliferative compartment.

It is from here that the tremendous cell turnover is provided as well as the protective niche for the intestinal stem cells (ISCs). As the cells begin to differentiate, they migrate up towards the lumen and are shed from the villus tip once they have performed their function, except for the Paneth cells which migrate downwards. Therefore, the crypt is predominantly a proliferative compartment, which is monoclonal and maintained by multipotent stem cells, whereas the villi comprise the differentiated compartment and are polyclonal, as they receive cells from multiple crypts (Wright, 2000).
**Intestinal epithelial renewal**

The small intestinal epithelium is the most vigorously self-renewing tissue of adult mammals (Crosnier et al., 2006). Stem cells in the proliferative crypts give rise to all of the epithelial cells to maintain the integrity of the intestinal epithelium and are defined by their ability to self-renew. In the mouse, the intestine alone has approximately a million crypts, which generate some 300 cells per crypt per day (Hagemann et al., 1970), as the average life cycle of an epithelial cell spans less than a week (Leblond and Stevens, 1948). This extreme production of cells is compensated by cell loss at the villus tip, as the epithelium is in a perpetual upward movement driven by the stem cells, which together with the Paneth cells escape this upward flow. The stem cells divide on average once every day (24-30hrs), giving rise to transit amplifying cells that divide more rapidly, approximately every 12-16hrs, while they migrate up to the crypt-villus junction, where they terminally differentiate. Once terminally differentiated, they support the villus with their individual function while they continue migrating towards the top of the villus, where they undergo apoptosis and are sloughed off into the lumen. Paneth cells on the other hand, are not as rapid in their renewal; they require 3-6 weeks (Garabedian et al., 1997; Ireland et al., 2005). Located at the base of the transit amplifying compartment are committed secretory progenitor cells that give rise to the Paneth cells. While migrating downwards, they differentiate and once mature, they provide the specialized microenvironment, the stem cell niche, with all the factors the stem cells require to survive and function (Sato et al., 2011).
Figure 1.1 Structural organization in the small intestine

(A) Scanning electron micrograph of the small intestine. The intestinal tissue is divided into villi and crypts (at least six crypts of Lieberkühn encircle one villus) (reproduced from Barker, 2014).

(B) Cartoon depiction of epithelial self-renewal in the intestinal epithelium. Lgr5+ (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) crypt base columnar (CBC) stem cells are intercalated with Paneth cells at the crypt base. These stem cells continuously generate rapidly proliferating transit-amplifying (TA) cells, which occupy the remainder of the crypt. TA cells differentiate into the various functional cells on the villi (enterocytes, tuft cells, goblet cells and enteroendocrine cells) to replace the epithelial cells being lost via anoikis at the villus tip. The +4 ‘reserve’ stem cells (which occupy the fourth position from the crypt base) can restore the Lgr5+ CBC stem cell compartment following injury (reproduced from Barker, 2014).
1.4 Intestinal stem cells

Small populations of either four to six (Potten and Loeffler, 1990) or one (Gordon et al., 1992) intestinal stem cell (ISC) is located in the crypt compartments. Multiple studies by numerous investigators exploring the location, number and behaviour of the ISCs within the base of the crypt have been characterized by varying methods (mouse models, following chemoradiation, somatic mutations, chimeric breeding, transgenic over-expression or ablation of specific regulatory genes). Interpretation of the data continues to be debated and as such two opposing stem cell models, which have been competing for legitimacy for over four decades, exist: the “stem cell zone model” and the “+4 model”. The “stem cell zone model” proposes that at the intestinal crypt base undifferentiated and mitotically active crypt base columnar (CBC) cells, which are intercalated between differentiated Paneth cells, give rise to all four cell lineages of the intestinal epithelium (Bjerknes and Cheng, 1999; Cheng and Leblond, 1974b). The “+4 model” on the other hand suggests that the stem cells reside directly above the uppermost Paneth cell positioned between +2 and +7, average +4, relative to the crypt base (Potten, 1977). Despite the recent discovery of specific markers for both candidates of the stem cell models, it has proven difficult to demonstrate which of the two models is correct. These are discussed in more detail in the relevant chapters.

**Figure 1.2 Intestinal stem cells**

(A) Summary of the current markers known for the +4 and CBC stem cells (reproduced from Barker, 2014).
Adapted from Barker, 2014
1.5 Brief summary of Wnt history

Wnt genes and proteins have now been studied for nearly 30+ years and the pace of discovery concerning components and regulators of the Wnt pathway continues to increase. Genes responsible for the patterning of larval body segments during development, e.g. Wingless (Wg), Armadillo (Arm) and Arrow, were originally discovered in Drosophila (Nusslein-Volhard and Wieschaus, 1980). The Int-1 gene, now known as Wnt1; which is an amalgam of Wg and Int-1, was shown to be favoured for insertion into the mouse mammary tumour virus (MMTV) proviral DNA (Nusse and Varmus, 1982). Discovery of the common origin of the Drosophila Wg and the murine Int-1 (Rijsewijk et al., 1987), laid the keystone of a signalling pathway now commonly referred to as the canonical Wnt cascade. Wnts and their downstream effectors, were then subsequently demonstrated to be conserved in all metazoans (Wodarz and Nusse, 1998). Additionally, studies in Xenopus supported the notion, that Wnt signalling is conserved between vertebrates and invertebrates (Nusse and Varmus, 2012). Gaps in understanding Wnt signal transduction were filled by the discovery of TCF/LEF transcription factors as Wnt nuclear effectors (Behrens et al., 1996), Frizzleds as Wnt receptors (Bhanot et al., 1996; Yang-Snyder et al., 1996) and Lrps (Wehrli et al., 2000) as co-receptors of Frizzled that help transduce the Wnt signal.

1.6 The Wnt signalling pathways

Wnts initiate signalling via binding to their Frizzled receptors. Wnt/Fzd mediated signalling branches into several pathways, whereby differential activation depends on specific Wnt ligands, Fzd isoforms and the cellular context. There are two types of pathways induced by Wnt/Fzd interaction; the canonical Wnt/β-catenin signalling pathway (β-catenin-dependent) and the non-canonical Wnt signalling pathways (β-catenin-independent), which are the Wnt/Ca++ and the vertebrate PCP pathway.

The canonical Wnt/β-catenin signalling pathway

The ultimate outcome of canonical Wnt signalling is the nuclear localization of β-catenin where it co-activates the transcription of target genes, hence β-catenin-
dependent. It governs cell differentiation, proliferation or apoptosis, depending on the cellular context and the surrounding microenvironment.

In the absence of Wnts, cytoplasmic β-catenin turns over rapidly; it is phosphorylated by a cytoplasmic multi-protein “destruction” complex containing: Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3β) and casein kinase I (CKI). The scaffolding proteins APC and Axin/Axin2 sequester β-catenin, thus allowing CKI to phosphorylate the N-terminus of β-catenin at serine (Ser) S45. Subsequently additional serine and threonine (Thr) residues N-terminal to S45 are phosphorylated by GSK3β. Once phosphorylated, β-catenin is quickly recognized by the F-box-containing protein β-TrCP, which mediates ubiquitination and degradation of β-catenin by proteasomes. Activation of the canonical pathway is initiated by Wnt binding to Fzd and low-density lipoprotein related protein (LRP) co-receptor. The binding of Wnt to the Fzd/LRP receptor complex induces the formation of receptor aggregates, which promote Dishevelled (Dsh)-dependent phosphorylation of the LRP. The phosphorylated LRP then recruits Axin to the receptor complex. This inhibits the multi-protein complex, thus allowing for the stabilization and accumulation of β-catenin due to its escape from phosphorylation and subsequent degradation. β-catenin then translocates to the nucleus, where it interacts with members of the LEF (lymphoid-enhancing factor)/TCF (T-cell factor) family of transcription factors to regulate the expression of certain β-catenin/TCF-responsive target genes. An inhibition of the canonical Wnt signal is possible due to naturally occurring secreted proteins, which can either bind to LRP, hindering its binding to Fzd [e.g. Dickkopf (DKK)] or bind to Wnt, hindering its binding to Fzd [e.g. secreted Fzd related proteins (sFRP)]; Wnt inhibitory factor (WIF) (Rubin et al., 2006).
Figure 1.3 Overview of canonical Wnt/β-catenin signalling

(A) In the absence of a Wnt ligand, cytoplasmic β-catenin is targeted for phosphorylation by a destruction complex consisting of Axin, APX, GSK3 and CK1. Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-Trcp, which targets β-catenin for proteosomal degradation. TCF-LEF/Groucho and histone deacetylases (HDAC) repress Wnt target gene transcription. (reproduced from MacDonald et al., 2009).

(B) When a Wnt ligand is present and binds to its receptors, Frizzled and Lrp5/6, Dvl recruitment by Frizzled, leads to phosphorylation of Lrp5/6 and Axin recruitment. Axin-mediated phosphorylation and subsequent degradation of β-catenin is disrupted, thus allowing β-catenin to accumulate in the nucleus, where it serves as a co-activator for TCF to activate Wnt responsive genes (reproduced from MacDonald et al., 2009).
Adapted from MacDonald et al., 2009
The non-canonical Wnt signalling pathway

The non-canonical Wnt signalling pathway, signals independent of β-catenin and is divided into two branches: the Planar Cell Polarity (PCP) pathway and the Wnt/Ca\(^{2+}\) pathway. PCP pathway activity depends on which Wnt/receptor complex is activated. A variety of cytoplasmic PCP pathway signal transduction components are then activated, including the small GTPases like Rac1, RhoA, and JUN-N-terminal kinase (JNK), which in turn activate downstream kinases to affect and control cell migration, polarity and morphogenetic movements (Niehrs, 2012). Recent data also suggest that PCP signalling can promote proliferation in several different cancers (Kikuchi et al., 2011). The Wnt/Ca\(^{2+}\) pathway is responsible for the release of intracellular Ca\(^{2+}\) and the activation of Ca\(^{2+}\)-sensitive kinases, PKC and CamKII, which activate the transcriptional regulator nuclear factor associated with T cells (NFAT), thus regulating the transcription of target genes required for cell fate and migration (Niehrs, 2012).

1.7 Wnt ligand synthesis, secretion and signalling

Wnt proteins, found to be conserved in all metazoan animals (Wodarz and Nusse, 1998), are approximately 40KDa in size and mediate relatively close-range signalling between cells. Wnt genes, of which there are 19 in the mammalian genome, encode for cysteine-rich glycolproteins of approximately 350-400 amino acids that contain an N-terminal signal peptide required for proper secretion. Wnts undergo two forms of lipid modification before their extracellular transport. For secretion of active Wnts from the endoplasmic reticulum (ER) it appears that acylation of a conserved serine residue (Ser209), via modification with a monounsaturated fatty acid, palmitoleic acid, is required (Takada et al., 2006). Porcupine (Porc), an ER multipass transmembrane protein and member of the membrane-bound O-acyltransferase (MBOAT) family, adds a palmitoyl group to Wnt proteins, which is both essential for their signalling ability and required for their secretion (Takada et al., 2006). The loss of Porc function, via inhibition with inhibitors of Wnt production (IWPs) (Chen et al., 2009), causes Wnts to accumulate in the ER (Kadowaki et al., 1996), completely abrogating Wnt secretion (Vandenheuvel et al., 1993). Acylation with palmitic acid at a conserved cysteine residue (Cys77) appears to be required for the extracellular transportation of Wnt
proteins (Willert et al., 2003). Wntless (Wls), a seven-span transmembrane protein, is present in the Golgi, plasma membrane and endosomes, cycling between these compartments, and exclusively binds only lipid modified Wnt proteins (Willert and Nusse, 2012). Wls facilitates the Golgi exit of Wnts, trafficking them from the Golgi to the plasma membrane, as suggested by the accumulation of Wnt in the Golgi in the absence of Wls (Port and Basler, 2010; Port et al., 2008).

The Wnt family of secreted signal proteins play key roles in numerous aspects of embryogenesis as well as in tumourigenesis (Logan and Nusse, 2004; Moon et al., 2004; Reya and Clevers, 2005). Although traditionally considered to act as long-range morphogens, Wnts are now considered to transmit signals locally, thus acting as short-range signals, due to the tight control over their secretion and transport. Indeed, in the intestine, Wnts in the stem cell niche have been shown to be secreted by either the Paneth cells (Wnt3) (Farin et al., 2016), between which the intestinal stem cells are sandwiched, or by mesenchymal cells adjacent to the crypts (Wnt2b) (Valenta et al., 2016). Thus, the distance between the Wnt-secreted and Wnt-receiving cells is found to be kept at a minimum in the intestinal crypt.

Initially, based on over-expression studies in Xenopus embryos, Wnt ligands were classified into two functional groups, e.g. expression of “Wnt1 class” Wnts 1, 3a, 8 and 8b is sufficient to induce a secondary dorsal-ventral axis in the Xenopus embryo, whereas “Wnt5a class” Wnt expression, including Wnts 4, 5a and 11, is insufficient (Du et al., 1995; Olson and Papkoff, 1994; Shimizu et al., 1997; Slusarski et al., 1997; Wong et al., 1994) thus suggesting that the two groups signal via different intracellular pathways; either the canonical or the non-canonical pathway. However this classification, based on signalling specificity, is considered an oversimplification and has been challenged by the finding that so-called non-canonical Wnts can activate β-catenin-dependent signalling when in the presence of appropriate Fzd receptors and vice versa. The bona fide “non-canonical” Wnt5a has been shown to induce axis duplication in Xenopus embryos when in the presence of Fzd5 (He et al., 1997) and more recently it has been demonstrated, that depending on receptor context, it can
either activate or inhibit β-catenin-dependent signalling (Mikels and Nusse, 2006) and in addition non-canonical Wnt11 has also been shown to activate β-catenin-dependent signalling during axis specification in *Xenopus* (Tao et al., 2005). Furthermore, the “canonical” Wnt3a protein is able to activate non-canonical Wnt signalling through G protein-linked PKCδ (Tu et al., 2007).

**Figure 1.4 The Wnt secretion machinery (reproduced from Clevers and Nusse, 2012)**

The Wnt proteins become lipid modified in the endoplasmic reticulum by the Porcupine (Porc) enzyme. Further transport and secretion is dependent on the Evi/Wntless multiple pass transmembrane protein. The retromer complex is necessary for recycling of the Evi/Wntless endosomal vesicles.
1.8 Wnt receptors

As mentioned above, Wnts can activate different signalling pathways, which is determined by the receptor they bind to. Both canonical and non-canonical Wnt signalling requires Wnt to bind with the receptor Fzd, thus Fzds function in all three pathways; the canonical Wnt/β-catenin pathway, the PCP pathway and the Wnt/Ca\(^{2+}\) pathway. Notably, the interaction between Wnt and Fzd is a promiscuous one, as a single Wnt can bind multiple Fzd proteins and vice versa to activate Wnt signalling. Fzd genes, originally discovered through genetic screens in *Drosophila*, while looking for mutations that disrupt cell polarity, encode receptors for Wnt proteins. The receptors have an extracellular N-terminus with a conserved cysteine rich domain (CRD), which is responsible for the binding of Wnt ligands, they span the membrane seven times, i.e. seven-span transmembrane domains, and have an intracellular C-terminus with a putative PDZ binding domain. There are 10 Fzds in the mammalian genome, which can be divided into 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 (Fredriksson et al., 2003; Sagara et al., 1998). Fzd receptors were, similarly to the Wnt ligands, assigned to either the Wnt/β-catenin or Wnt/Ca\(^{2+}\) pathways based on their basal signalling abilities (Kuhl et al., 2000). This preliminary assignment has since been shown to be too general as well, as it has been demonstrated that Fzd4 can activate either β-catenin-dependent signalling (Ye et al., 2009) or non-canonical Wnt signalling branches (Robitaille et al., 2002). Also, in colorectal cancer Fzd7 has also been shown to signal via both the canonical and non-canonical pathways (Ueno et al., 2009).

The canonical Wnt pathway is activated when Wnt binds to both Fzd and its co-receptor LRP5/6. The single-span low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) was initially discovered when screening for *Drosophila* embryonic lethal mutants (Nusslein-Volhard and Wieschaus, 1980). Following the binding of Wnt, LRP5/6 on the cytoplasmic side, participates in key molecular interactions with the scaffolding protein Axin and the kinases CKI and GSK3β of the destruction pathway.
The non-canonical PCP pathway is activated following the binding of Wnt and Fzd and the associated receptor tyrosine kinases such as Ror2 or Ryk (Sebbagh and Borg, 2014) and Wnt/Ca\(^{2+}\) signalling is activated when Wnt, Fzd and the co-receptor Ror1/2 interact. Ror receptors (Ror1 and Ror2) can bind directly to Wnt ligands, suggesting that they can both function as Wnt receptors (Mikels et al., 2009). Ryk receptors on the other hand, do not bind directly to Wnt ligands. They are considered to be catalytically inactive and have been shown to bind to Frizzled, suggesting a potential co-receptor role in Wnt signalling, however this function is cell-context dependent (Berndt et al., 2011; Lu et al., 2004).

**Figure 1.5 Wnt receptor: Frizzleds (reproduced from Kaykas et al., 2004)**

A phylogenetic tree of human Frizzled. More related Frizzleds are shown closer together in the tree. 0.1 represents the number of changes per residue per unit length.
1.9 Regulators of Wnt/Frizzled receptor interaction

Regulation of the interaction between Wnt ligands and Fzd receptors is controlled by several proteins, to ensure that Wnt signalling is tightly regulated. Wnt regulators, both agonists and antagonists play important roles during development and continue function through to adulthood and have even been implicated in cancer. The secreted frizzled-related proteins (sFRPs) are a family of five secreted glycoproteins (sFRP-1, -2, -3, -4 and -5), which bind directly to Wnts, thereby altering their ability to bind to the Wnt receptor complex (Jones and Jomary, 2002). All sFRP family members contain an N-terminal domain, which is 30-50% homologous to the CRD of Fzd receptors, and thus considered both necessary and sufficient for Wnt binding and inhibition (Lin et al., 1997; Rehn et al., 1998). Similar to the sFRPs, the Wnt inhibitory factor 1 (WIF-1), also binds to Wnt ligands and inhibits Wnt signalling (Hsieh et al., 1999). WIF-1 consists of a N-terminal secretion signal sequence, the WIF domain, five Epidermal growth factor (EGF)-like domains and a hydrophilic C-terminus (Malinauskas et al., 2011). Wnt has been shown to bind to the WIF domain and the EGF-like domain (Malinauskas et al., 2011). The Dickkopf (Dkk) proteins selectively antagonise canonical Wnt signalling by binding to LRP5/6, thereby preventing Wnt and Fzd from forming a tertiary complex with it (MacDonald et al., 2004). Importantly, all three of these regulators are epigenetically silenced in colorectal cancer, via promoter hypermethylation (Aguilera et al., 2006; Caldwell et al., 2004; Suzuki et al., 2004; Taniguchi et al., 2005).

Figure 1.6 Regulators of Wnt/Frizzled receptor interaction (reproduced from Kypta and Waxman, 2012)

Wnt/β-catenin signalling is switched off, when inhibitors of Wnt/Frizzled interaction are present. The proteins sFRP or WIF-1 bind to Wnt and Dkk binds to the co-receptor Lrp5/6.
1.10 The role of Wnt signalling in the development of the gastrointestinal tract

Development of the GIT is conserved across species and develops by using a basic mechanism of development, the epithelial-mesenchymal interaction. Initially the embryonic gut forms as a simple tube that undergoes regional specialization followed by morphogenesis and differentiation, thus creating the different organs of the gastrointestinal tract. This primitive tube forms from two of the three primary germ layers established during gastrulation. The endoderm gives rise to the epithelium and the mesoderm develops into smooth muscle, the mesenchyme and numerous other cell types. The formation of the primitive gut tube and its subsequent regionalization involves cross-talk between the two layers, which is essential for normal gut development. Cross-talk between several morphogenetic pathways also contributes to the development of the GIT. The Wnt pathway is one of these. Initial studies of ascidian embryos, in which β-catenin was found to be essential for endoderm formation, revealed a role for Wnt (Imai et al., 2000). Studies in mice also demonstrated a role for β-catenin in endoderm formation (Lickert et al., 2002). Furthermore, expression of Wnt11 and Wnt5a were localized in the underlying mesenchyme throughout the anterior-posterior (A-P) axis (Lickert et al., 2001), although whether they function in the development of the GIT has not yet been established.

1.11 The role of Wnt signalling in intestinal epithelial homeostasis

Wnt signalling is also critical for adult homeostasis in the intestine and plays a crucial role as a regulator in stem cell proliferation and differentiation (Korinek et al., 1998; Sato et al., 2009; Van der Flier et al., 2007). An indication that Wnt signalling played a critical role in intestinal homeostasis was the intestinal phenotype of TCF-4 knockout mice. The intestinal epithelium of the TCF-4 knockout mice lacked proliferating crypts; that is, there was no stem-cell compartment, and consequently the epithelium was only composed of differentiated/non-dividing villus cells. Due to this aberration the mice died shortly after birth (Korinek et al., 1998). Furthermore, following the targeted disruption of β-catenin/TCF-4 activity in intestinal epithelial cells, c-Myc (a Wnt target gene) expression was decreased and as a consequence, the cell-cycle inhibitor p21 was
up-regulated resulting in abrupt arrest of proliferation in the crypt cells. Wnt signalling also plays a role in providing essential signals to the stem cell niche. Expression analysis of Paneth cells revealed that they provide the adjacent stem cells with a cocktail of niche factors such as Wnt3, EGF and Notch (Sato et al., 2011). Wnt3 was demonstrated to be essential for Lgr5+ stem cells, when grown in vitro as single cells, i.e. without their adjacent Paneth cells, as they did not survive (Farin et al., 2012). In addition, Wnt2b, which is secreted from subepithelial mesenchymal cells adjacent to the crypts, can compensate for the loss of Wnt3 (Farin et al., 2012; Valenta et al., 2016). Fzd receptors also have a role in the regulation of intestinal stem cells. Paneth cells are regulated by Wnt/Fzd5 interaction (van Es et al., 2005a) and our recent research has demonstrated a critical role for Fzd7 as a Wnt receptor for Lgr5+ stem cells (Flanagan et al., 2015).

1.12 Aberrant Wnt signalling activity and cancer

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the Western world (Jemal et al., 2011), with approximately 4,000 deaths each year in Australia alone. It has risen from being the 3rd most common cancer in 2012 to the second most common in 2016 as stated by Bowel Cancer Australia. In approximately 90% of fatal cases, metastasis is the cause of mortality.

A link between the Wnt pathway and human disease was first established by the discovery that patients with the hereditary form of CRC, known as familial adenomatous polyposis (FAP) had mutations in the APC gene (Kinzler et al., 1991a). This discovery was followed by the revelation, that the large APC protein (2843 amino acids) (Nathke, 2004) interacts with β-catenin and can subsequently activate Wnt/β-catenin activity (Korinek et al., 1997b; Morin et al., 1997a; Su et al., 1992). Thus, the initiation of CRCs evolves from the dysregulation of the Wnt signalling pathway. In 80-90% of CRCs, it develops from the mutant truncated tumour suppressor protein APC. In a normal epithelial cell, without Wnt exposure, APC helps GSK3 phosphorylate β-catenin. But when the APC gene is mutated, the truncated protein is less efficient at
facilitating phosphorylation of β-catenin. Thus, β-catenin is not phosphorylated, therefore it is not degraded by the proteasomes and accumulates in the cytoplasm and enters the nucleus, where it activates the transcription of target genes by binding to TCF/LEF. The canonical Wnt pathway becomes constitutively active, without the need for signalling between Wnts and Fzds. Consequently, when APC is mutated, Wnt signalling does not turn off. The cells fail to terminally differentiate and stop their migration towards the intestinal lumen; they also do not stop proliferating, thereby forming polyps. It is then possible for the polyps to undergo changes, causing them to transform from benign to malignant tumours.

Because of this constitutive activation of β-catenin/TCF/LEF transcription, it was assumed, that Wnt and Fzd molecules would not have a role in CRC. However, it has been shown, that Wnts and Fzds are over-expressed in CRC and also that inhibitors of Wnt-Fzd interaction are silenced in CRC (Aguilera et al., 2006; Caldwell et al., 2004; Suzuki et al., 2004). Both the over-expression of Wnts and Fzds and the loss of inhibitors indicate additional activity of Wnt-Fzd signalling mediated by the receptor-ligand complex.

1.13 Tumour progression of colorectal cancer involves EMT and MET

As mentioned before, when APC is mutated, Wnt signalling does not turn off. However, although all the malignant cells harbor truncated APC protein, nuclear β-catenin is not always detected. Detailed analysis of carcinoma tissue sections by immunohistochemistry revealed variable patterns of β-catenin localization; it was shown, that in some areas β-catenin was membrane-bound and in others it was nuclear. The lack of detectable nuclear β-catenin in areas of the carcinoma may be the result of a varying amount of nuclear β-catenin, which is below the detection limit in some CRC cells. Nuclear over-expression of β-catenin, the hallmark of active canonical Wnt signalling, was predominantly at the invasive front of carcinomas. In contrast, β-catenin was membrane-bound in the more polarized tumour cells in the central areas of the carcinoma, even though all the tumour cells harbor truncating APC mutations.
(Brabletz et al., 2001). This indicates that there is additional regulation of Wnt signalling in CRC cells, even though mutations in the downstream components of the pathway exist and turn on the pathway.

The cells at the invasive front are thought to be the beginning of metastasis, the dissemination of tumour cells to other organs in the body. This is due to the recognition that the observed distribution of β-catenin in the malignant tumour tissues bore many analogies to tissue patterning during embryogenesis and thus the link between canonical Wnt signalling and epithelial-mesenchymal transition (EMT) in CRC progression was made (Kirchner and Brabletz, 2000). As Wnt signalling is involved in both stem-cell function and the induction of EMT, it is a key factor for both the initiation and progression of CRC tumours (Brabletz et al., 2005).

It has also been observed, that in most CRC cases where metastasis has taken place, the metastases recapitulate the pathology of the primary tumour. This observation gave way to the recognition, that the EMT at the invasive front is dynamically regulated and that the reverse transition, mesenchymal-epithelial transition (MET) must occur at the secondary tumour site. That is, the de-differentiated mesenchymal cells at the invasive front revert back to a differentiated phenotype to build the tumour mass at the secondary site. Moreover, the secondary tumours also have invasive fronts. Analysing the staining of three proteins (β-catenin, E-cadherin, Ki-67), the pathology of the metastases is indistinguishable from the primary tumour. The de-differentiation and the reversion back to a differentiated state is a dynamic process (Brabletz et al., 2001).

The cells can either be epithelial and engaged in tubular patterning, or mesenchymal and migrate with invasiveness. It is possible for the cells to transdifferentiate between the two. Importantly, the initiation of tumour growth at the secondary site is the rate-limiting step to metastasis, thus the ability to block this step offers novel avenues for cancer therapy (Chambers et al., 2002; Vincan and Barker, 2008).
1.14 The Frizzled-7 receptor in the normal intestinal epithelium and cancer

Homologues of the Frizzled-7 receptor gene have been found in a range of organisms, from sponges (Adell et al., 2003) and hydra (Minobe et al., 2000) to vertebrates (Wang et al., 1996). The human Fzd7 gene was first identified and characterised in 1998 (Sagara et al., 1998). In the same year, it was also discovered to be up-regulated in oesophageal cancer and named FzE3, by a different group (Tanaka et al., 1998). Following the successful cloning of Xenopus Fzd7 (Xfz7), roles for Wnt/Fzd7 signalling during various developmental and adult processes such as neural crest induction (Abu-Elmagd et al., 2006), gastrulation (Winklbauer et al., 2001) and intestinal homeostasis (Flanagan et al., 2015) were identified. In the adult intestinal epithelium, expression studies via in situ hybridization for mRNA, demonstrated that Fzd7 expression was predominantly located at the base of the crypt in the CBC stem cell compartment, with a gradual decrease in expression in the cells going up the crypt and no detection in the villi (Gregorieff et al., 2005). Enrichment of Fzd7 in the CBC stem cell compartment was further supported by a Frizzled gene-profiling assay of crypts from Lgr5CreER\textsuperscript{ERT2} mice, which were arbitrarily sorted into five populations, with 5+ (the CBC stem cells) having the highest and 1+ having the lowest EGFP expression. Fzd7 which was shown to track with Fzd2, a member of the Fzd7 subclass, with the highest relative expression seen in the CBC stem cells and a noticeable decrease while moving away from the crypt base, along the crypt axis (Flanagan et al., 2015). This localized expression, suggests a role in Wnt signal transduction to intestinal stem cells. Indeed, Fzd7 was demonstrated to be necessary for regeneration following whole body ionizing irradiation and lineage tracing studies showed that following deletion of Fzd7 in the Lgr5\textsuperscript{+} cells, the stem cells that had Fzd7 deleted were lost from the epithelium and triggered rapid repopulation (Flanagan et al., 2015). Thus, revealing Fzd7 to be a Wnt receptor for the Lgr5\textsuperscript{+} stem cells.

In CRC, commonly over-expressed Fzd receptors with elevated Wnt activity are Fzd3, Fzd6 and Fzd7 (Ueno et al., 2008; Vincan et al., 2007b). Fzd7 was shown to be commonly up regulated in colon, gastric and hepatocellular carcinomas and is also associated with poor prognosis and survival (Merle et al., 2004; Merle et al., 2005;
Furthermore, down regulating Fzd7 reduces cell viability, cell migration and cell invasion within various cancers types both *in vivo* and *in vitro* (Merle et al., 2004; Merle et al., 2005; Ueno et al., 2009; Ueno et al., 2008; Vincan et al., 2007b). Additionally, Fzd7 is one of the few Fzds capable of transmitting both canonical and non-canonical Wnt signals, both of which, play critical roles during tumourigenesis and tumour progression (Asad et al., 2014; Ueno et al., 2009; Vincan et al., 2007b).
1.15 Aims of thesis

The principal aim of this thesis is to examine the function of a Wnt receptor, Frizzled-7, in varying intestinal contexts. These studies will provide valuable insight into the role of Wnt/Frizzled-7 signalling in intestinal homeostasis, cancer and cancer progression. The research aims of this thesis are as follows:

**Aim 1.** Due to the well established role of Frizzled-7 in colorectal cancer morphogenesis (Vincan et al., 2007b), in Chapter 2 we aim to examine the Wnt ligand which interacts with it. The human colorectal cancer cell line LIM1863-Mph was employed for this study.

**Aim 2.** We have demonstrated *in vivo* that Frizzled-7 is responsible for transmitting the critical Wnt signal in the intestinal crypt base columnar stem cells and that it is an essential Wnt receptor in regeneration of the intestinal epithelium (Flanagan et al., 2015). Using the same conditional genetic approach, in Chapter 3 utilizing 3D “mini-gut” organoid cultures, we aim to further investigate the functional requirement of Frizzled-7 in Lgr5+ intestinal stem cells *in vitro*.

**Aim 3.** A role for Frizzled-7 in both the genesis and progression of many cancers of epithelial-origin has been well established; furthermore a role for Frizzled-7 as a Wnt receptor in the crypt base columnar stem cells has also been demonstrated. Thus in Chapter 4 we aim to examine the potential therapeutic benefit of Frizzled-7 inhibition as well as investigate a role for Frizzled-7 in cancer stem cells using *in vitro* adenoma organoid cultures from a mouse model of colorectal cancer.
Chapter 2

Wnt(s)/Frizzled-7 in the progression of colorectal cancer
2.1 Introduction

Colorectal cancer (CRC) is a major health problem worldwide and similar to other forms of cancer, it is much harder to treat once it has spread to other organs in the body. A necessary initiating step in the genesis of most CRCs is the deregulation of the β-catenin-dependent (also known as “canonical”) Wnt signalling pathway, which occurs through genetic mutation of downstream pathway components, primarily APC or less frequently CTNNB1 (the gene that codes for β-catenin) or Axin. Aberrant Wnt pathway activation has been linked to many different forms of cancer. Interestingly, what we understand about Wnt signalling, stems from the discovery of Wnt1/Int1 as a proto-oncogene activated in mammary gland tumours by the mouse mammary tumour virus (MMTV) and from investigations into its role in cancer progression. In brief, β-catenin-dependent Wnt signalling, also known as Wnt/β-catenin signalling, is activated by a Wnt ligand binding with its seven-span transmembrane protein Frizzled (Fzd) and co-receptor, the single-span transmembrane protein, low-density lipoprotein receptor-related protein (LRP). Wnt binding to Fzd inhibits the action of a cytoplasmic multi-protein complex, the so-called β-catenin destruction complex, which is comprised of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 beta (GSK3β) and casein kinase I (CKI), tasked with the role of phosphorylating β-catenin, which is then targeted for proteosomal degradation. Inhibition of the destruction complex leads to the accumulation of β-catenin, thus allowing for it to translocate to the nucleus and associate with members of the TCF/LEF (T-cell factor/lymphoid-enhancing factor) family of transcription factors to activate transcription of Wnt target genes (Clevers and Nusse, 2012). In CRC, mutations to the tumour suppressor gene APC, a negative regulator of Wnt/β-catenin signalling, are present in approximately 90% of familial and sporadic patients (Kinzler et al., 1991b; Kinzler and Vogelstein, 1996; Korinek et al., 1997a).

Alternatively to β-catenin-dependent Wnt signalling, there is also the β-catenin-independent Wnt pathway, also known as the “non-canonical” pathway. The β-catenin-independent Wnt pathway has two branches, the Planar Cell Polarity (PCP) and the Wnt/Ca\textsuperscript{2+} pathways, which have been described and found to play key roles in cancer progression (Anastas and Moon, 2013; Niehrs, 2012; van Amerongen, 2012).
The pathways are also activated by Wnt binding to Fzd, however, they do not require β-catenin or the co-receptor LRP, but instead use the receptor Ror1/2 (Niehrs, 2012; Phesse et al., 2016). The PCP pathway involves activation of small GTPases like Rac1, RhoA, and JUN-N-terminal kinase (JNK), which in turn activate downstream kinases to affect and control cell migration, polarity and morphogenetic movements (Niehrs, 2012). The key event in the Wnt/Ca\(^{2+}\) pathway is the release of intracellular Ca\(^{2+}\) and consequent activation of effectors such as calmodulin-dependent kinase II (CamKII), protein kinase C (PKC) and calcineurin, which activate the transcriptional regulator nuclear factor associated with T cells (NFAT), thus regulating the transcription of target genes required for cell fate and migration (Niehrs, 2012).

The β-catenin-dependent Wnt pathway is generally considered to drive proliferation and neoplastic transformation of cells, while the β-catenin-independent Wnt pathways have been shown to be involved in tumour progression such as proliferation, angiogenesis and metastasis (Anastas and Moon, 2013). Deregulation of components from these pathways leads to aberrant induction of Wnt signalling and thus gene transcription, which can rapidly transform cells sufficient to drive tumourigenesis.

Aberrant activation of the β-catenin-dependent Wnt pathway, via mutations to downstream components, leads to the constitutive activation of β-catenin/TCF mediated transcription in CRC, for which additional regulation was presumed unnecessary. Due to the constitutive activation of the pathway as a result of genetic mutations, it is as such irreversible. However, staining for β-catenin, whose nuclear expression is the hallmark of β-catenin-dependent Wnt signalling, was found to be variable in carcinoma tissue sections. Nuclear expression was seen predominantly at the invasive front, whereas in contrast, the tumour centre often showed no nuclear staining, but retained a membranous expression of β-catenin (Brabletz et al., 1998), despite all cells harbouring APC mutations, indicating regulation by the tumour microenvironment. The lack of homogenous expression of β-catenin in the CRC tissues was the first indication, that there was further regulation of the β-catenin-dependent Wnt pathway, despite the mutations in the downstream components. Additionally, it was recognized, that the distribution of β-catenin in the colorectal carcinoma tissues, resembled that of tissue patterning during embryogenesis (Kirchner and Brabletz,
2000) and thus the link between the β-catenin-dependent Wnt pathway and epithelial-mesenchymal transition (EMT) in CRC was made. Then came the discovery that when comparing central areas of primary colorectal carcinomas and corresponding metastases, the same patterns of β-catenin expression, i.e. the invasive front had nuclear expression and the tumour centre had membranous expression (Brabletz et al., 2001), were visible. In addition to the variations noticed in β-catenin expression, cells at the invasive front decrease in E-cadherin and dramatically up-regulate several markers associated with EMT such as LamG2 (Brabletz et al., 2001; Hlubek et al., 2001). Also, the cells shut down cell proliferation and become Ki-67 negative (Brabletz et al., 2001). The cells at the tumour centre in contrast, are dividing and expressing Ki-67. Thus, the progression of tumours, which is associated with the loss of epithelial differentiation and the gain of mesenchymal-like properties, i.e. EMT, is dynamically regulated and a reverse transition, mesenchymal-epithelial transition MET, must occur at the secondary site, since most metastases recapitulate the pathology of the primary tumour. This is not only unique to CRC as it was formally proven using genetic tracing in a transgene mouse model using a mesenchymal-specific Cre-mediated fluorescent marker switch in spontaneous breast-to-lung metastasis (Fischer et al., 2015). It was demonstrated that within a predominantly epithelial primary tumour, a small portion of tumour cells undergo EMT.

EMT is an evolutionarily conserved developmental process that contributes to the formation of the body plan, histogenesis and organogenesis (Lim and Thiery, 2012). Pioneering work by Elizabeth Hay (Greenburg and Hay, 1982; Hay, 2005) revealed that during EMT, epithelial cells lose apicobasal polarity and intercellular junctions. The epithelial basement membrane is disrupted due to the changes in cell polarity and adhesion, thus allowing cellular penetration into an extracellular matrix (ECM)-rich compartment. This process is referred to as delamination. The newly formed mesenchymal cells transiently express distinct mesenchymal markers associated with migration and invasion, they acquire a front-rear polarity while becoming invasive and favour cell-ECM rather than cell-cell adhesions (Lim and Thiery, 2012). EMT is reversible and cells frequently cycle between epithelial and mesenchymal state. Of significance, is that EMT has not only been implicated in pathological conditions, such
as organ fibrosis but in cancers as well, where it contributes to tumour progression and metastasis (Kalluri and Weinberg, 2009; Nieto et al., 2016; Thiery et al., 2009). The genes associated with developmental EMT and MET are also involved in cancer metastasis, hence EMT and MET have been used to describe the phenotype transitions that lead to tumour metastasis (Nieto et al., 2016; Thiery, 2002; Thiery et al., 2009).

As mentioned before, additional modulation of β-catenin-dependent Wnt signalling via the upstream components of the pathway, i.e. at the plasma membrane, Wnt and Fzd per se, was initially deemed inconsequential, however it has since been accepted by the field, that Wnts and Fzds have a functional role in CRC, despite an already active pathway. The discovery of naturally occurring inhibitors of Wnt-Fzd interaction, the sFRPs, as bona fide tumour suppressors, that both Wnts and Fzds are over-expressed in CRC and that they have the ability to additionally modulate the pathway has proven hard to disagree with. And as such, it is now widely accepted in the field, that additional modulation of the pathway is involved in tumour progression.

Understanding the transition of tumour cells from epithelial, with tubular patterning, to mesenchymal, with migratory invasiveness, is an important process to study. However, researching this process is difficult to perform in vivo; thus a cell culture model was developed by our lab. It is a unique CRC morphogenesis culture system that has been established by adapting the human CRC cell line LIM1863 that grows as three-dimensional (3D) spheres; it is referred to as LIM1863-Mph (morphogenetic). The cell line recapitulates the dynamic phenotypic transitions, which are characteristic of the human disease state (Vincan et al., 2007a; Vincan et al., 2007b). The LIM1863-Mph organoid cells form free-floating multicellular spheres in which polarized cells are arranged around a central lumen (Whitehead et al., 1987). The spheres contain morphologically differentiated epithelial cells, namely goblet and enterocytes, that are found in the colonic crypt and all cells within the spheres proliferate. Although adjacent cells in a free-floating organoid are tightly associated with each other through cell-cell junctions, they can spontaneously anchor to the tissue culture plastic and form adherent monolayer patches. Since the EMT of organoids is reversible; the monolayer cells will subsequently spontaneously transdifferentiate into polarized/differentiated cells, which again assemble into free-floating organoids. That is, after several days of
incubation, the cells at the centre of a monolayer patch will begin to reorganize and re-assemble to form organoids (Vincan et al., 2007a; Vincan et al., 2007b). Thus, a spontaneous transformation of the cell between monolayer and organoid takes place. The LIM1863-Mph organoid cell model allows the study of regulators of EMT and MET to be possible. Importantly, the monolayer cells not only mimic the invasive front by expressing genes associated with EMT (e.g. LamG2, SLUG, etc.) (Vincan et al., 2007a), they also down regulate cell proliferation, a key feature of CRC invasive front cells. Notably, the EMT is partial as the cells maintain E-cadherin expression and migrate as a sheet on the tissue culture plastic, rather than individual cells (Vincan et al., 2007b). Experiments with this model demonstrated that RNAi mediated depletion of Fzd7 resulted in the inhibition of organoids re-assembling, thus suggesting that Fzd7 is necessary for organoid formation to take place (Vincan et al., 2007b) and as such is implicated in CRC morphogenesis. In addition, Fzd7 was shown to be required by the de-differentiated cells to migrate on the tissue culture plastic. This function of Fzd7 appeared to be via the non-canonical Wnt pathways (Vincan et al., 2007b), which was later formally demonstrated by Ueno et al.. Furthermore, previous work in our lab has shown a function for Fzd7 in CRC, by employing a dominant-negative receptor ectodomain of Fzd7 that had potent anti-tumour effects in vivo in mouse Xenografts. Thus Fzd7 functions in CRC, however, it remains to be determined which Wnt is orchestrating organoid assembly, which is the focus of the current study.
Figure 2.1 Colorectal cancer morphogenesis

(A) Morphogenesis in colorectal cancer. Tumour cells are in a signal responsive state and can transdifferentiate to either form tubular structures or dissociate from the tumour mass and migrate away. Notably, tubular patterns are associated with cell proliferation, while invasion and migration are associated with cell dormancy. (reproduced from Brabletz et al., 2005)

(B) Cell culture model: LIM1863-Mph cells are a dynamic model of CRC morphogenesis. They can undergo spontaneous cyclic transitions between two-dimensional and three-dimensional states. They can anchor and shed cells to form monolayer patches (EMT) and then subsequently repolarize (MET) and form free-floating organoids (red circle indicates one organoid). The organoid represents the tumour center, while the monolayer patch represents the invasive front. Scale bar represents 100µm. (reproduced from Vincan et al., 2007a)
A
Tubular pattern formation
Cell proliferation

MET
Epithelial transition

Signal susceptibility

EMT
Mesenchymal transition

Migration with invasiveness
Cell dormancy

Adapted from Brabletz et al., 2005

B
Tumour center

Invasive front

Adapted from Vincan et al., 2007a
2.2 Materials and Methods

Cell culture

The LIM1863-Mph cell line is a human colon carcinoma cell line, which was established in our lab (Vincan et al., 2007a). It is an adaptation from the original cell line, which was derived by Dr. Robert Whitehead at the Ludwig Institute Melbourne from a colonic carcinoma (Whitehead et al., 1987). The cell line is maintained with RF-10+ media: RPMI 1640 medium (GIBCO, #11875-119), supplemented with 10% heat inactivated fetal calf serum (Chemicon International, Embryo Max FCS, ES qualified #ES-009-A), 1% Penicillin/Streptomycin, 1% Glutamine, 1µg/mL hydrocortisone (Solu-Cortef for injection), ~0.9-1µg/mL insulin (Insulin for injection, 100U/mL, from Pharmacy) and 10⁻⁴M 1-thioglycerol (Sigma, #M6145). The cells were incubated at 37°C in a humidified atmosphere containing 10% (V/V) CO₂ in air. Adherent LIM1863-Mph cells were detached by removing all supernatant, washing off all medium with Dulbecco’s PBS⁻ (phosphate buffered saline without Mg²⁺/Ca²⁺) and the addition of Trypsin/EDTA solution (0.05% (w/v trypsin 1:250, 0.02% (w/v) EDTA, pH7.0). The cells were incubated for approximately 2-4min and once dislodged, with a sharp tap to the tissue culture plastic; serum-containing medium was added to inhibit further Trypsin/EDTA activity.

L-cells and HEK293T cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% Penicillin/Streptomycin and 1% Glutamine. The cells were also cultured at 37°C in 10% CO₂. Adherent cells were detached following the same method as the LIM1863-Mph cells above.

Imaging LIM1863-Mph cultures

Differential interference contrast (DIC) images of LIM1863-Mph cultures were taken on a Nikon Ti-E microscope using either a 4× Plan Fluor NA 0.13 objective or a 10× Plan Fluor NA 0.3 objective. A focal stack of images was collected 10µm apart and processed through the “Best Focus” function of MetaMorph v7.7.7 (Molecular Devices) to generate the final image.
**Transfection**

HEK293T cells were transfected with various expression plasmids using Liopfectamine LTX Reagent and PLUS Reagent (Invitrogen, #15338-100). Transfection with Lipofectamine was conducted in 6-well plates according to the manufacturer’s instructions with the mammalian plasmids: tagged protein constructs in pcDNA3.1 expression vector (Invitrogen); FZD7-ecto-V5-HIS, Wnt3-FLAG, Wnt2b-FLAG or the empty vector (EV); all have a concentration of 1µg/µL (Struewing et al., 2007; Struewing et al., 2006). The cells were seeded at 4x10^5 per well in antibiotic free medium. For each transfection sample, a complex of DNA and transfection reagent was prepared. The Plus Reagent is used at a ratio of 1:1 (Plus Reagent:DNA) and Lipofectamine LTX is used at a ratio of 1:3 (Lipofectamine LTX:DNA). OPTI-MEM I Reduced Serum Medium (GIBCO, invitrogen, Cat. No.11058), DNA and PLUS Reagent were incubated for 5min at room temperature (RT). Lipofectamine was then added to the mixture and incubated for 30min at RT. The DNA-Lipid complex is then added drop-wise to the wells and incubated for 48hrs at 37°C in 10% CO₂ before harvesting. A media change takes place 24hrs after transfection to remove extra transfection reagent and dead cells.

**Harvesting cells and protein lysis**

The transfected HEK293T cells were washed with PBS⁺ and then lysed with passive lysis buffer (Promega, #E194A) supplemented with 5U/µL Benzonase Nuclease (Sigma, #E1014) to degrade DNA and RNA in the whole cell lysates. The cell lysates were then subjected to immunoprecipitation.

L2-cells treated with conditioned medium were washed with warm PBS⁺ and then scraped from the well in PBS⁺. The cells were then spun down at 1200rpm for 2min and resuspended in PBS, followed immediately with the addition of hot (≥95°C) 2x Laemmli Sample Buffer (Bio-Rad Laboratories, #161-0737) (with 0.5M β-mercaptoethanol) and incubated at ≥95°C for 5min. The samples were then stored respectively at either 4°C (short term) or at -20°C (long term) until a western blot was conducted.
**Immunoprecipitation and Co-immunoprecipitation**

The HEK293T cells transfected with the mammalian plasmids: FZD7-HIS-V5, Wnt3-FLAG and Wnt2b-FLAG were subjected to immunoprecipitation using the Anti-FLAG M2 Affinity Gel (SIGMA, Cat. No. A2220), according to the manufacturer’s instructions. The Anti-FLAG M2 Affinity Gel is a mouse monoclonal antibody covalently attached to agarose, supplied as a suspension in 50% glycerol and must therefore be thoroughly suspended for a uniform suspension of the resin and then immediately transferred to a fresh eppendorf tube. The gel suspension was washed to remove the glycerol and equilibrated with TBS (Tris buffered saline; Tris HCl, with 150mM NaCl, pH7.4). To remove any traces of unbound Anti-FLAG antibody from the resin it was washed with 0.1M glycine HCl (pH3.5). Following washes to remove the glycine HCl solution, the cell lysate was then added to the washed resin and incubated overnight at 4°C on a rotor. The next day, the resin was centrifuged at 5,000g for 30sec followed by three washes with TBS. After the removal of the supernatant 2x Laemmli Sample Buffer (Bio-Rad Laboratories, #161-0737) (without 2-mercaptoethanol) was added and heated at 65°C (over 65°C cooks the Fzd7 proteins) for 3min. The samples are then centrifuged at 5,000g for 30sec to pellet any undissolved agarose. The supernatant was then transferred to a new tube and stored at -20°C. The eluted samples were then subjected to immunoblot analysis.

**Western blot**

The protein samples were separated electrophoretically by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) on a 10-well Mini-PROTEAN TGX precast 4–20% gel (Bio-Rad, Cat. No. 456-1093). The gel was loaded with a molecular marker (Precision Plus Protein Dual Color Standards, Bio-Rad, #161-0374 or Precision Plus Protein WesternC Standards, Bio-Rad, #161-0376), the samples and controls. The power-pack was set at 200V and run for 30-40min. Following electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Hybond C-Extra, Amersham, Cat. No. RPN303E). The transfer cassette(s) were assembled with the gel and membrane as such: Pad; x3 MM paper; Membrane; Gel; x3 MM paper; Pad. While assembling the cassette(s), the pads and membrane were kept wet with transfer buffer. A pipette was rolled over the finished “sandwich” to ensure contact between
the membrane and gel. The cassette(s) were placed in the tank along with a freeze tub and the power-pack was set at 100V for 1hr. After transfer, the membrane was checked for the pre-stained molecular markers and then placed in either 5% (w/v) skim milk powder in TBS or 5% (w/v) skim milk powder in 0.1% PBT (PBS containing 0.1% (v/v) Tween-20) (for detection with chemiluminescence) to eliminate non-specific binding of the antibodies to the membrane.

**Immunoblotting**

The membrane was blocked for 1hr at room temperature (RT) on a rocker. It was then rinsed in 0.05% TBS-T/0.1% PBT and placed in a tub with the primary antibody overnight (O/N) at 4°C on a rocker. Following the O/N incubation, the primary antibody was washed off with 0.05% TBS-T/0.1% PBT and then the secondary antibody was added to the membrane. It was incubated at RT for 1hr on a rocker, protected from light. The secondary antibody was washed off with 0.05% TBS-T/0.1% PBT and detection of the antibodies ensued.

The membranes for the co-immunoprecipitation experiment were treated with the following primary and secondary antibodies: mouse anti-FLAG (M2) (Sigma, #F1804) and Alexa anti mouse 680. The membranes were then stripped with NaOH and reprobed with the primary mouse anti-V5 (Invitrogen, #771149) and secondary Alexa anti mouse 680.

The membranes for the conditioned medium experiment were treated with the following primary and secondary antibodies: mouse anti-β-catenin (BD Transduction Labs, #610154) and anti-mouse-HRP (DAKO, #P0161) with streptactin-HRP (Bio-Rad, #161-0381).

**Detection**

Detection of the co-immunoprecipitation membranes were completed using the Odyssey Infrared Imaging System (Li-Cor Biosciences). The membranes were scanned in the channels 700 and 800 with an intensity of 5.0.

The membrane with the L2-cell samples were detected via Enhanced Chemiluminescence (ECL), a non-radioactive light-emitting system. The membranes were incubated for 1min in equal volumes of Clarity Western ECL Substrates;
Luminol/enhancer reagent and Peroxide reagent (Bio-Rad, #1705060). An oxidative degradation of the luminol occurs, resulting in light emission at a wavelength of 428nm. The membrane is removed from the substrate solution and drained of excess solution, then placed on a plastic sheet protector and imaged using a digital imager (Bio-Rad ChemiDoc).

**Immunofluorescence staining**

LIM1863-Mph cells were seeded at $5 \times 10^3$/cm$^2$ in 2-chamber slides (Lab-Tek, #177429). Following treatment, the growth medium was removed and the cells were washed twice in PBS$^{++}$ (phosphate buffered saline with Mg$^{++}$/Ca$^{++}$). The cells were then fixed *in situ* with 100% Methanol at -20°C for 20min; they were then rinsed in PBS to remove the excess fixative. To permeabilize the cells, they were incubated for 30min at room temperature (RT) in 0.2% PBST (phosphate buffered saline + 0.2% Triton X-100). Following removal of the PBST the cells were incubated for 1hr in 5% FCS in 0.2% PBST to block nonspecific binding of the antibodies. After removing the block, the primary antibody mix, made up in 5% FCS in 0.05% PBST (PBS + 0.05% Triton X-100), was added and left overnight (O/N) at 4°C. After the O/N incubation, the cells were rinsed and then washed in 0.05% PBST, removing any excess primary antibody mix. The cells were then incubated in the secondary antibody mix for 1hr. DAPI (4′-6-Diamidino-2-phenylindole) was then added at a dilution of 1/1000 for 5min to stain the nuclei, followed by three washes in 0.05% PBST, after which 70% glycerol was added and covered with a cover slip, then sealed with nail varnish. Detection of the stained cells was conducted by confocal microscopy.

The primary antibodies used were mouse anti-E-cadherin (BD Transduction Labs, #610181) and mouse anti-ZO-1 (Invitrogen, #339100) plus the secondary antibody Alexa anti mouse 568. Detection of the staining for E-cadherin and ZO-1 was captured on a Leica LAS AF SP5 microscope using a 20× HCX Plan Apo NA 0.7 IMM objective. A Z-stack of images was collected and processed in ImageJ v1.43u, then finalized in Adobe Photoshop CS4 v11.0.2 to generate the final image.
RNA extraction and cDNA synthesis

Total RNA extraction and cDNA synthesis was as previously described (Vincan et al., 2007b). Briefly, following treatment, the organoids were handled as when passaging, except the clean pellets were resuspended and homogenised in TRIzol (Invitrogen #15596-018) and incubated for 30mins at room temperature. Phenol:Chloroform:isoamyl alcohol was added to the homogenised samples and mixed well. After 5mins at room temperature, the samples were centrifuged (10,000rpm) for 15mins in order to separate the upper clear aqueous phase (nucleic acid containing fraction) from the lower pink organic phase (protein containing fraction). The clear aqueous phase was removed and mixed with an equal volume of 70% Ethanol. The samples were purified and DNase treated using RNeasy mini-prep columns (Qiagen #74104) following the manufacturers’ instructions. RNA samples were then quantified using a DNA/RNA nanodrop spectrophotometer (Thermo Scientific Nanodrop 1000). cDNA was synthesized with 4µg of each RNA sample. Samples were reverse transcribed using anchored oligo(dT) primers (Promega) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega #M1705), in a final volume of 100µL.

RNA analysis

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the 2x SYBR green PCR master mix (Invitrogen #4309155) and ABI PRISM 7500 Sequence Detector (Applied Biosystems) as previously described (Vincan et al., 2007b). In brief, final volumes of 20µl PCR amplification mixture (2x SYBR green I Master Mix buffer (10µL), sterile water (7µL), template cDNA (1µL) and forward and reverse primer mix (2µL)) were set up in triplicate wells of a 96-well tray (Applied Biosystems MicroAmp #4346907). The cycling conditions compromised 2min at 50°C, 10min polymerase activation at 95°C, 40 two-step cycles of 15sec at 95°C and of 1min at 60°C and a dissociation stage of 15sec at 95°C, 1min at 60°C, 15sec at 95°C and 15sec at 60°C. The housekeeping gene hydroxymethylbilane synthase (HMBS) (Vandesompele et al., 2002) primers were used to equalize the data; with threshold cycle (CT) values, the $2^{\Delta \Delta CT}$ method (Vincan et al., 2007b) was used to calculate the fold change. For primer sequences see appendix Table 1.
**Statistical analysis**

Data are expressed as mean ± SEM, where mean represents number of experiments (three or more). Statistical tests used are Student’s t test or Mann-Whitney with Prism6 (GraphPad software) where *P* values of ≤ 0.05 were considered significant.
2.3 Results

Wnt signalling in LIM1863-Mph cells

The first question to answer when investigating which Wnt is involved in the reversible EMT/MET is which Wnt family members are expressed and are any differentially expressed between the epithelial and mesenchymal states (Figure 2.2-A). Employing a Human Wnt Signalling Pathway Array (Qiagen, #PAHS-043YA), total RNA from LIM1863-Mph monolayers and organoids were run on four plates each and the expression profiles compared. The results revealed that Wnt2b was significantly expressed in the organoids as well as elevated expression levels of Wnt3, Wnt5b, Wnt7b and Wnt10a in the organoids compared to the monolayer cells (Figure 2.2-B). Wnt3 and Wnt10a had comparable expression levels, whereas Wnt5b and Wnt7b were much lower.

Looking at expression levels of Wnt target genes (Figure 2.2-C) in the monolayer cells and organoids, the following had significantly higher levels in the organoids: Disabled-2 (DAB2), paired-like homeodomain transcription factor 2 (PITX2), JUN (encodes c-Jun), CCND1 (Cyclin D1), CCND2 (Cyclin D2) and MYC (encodes c-Myc). DAB2 has been demonstrated to be required for the epithelial-mesenchymal transition (EMT), induced by transforming growth-factor β (TGF-β). Induced expression of DAB2 was found to be concomitant with EMT promotion (Prunier and Howe, 2005). The PITX2 gene has been shown to regulate the expression of MYC, CCND1 and CCND2 (Baek et al., 2003; Kioussi et al., 2002), thus the elevated mRNA expression levels of all four genes. Furthermore, c-Myc and Cyclin D1, the first oncogenes identified as being regulated by β-catenin (Batsche et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999), are effectors of proliferation in cancers.

The expression patterns of the Wnts and Wnt target genes shown to be significantly different, between the monolayer cells and organoids in the array, were confirmed by independent qRT-PCRs comparing EMT to MET and organoids (Figure 2.3-A). Laminin-5 γ2 chain (LamG2), an epithelial basement membrane protein, and Claudin-1 (Cldn1), a tight junction protein, known genes up-regulated during EMT, were dramatically upregulated (Figure 2.3-B) in the monolayer cells with EMT characteristics (hence forth referred to as EMT cells), whereas the Wnt target genes (Figure 2.3-C), c-Myc and
Cyclin D1, as well as CD44 (not part of the array) were all significantly up in the organoids, as demonstrated by the array results. Notably, these genes (c-Myc, Cyclin D1 and CD44) normally track with cell proliferation, thus in this case as well, they are tracking with cell proliferation. Furthermore, the expression levels for the Wnts were also confirmed, both Wnt2b and Wnt3 have higher expression levels in the organoids than in the EMT cells as demonstrated by the array.

Figure 2.2 Wnt signalling pathway gene expression in LIM1863-Mph cells

(A) Representative differential interference contrast (DIC) images of LIM1863-Mph monolayer (Mono) (left image) and organoids (right image).

Scale bar represents 100µm.

(B) Human Wnt Signalling Pathway Array: Wnt gene expression levels, determined by qRT-PCR and normalized to HMBS, of LIM1863-Mph cells, monolayers (Mono) versus organoids (mean ± SEM, *p < 0.05, n = 4 array plates per cell type).

(C) Human Wnt Signalling Pathway Array: Wnt target gene expression levels, determined by qRT-PCR and normalized to HMBS, of LIM1863-Mph cells, monolayers (Mono) versus organoids (mean ± SEM, *p < 0.05, n = 4 array plates).
Figure 2.3 Gene expression of Wnt signalling pathway components in LIM1863-Mph cells

(A) Representative differential interference contrast (DIC) images of LIM1863-Mph cells; EMT (left image), MET (middle) and organoid (right image). Scale bar represents 100µm.

(B) Various Wnt gene expression levels, determined by qRT-PCR and normalized to HMBS, of LIM1863-Mph cells; EMT, MET and organoid (mean ± SEM, *p < 0.05, n = 3 populations).

(C) Wnt target gene expression levels, determined by qRT-PCR and normalized to HMBS, of LIM1863-Mph cells; EMT, MET and organoid (mean ± SEM, *p < 0.05, n = 3 populations).
A

EMT  MET  ORG

B

Wnt expression (mRNA) in LIM1863-Mph cells

Fold Change

* * *

Wnt2b  Wnt3  Wnt3a

C

Gene expression (mRNA) in LIM1863-Mph cells

Fold Change

* * *

LamG2  Cldn1  c-Myc  Cyclin D1  CD44
Inhibiting Wnt secretion impedes LIM1863-Mph cells from forming organoids

Having established an expression profile for Wnts in the LIM1863-Mph cells, the next question asked was whether they have a role in EMT/MET. To investigate which of the Wnts identified, with significant expression levels in the organoids, potentially participate in LIM1863-Mph morphogenesis, the Porcupine (Porc) inhibitor IWP2 was employed. The aim was to use IWP2 to inhibit all Wnt secretion and then add recombinant Wnts to the cells and determine if they could rescue the phenotype. To demonstrate the functionality of IWP2, L2-cells were either treated with conditioned medium harvested from L2-cells (negative control), Wnt3a conditioned medium harvested from L3a cells (positive control) or Wnt3a conditioned medium co-treated with 5µM IWP2. After treatment, the L2-cells were harvested for protein and separated electrophoretically by SDS-PAGE on a 4–20% gel, then transferred to a membrane and detection was via immunoblot (Figure 2.4-A). The membranes were probed for total β-catenin. The immunoblots demonstrate that the L2-cells treated with Wnt3a conditioned medium had an increase in β-catenin compared to the control treated cells. Whereas the L2-cells co-treated with Wnt3a conditioned medium and IWP2 did not have an increase in β-catenin, thereby confirming that IWP2 inhibited Wnt secretion. Thus, initial experiments to demonstrate the effects of inhibiting Wnt secretion in the LIM1863-Mph cells were conducted. LIM1863-Mph cells were plated for imaging (Figure 2.4-B) and once monolayer patches were established, they were treated with either the vehicle (DMSO) or IWP2. The DIC images show that by Day 8, the cells treated with the IWP2, were cottonwool-ish in appearance, whereas the DMSO treated cells have noticeable organoids forming (Figure 2.4-C; circles). LamG2 and Cldn1 expression (Figure 2.4-D) is significantly up in the EMT cells treated with IWP2 compared to those treated with DMSO, suggesting that the cells are not undergoing MET. The lack of significance between the cells treated with IWP2 and DMSO in regard to the Wnt target genes: c-Myc, Cycclin D1 and CD44 (Figure 2.4-D), is not surprising as the fold change between DMSO and IWP2 of LamG2 and Cldn1 expression, albeit significant, is not as marked as that of the independent study comparing EMT vs. MET vs. ORG populations previously (Figure 2.3-C). The fold change for LamG2 between the EMT and MET populations was 20 fold (Figure 2.3-C), whereas
it is only 2 fold in the IWP2 compared to DMSO treated cells. Furthermore, as the
immunoblot showed, not all Wnts are inhibited. To investigate whether we could
restore organoid assembly, we conducted experiments with the addition of
CHIR99021, a GSK3β inhibitor, i.e. it activates the pathway downstream of Wnts and
Fzds. The addition of CHIR had no effect on the cells (data not shown).

Figure 2.4 Treatment of LIM1863-Mph cells with Porc inhibitor (IWP2)

(A) Full Immunoblot of lysates from L-cells: untreated, treated with Wnt3a conditioned medium (CM) or co-treated with Wnt3a conditioned medium and the Porc inhibitor IWP2. The Immunoblot shows detection of total β-catenin.

Arrow: the band of interest.

Molecular weight markers are shown on the left.

(B) Representative differential interference contrast (DIC) images of LIM1863-Mph monolayer cells imaged at Day 0, 4, 6 and 8 post IWP2 or DMSO treatment.

Scale bar represents 100µm.

(C) Representative differential interference contrast (DIC) images of LIM1863-Mph monolayer cells imaged at Day 8 post IWP2 or DMSO treatment.

Circle: organoid

Scale bar represents 100µm.

(D) Gene expression levels determined by qRT-PCR and normalized to HMBS. Cells were harvested 8 days after treatment with IWP2 and compared to the vehicle DMSO treated cells (mean ± SEM, *p < 0.05, n = 3 experiments).
Recombinant Wnts potentially restore MET

Following the demonstration that inhibiting Wnt secretion had stopped the monolayer cells from forming organoids, the next question was could we rescue/restore MET by adding recombinant Wnts. As the array showed a marked expression for Wnt2b and a significant level of expression for Wnt3, it was decided to focus on those two, especially as it has been shown, that Wnt2b can compensate for Wnt3 loss in intestinal stem cells in vitro (Farin et al., 2012). LiM1863-Mph cells were grown in 2-chamber slides and treated with the following combinations: DMSO (vehicle for IWP2) with either 0.1% BSA (vehicle for the recombinant Wnts), Wnt2b or Wnt3a; IWP2 with either 0.1% BSA, Wnt2b or Wnt3a. The concentration for IWP2 was 5µM and the recombinant Wnts were 100nM. To better visualize the difference in the morphology of the cells following treatment, immunofluorescence (IF) images were taken. The cells were probed with either E-cadherin or ZO-1, to demonstrate the cell architecture. The DMSO treated and IWP2 co-treated with either Wnt2b or Wnt3a, show that organoids are re-assembling because the monolayer cell patches have “holes”, i.e. areas where the cells are lifting off (arrows) and starting to re-organize themselves into organoids (circles) (Figures 2.5-A and 2.6-A, -B). Staining for ZO-1 in the organoids that have started to form was seen in the centre of the organoids (Figure 2.5-A top row, 2.6-A top row, 2.6-B top row), whereas the IWP2 treated cells are a tight patch and the staining does not distinguish any organoids forming or cells lifting off (Figure 2.5-B top row). The cells treated with IWP2 + Wnt2b that were probed for E-cadherin are a prime example of a cell lifting off (Figure 2.6-A bottom row). The 3D effect of it is well defined by the staining. Simultaneously, cells were also set up in 6-well plates and treated the same as mentioned above. The cells were then harvested for qRT-PCR analysis (Figure 2.7). When we compared the control (DMSO+0.1% BSA) treated cells with the IWP2+0.1% BSA treated ones, there was no difference in the expression levels between LamG2 or Cldn1 (Figure 2.7-A) indeed there was no difference in their expression level in most of the treatments, except between the treatment of the cells with the control (DMSO+0.1% BSA) and DMSO+Wnt2b (Figure 2.7-B), which albeit significant is very minimal. In the control experiments, DMSO+Wnt2b and DMSO+Wnt3a (Figure 2.7-B, -C), expression of the Wnt target genes increased,
whereas in the IWP2+Wnt2b and IWP2+Wnt3a treated cells (Figure 2.7-D, -E), the expression was significantly decreased, albeit with a fold change of less than 0.5, which is negligible. The cells treated for IF demonstrate that when co-treated with IWP2+Wnt2b or IWP2+Wnt3a the cells are again able to form organoids and thus undergo MET.
**Figure 2.5 Immunofluorescence of treated LIM1863-Mph cells**

(A) Immunofluorescence analysis of LIM1863-\textit{Mph} cells treated with DMSO+0.1\%BSA.

(Top row) Blue: nuclei - DAPI; Red: ZO-1

(Bottom row) Blue: nuclei - DAPI; Red: E-cadherin

Arrows: indicates areas where cells are lifting off; circles: organoids

(B) Immunofluorescence analysis of LIM1863-\textit{Mph} cells treated with IWP2+0.1\%BSA.

(Top row) Blue: nuclei - DAPI; Red: ZO-1

(Bottom row) Blue: nuclei - DAPI; Red: E-Cadherin

Scale bar represents 50µm.

**Figure 2.6 Immunofluorescence of LIM1863-Mph cells treated with IWP2+Wnt3a or Wnt2b**

(A) Immunofluorescence analysis of LIM1863-\textit{Mph} cells treated with IWP2+Wnt3a.

(Top row) Blue: nuclei - DAPI; Red: ZO-1

(Bottom row) Blue: nuclei - DAPI; Red: E-cadherin

(B) Immunofluorescence analysis of LIM1863-\textit{Mph} cells treated with IWP2+Wnt2b.

(Top row) Blue: nuclei - DAPI; Red: ZO-1

(Bottom row) Blue: nuclei - DAPI; Red: E-cadherin

Arrows: indicates areas where cells are lifting off; circles: organoids

Scale bar represents 50µm.
Figure 2.7 Gene expression analysis of treated LIM1863-Mph cells

(A) Gene expression levels determined by qRT-PCR and normalized to HMBS. Cells were harvested 8 days after co-treatment in 0.1%BSA with either IWP2 or DMSO (mean ± SEM, n = 3 experiments).

(B) Gene expression levels determined by qRT-PCR and normalized to HMBS. Cells were harvested 8 days after co-treatment in DMSO with either 0.1%BSA or Wnt2b (mean ± SEM, *p < 0.05, n = 3 experiments).

(C) Gene expression levels determined by qRT-PCR and normalized to HMBS. Cells were harvested 8 days after co-treatment in DMSO with either 0.1%BSA or Wnt3a (mean ± SEM, *p < 0.05, n = 3 experiments).

(D) Gene expression levels determined by qRT-PCR and normalized to HMBS. Cells were harvested 8 days after co-treatment in IWP2 with either 0.1%BSA or Wnt2b (mean ± SEM, *p < 0.05, n = 3 experiments).

(E) Gene expression levels determined by qRT-PCR and normalized to HMBS. Cells were harvested 8 days after co-treatment in IWP2 with either 0.1%BSA or Wnt3a (mean ± SEM, *p < 0.05, n = 3 experiments).
A
Gene expression (mRNA) in LIM1863-Mph cells co-treated with the vehicle 0.1%BSA

B
Gene expression (mRNA) in LIM1863-Mph cells co-treated with DMSO

C
Gene expression (mRNA) in LIM1863-Mph cells co-treated with DMSO

D
Gene expression (mRNA) in LIM1863-Mph cells co-treated with IWP2

E
Gene expression (mRNA) in LIM1863-Mph cells co-treated with IWP2
Frizzled-7 interacts with both Wnt2b and Wnt3

The results of the previous experiments suggest a role for Wnt2b and Wnt3a, in restoring the ability of the LIM1863-Mph cells to re-assemble into organoids, i.e. undergo MET, when treated with the Porc inhibitor, IWP2, which blocks all Wnt secretion. As mentioned before, our lab has shown that the when Fzd7 expression is decreased, organoid assembly is halted (Vincan et al., 2007b). Thus, we next employed co-immunoprecipitation (Co-IP) to investigate a potential interaction between Fzd7 and Wnt2b as well as confirm findings, which previously demonstrated an interaction between Fzd7 and Wnt3 via Co-IP in hepatocellular carcinoma cells (Kim et al., 2008). HEK293T cells were co-transfected with epitope V5-tagged Fzd7 and either epitope FLAG-tagged Wnt2b or FLAG-tagged Wnt3. The cells were then harvested for protein and the FLAG-tagged proteins were subjected to immunoprecipitation using Anti-FLAG M2 Affinity gel. Following the Co-IP, the proteins were separated electrophoretically by SDS-PAGE on a 4–20% gel, then transferred to a membrane and detection was via immunoblot (Figure 2.8). The membranes were first probed for the V5-tag, then stripped and re-probed for the FLAG-tag. The immunoblots showed that Fzd7 interacted with both Wnt2b and Wnt3.

Figure 2.8 Fzd7 interacts with both Wnt2b and Wnt3

Full Immunoblot (IB) of FLAG immunoprecipitates (IP) from HEK293T cells transfected with either WNT2b-FLAG or WNT3-FLAG. The Immunoblots either show detection of the FLAG-tag of WNT2b (left) and WNT3 (right) or the V5-tag of FZD7. Arrows: the bands of interest.

Molecular weight markers are shown on the left of each blot.
2.4 Discussion

In CRC, aberrant activation of the Wnt/β-catenin signalling pathway leads to the accumulation of β-catenin in the nucleus and thus the hyperactivation of β-catenin-TCF/LEF gene transcription. Indeed, the over-activation of the Wnt signalling pathway, caused by mutations to some of its intracellular components, is found in nearly all CRCs, both sporadic and familial (Kinzler et al., 1991a). Most notably, is the tumour suppressor gene APC, which is found to be mutated in approximately 80-90% of CRCs. However, in addition to these APC mutations, the over-expression of certain upstream signalling components, such as Wnts and Frizzleds, has also demonstrated that Wnt activity can be regulated upstream, irrespective of the downstream pathway-activating mutations (Caldwell et al., 2004; Suzuki et al., 2004; Ueno et al., 2009; Vincan et al., 2007b; Vincan et al., 2005). Importantly, CRC, despite its potential to effectively be cured if it is detected early, remains localized and is surgically resected, is still statistically the second leading cause of cancer mortality, which is due to the formation of metastasis, primarily located in the liver. The malignant progression of gaining invasive capabilities and metastasis relies on dynamic reversible processes, EMT and MET. It is of great importance to understand how these processes are coordinated and regulated, as it may provide novel avenues to block metastasis.

The results presented in this study using a human CRC cell line, demonstrate the potential for two Wnt ligands, namely Wnt2b and Wnt3/3a that interact with Fzd7, to facilitate MET.

Although initially considered unnecessary for regulating the pathway additionally, it is now accepted, that the upstream components participate in the progression of CRC. This is primarily due to the demonstration that naturally occurring inhibitors of Wnt-Fzd interaction, the sFRPs, are bona fide tumour suppressors in CRC (Caldwell et al., 2004; Suzuki et al., 2004), suggesting that the additional modulation of the β-catenin-independent Wnt signalling pathway is via the upstream components. Although a role for Wnts and Fzds was dismissed by the field, it has been demonstrated that both Wnts (Dimitriadis et al., 2001; Katoh et al., 1996; Kirikoshi et al., 2001; Ru et al., 2008) and Fzds (He et al., 2011; Holcombe et al., 2002; Sagara et al., 1998; Ueno et al., 2008; Vincan, 2004; Vincan et al., 2007b) are over-expressed in CRC tissues and cell lines and
that they can additionally modulate the β-catenin-dependent Wnt signalling pathway (Ueno et al., 2009; Vincan et al., 2007b; Vincan et al., 2005). This additional modulation of the pathway has since been recognized as being involved in tumour progression (Brabletz et al., 2005). The dynamic EMT and MET are also associated with CRC progression. Indeed, aberrant activation of the Wnt pathway can induce EMT in tumour cells (Kim et al., 2002; Mariadason et al., 2001; Muller et al., 2002).

In our lab a role for the Fzd receptor, Fzd7, in the LIM1863-Mph cells which undergo spontaneous EMT and MET, demonstrated a requirement for it in MET and migration (Vincan et al., 2007b). However, the Wnt that interacts with Fzd7 to activate MET was unknown. Employing a gene array, we compared the expression levels of the two populations; organoids which are in an epithelial state and monolayers which are in a mesenchymal state. Five Wnts, namely Wnt2b, Wnt3, Wnt5b, Wnt7b and Wnt10a, were found to be significantly expressed in the organoids compared to the monolayers. Wnt2b had a marked level of expression as did Wnt3 and Wnt10a. Whereas, although significant, Wnt5b and Wnt7b were not as prominent in their expression levels as the other three. Following the treatment of the LIM1863-Mph cells with IWP2, an inhibitor of Wnt secretion, we demonstrated a requirement for Wnts in the assembly of organoids, i.e. MET and thus continued the investigated of which Wnt could restore this.

Due to their minimal levels of expression, despite being significant, Wnt5b and Wnt7b were not considered for further experiments. However, it is worth mentioning, that Wnt5b and Wnt7b, which are known non-canonical Wnt ligands, have been shown to induce cell transformation in vitro (Wong et al., 1994) also Wnt5b together with the receptor Fzd2, which is a member of the Fzd7 subclass, has been shown to be highly expressed in KRAS-mutated CRC cell lines resistant to the MEK1/2 (mitogen-activated protein kinase/ERK (extracellular-signal-regulated kinase) kinase 1/2) inhibitor Selumetinib (AZD6244) (Tentler et al., 2010). In addition, Wnt7b, as well as Fzd1 (another member of the Fzd7 subclass), was revealed to be part of the gene expression program associated with oncogenic Ras-induced and TGF-β-induced EMT (Kanies et al., 2008). Furthermore, studies have reported that expression of the canonical Wnt10a ligand is up-regulated in CRC cell lines (Kirikoshi et al., 2001).
We chose to investigate the role of Wnt2b and Wnt3/3a in the LIM1863-Mph cells. The decision to use those two was based on the following. β-catenin localization in the LIM1863-Mph cells shows, that there is a sharp increase in nuclear β-catenin as the cells start to lift off the tissue culture plastic. The cells undergo MET and thus cell proliferation, indicated by Ki-67 staining, is dramatically up-regulated at the same time. This increased Ki-67 staining and intense nuclear β-catenin staining, did not occur when Fzd7 expression was down-regulated using RNAi (Vincan et al., 2007b), indicating that Fzd7-mediated canonical signalling was inducing MET; thus the “canonical” Wnts (Wnt2b, Wnt3 and Wnt10a) are more likely to be involved. Interestingly, Wnt2b mediated canonical signalling has been implicated during the morphogenesis of renal tubes (Iglesias et al., 2007), which is one of the key MET events during development. Thus a role for Wnt2b in promoting the formation of glandular structures in CRC is consistent with its role during development. Furthermore, it was also one of the first Wnts demonstrated to be over-expressed in CRC cell lines (Katoh et al., 1996). Studies in hepatocellular carcinoma cells reported that Wnt3 directly interacts with Fzd7 (Kim et al., 2008). It was also shown that Wnt2, Wnt3 and Wnt3a were detected in the human colon cancer cell line HCT-116 by qRT-PCR (Bafico et al., 2004) and in colorectal tumours elevated levels of Wnt3 expression were demonstrated via immunohistochemical staining comparing normal colon epithelium to colon carcinomas (Voloshanenko et al., 2013). In addition, Wnt3, produced by Paneth cells in the intestinal epithelium, is essential for Lgr5 stem cells. The lack of Wnt3 in single Lgr5 stem cell cultures, i.e. lacking Paneth cells and Cre-mediated deletion of Wnt3 in vitro in organoid cultures can be overcome by the addition of recombinant Wnt3a or Wnt3a conditioned medium (Farin et al., 2012; Sato et al., 2011). Furthermore, it was demonstrated in vivo that Wnt3 was dispensable for adult intestinal homeostasis, indicating a redundancy of Wnt signals; in contrast in vitro studies showed that Wnt3 deleted organoid cultures did not survive, suggesting that a mesenchymal Wnt, for example Wnt2b or Wnt5a, which are highly expressed outside of the intestinal epithelium (Farin et al., 2012; Klostermeier et al., 2011), was required. Importantly, Wnt2b was shown to be a potent Wnt/β-catenin signalling activator (Goss et al., 2009). Indeed, by employing retroviral transduction, it was revealed that Wnt2b could restore growth of Wnt3 deleted organoids (Farin et al., 2012). More recently, it was
shown, that high levels of Wnt2b are predominantly expressed in Gli1 positive subepithelial mesenchymal cells, which are adjacent to the crypts (Valenta et al., 2016).

Immunofluorescence of the cells treated with either IWP2+Wnt2b or IWP2+Wnt3a demonstrated that the recombinant Wnts were able to restore MET, i.e. the cells were lifting off of the tissue culture plastic and organoids were forming, thus suggesting, that they may be the Wnts to interact with Fzd7 during MET. Notably, the gene expression results for LamG2, which were dramatically increased in the monolayer cells of the independent qRT-PCR, only had a slight significant increase in the IWP2 treated experiments. Whereas there was little to no difference in expression detected in the experiments looking at the ability of the recombinant Wnts to restore MET. This lack of expression and yet the morphological examination of the cells revealing that organoid assembly was halted when treated with IWP2 and yet restored when co-treated with IWP2 and the recombinant Wnts, is similar to that of the experiment in our lab looking at the role of Fzd7 in the LIM1863-Mph cells (Vincan et al., 2007b). Indeed, despite the clear lack of organoid assembly following Fzd7-depletion, as seen in this study as well following IWP2 treatment, the expression level of LamG2, was not increased. In this study as well as in the Fzd7 study, the cells were not migrating and thus the lack of induction in LamG2, which is a basement membrane adhesion protein that is important for epithelial cell attachment and cell migration, is consistent with the observed decrease in cell migration.

As the studies in hepatocellular carcinoma cells reported that Wnt3 directly interacts with Fzd7 via Co-IP (Kim et al., 2008) and the IF study suggested that Wnt2b and Wnt3a were restoring MET, we confirmed that Fz7 and Wnt3 do interact and also that Wnt2b Co-IPs with Fzd7. These findings, further suggest that both Wnt2b and Wnt3a interact with Fzd7 during MET.
2.5 Conclusion

In this study, we have shown that Wnt secretion is required for organoid assembly; furthermore we have demonstrated that Wnt2b and Wnt3a are capable of restoring organoid assembly, when it is inhibited. In addition, we showed that Wnt2b and Wnt3 both interact with Fzd7. Thus, these results taken together with the findings from previous work in our lab focused on the role of Fzd7 in organoid formation, suggest that the interaction between either Fzd7 and Wnt2b or Fzd7 and Wnt3a is required for organoid assembly, i.e. MET.
Chapter 3

The role of Frizzled-7 in the normal small intestinal epithelium
3.1 Introduction

The small intestine of the mouse is lined by a simple absorptive epithelium, which is organized into crypt-villus units. The epithelium is continuously and rapidly turned over and replaced every 3-5 days (Leblond and Stevens, 1948). For this to take place, some 300 cells are generated per crypt every day in the mouse (Marshman et al., 2002). The exception to this rapid turnover are the Paneth cells, which are renewed only every 3-6 weeks (Bjerknes and Cheng, 2005; Garabedian et al., 1997; Ireland et al., 2005). This massive cell production is maintained by proliferative adult stem cells, called crypt base columnar (CBC) stem cells, which are located, as the name says, at the base of the crypt, known as crypts of Lieberkühn. The CBC stem cells by definition self-renew and give rise to all the cell types in the crypt. Their highly proliferative progenitors, known as transit amplifying cells, migrate up the crypt as they expand and then undergo differentiation to become the various cell types, for example absorptive or secretory cells. The terminally differentiated cells make up the villus, where they continue migrating upward while performing their specific functions, then die by apoptosis and are sloughed off into the lumen once they reach the villus tip. An exception to this upward migration is the Paneth cell lineage. The Paneth cells are progeny of the CBC stem cells, which complete their differentiation process via a bidirectional migration; the terminally differentiated Paneth cells maintain residence at the base of the crypt nestled between the stem cells. They provide the specialized microenvironment at the base of the crypt, called the stem cell niche, with all the factors they require to survive and function: Wnt3, EGF, TGF-α and the Notch ligand Dll4 (Sato et al., 2011).

Cell number, cell proliferating vs. cell differentiating and loss of cells at the villus tip, is under strict homeostatic control. It has been shown that the Wnt/β-catenin pathway is critical for this control (Pinto et al., 2003). In summary, Wnt/β-catenin signalling is activated when a Wnt ligand binds to its receptor complex composed of Frizzled (Fzd) and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), which triggers a series of events that disrupts a cytoplasmic multi-component destruction complex. This destruction complex includes Axin, adenomatous polyposis coli (APC), glycogen synthase kinase-3 beta (GSK3β) and casein kinase-1 (CK1), which are required for the
targeted proteosomal degradation of the transcriptional activator β-catenin. Disruption of the destruction complex leads to the accumulation and nuclear translocation of β-catenin. In the nucleus, β-catenin engages the N terminus of DNA-binding proteins of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, to activate the transcription of Wnt/β-catenin target genes (Clevers and Nusse, 2012). Wnt signalling is turned “on” in the stem cells and proliferating, undifferentiated precursor cells and “off” once the cells terminally differentiate. In the transit-amplifying compartment, it is also necessary for the upward migration of the cells and for the positioning of the Paneth cells at the crypt base (Batlle et al., 2002).

Wnt signalling also plays a crucial role as a regulator in stem cell proliferation and differentiation (Korinek et al., 1998; Sato et al., 2009; Van der Flier et al., 2007). Seminal work from the Clevers Lab identified Lgr5 (leucine-rich-repeat-containing G protein-coupled receptor 5), which is a Wnt/β-catenin target gene, as an exclusive marker of the CBC stem cells, in both mouse and human intestine (Barker, 2014; Barker et al., 2007; Itzkovitz et al., 2012). Barker and colleagues (Barker et al., 2007) demonstrated that Lgr5 specifically marks the CBC stem cells in the intestine, by using a novel transgenic mouse (Figure 3.1 A). In this mouse one allele of Lgr5 is replaced by a transgene that codes for an enhanced green fluorescent protein (EGFP) followed by an internal ribosome entry site (IRES) and a Cre-recombinase (CreERT2) Lgr5EGFP-IRES-CreERT2 (Lgr5CreERT2 for simplicity). Thus the mouse constitutively expresses EGFP in cells where the Lgr5 promoter is active as well as a Cre-recombinase enzyme that requires tamoxifen induction for enzyme activity (Figure 3.1 A). The Cre-recombinase (Cre: cyclization recombination) is a bacterial (bacteriophage P1) enzyme that catalyses recombination between two 34-bp DNA recognition sites named loxP (locus of crossing [x-ing]-over of bacteriophage P1) (Feil et al., 2009). Thus if loxP sites are on either side of a gene, Cre-recombinase will cut out that gene, generating a conditional knockout mouse. Cre-recombination can be traced using the bacterial (Escherichia coli) LacZ gene which encodes the β-galactosidase enzyme (Burn, 2012). The LacZ reporter mouse has a “stop” cassette that is flanked by loxP sites so that when it is crossed with a Cre mouse, the “stop” cassette is excised and β-galactosidase is expressed in cells where Cre is expressed and active. This expression can be detected histochemically via
X-gal staining, which turns blue when the enzyme is active. Barker and colleagues (Barker et al., 2007) showed that when low level induction of Cre-recombination is used in the Lgr5-LacZ mouse, so that only one stem cell is recombined per crypt, over time this induced mouse yields ribbons of blue cells that span from the crypt base to the villus for the life of the animal. All the cells carrying the genetic trace that originated from the recombined stem cell. Lineage tracing where the LacZ reporter is under the control of the Lgr5 promoter demonstrated that all the different cell types originate from the Lgr5+ stem cells and carry the genetic trace as all the cells are blue. Another Cre, the AhCre, which is induced with β-naphthoflavone (β-NF), also targets the stem cells as well as all of the other cell types in the crypt and villus epithelium of the small intestine, however not the Paneth cells (Ireland et al., 2004). The blue non-stem cells are “washed” off the epithelium by normal turnover. However, when a stem cell is targeted, all cells are blue, from the crypt to the villus, within 7 days (Flanagan et al., 2015).

The above-mentioned CBC stem cells have been in direct competition, for the past four decades, with a second stem cell model, the ‘+4 model’, by Potten (Potten, 1977). The ‘stem cell zone model’ by Leblond, Cheng and Bjerknes (Bjerknes and Cheng, 1999; Cheng and Leblond, 1974a), suggests that the CBC cells are the resident stem cells. Whereas the +4 model (+4, the fourth position from the crypt base) advocates that a ring of 16 cells sitting immediately above the Paneth cells are the stem cells. Although specific markers for the two candidate stem cell populations have been discovered; +4 stem cell markers: B cell-specific Moloney murine leukemia virus integration site 1 (Bmi1) (Sangioergi and Capechi, 2008), Leu-rich repeats and immunoglobulin-like domains 1 (Lrig1) (Powell et al., 2012; Wong et al., 2012), Homeodomain-only (Hopx) (Takeda et al., 2011) and Telomerase reverse transcriptase (Tert) (Montgomery et al., 2011); and CBC stem cell markers: Lgr5 (Barker et al., 2007), Achaete Scute-like homologue 2 (Ascl2) (Jubb et al., 2006; Ziskin et al., 2013), Olfactomedin 4 (OLFM4) (Van der Flier et al., 2009a), Musashi homologue 1 (MSI1) (Cambuli et al., 2013; Nishimura et al., 2003; Potten et al., 2003), SPARC-related modular calcium-binding 2 (SMOC2) (Munoz et al., 2012), Prominin-1 (PROM1) and sex-determining region y-box 9 (Sox9) (Formeister et al., 2009; Ramalingam et al., 2012), it has proven difficult to
conclude which model is correct, as there is considerable overlap in marker expression. Notably, multicolour mRNA fluorescence in situ hybridization (FISH) assays (Itzkovitz et al., 2012) and expression profiling (Munoz et al., 2012) revealed that the prospective +4 markers Bmi1, Hopx and Tert are broadly expressed in CBC cells and do not uniquely mark a cell population within the crypt. In addition, Single-molecule FISH revealed that some proposed CBC stem cell markers such as PROM1 and MSI1 are also not restricted to CBC cells and display expression along the crypt axis. In comparison, Lgr5 identifies the CBC stem cells with exquisite specificity. Potentially, neither model is correct per se, as a unifying theory incorporating aspects of both models is emerging, based on the existence of distinct stem cell pools involved in epithelial homeostasis and regeneration.

Indeed, recent work in the field of intestinal epithelial regeneration has demonstrated a high degree of crypt plasticity. Lgr5+ CBC stem cells, which drive intestinal epithelial homeostasis (Barker et al., 2007), when targeted for conditional ablation revealed that homeostasis is unaffected and the intestine can survive the short-term loss of the CBC stem cell pool in vivo (Tian et al., 2011). In brief, the Lgr5+ CBC stem cell compartment was ablated in mice using a human diphtheria toxin receptor (DTR) gene knocked into the endogenous Lgr5 locus (LGR5DTR). It was shown that Bmi1-expressing stem cells, which reside above the crypt base at the +4 position, could compensate for the loss of the Lgr5+ CBC stem cells under basal conditions. By in vivo lineage tracing from the Bmi1 locus following the acute ablation of the Lgr5+ CBC stem cell compartment, it was demonstrated that Bmi1+ cells could give rise to Lgr5-expressing cells, contributing to the repopulation of the Lgr5+ stem cell pool during the subsequent regeneration phase, thus serving as a reserve stem cell pool. Supporting this finding was the demonstration that the +4 Hopx-expressing cells can give rise to Lgr5+ CBC stem cells and vice-versa (Takeda et al., 2011). However, in stark contrast, the depletion of LGR5+ CBC stem cells when combining the LGR5DTR model with ionizing radiation dramatically impaired the regenerative capability of the epithelium (Metcalfe et al., 2014). Suggesting that the cells, which can contribute to maintaining intestinal homeostasis in the absence of LGR5+ CBC stem cells and efficiently regenerate the LGR5-expressing cells under basal conditions, are ablated or functionally altered following radiation-
induced damage. Thus Lgr5+ cells per se are absolutely required for regeneration of the epithelium following damage (Metcalfe et al., 2014). Furthermore, in another study it was shown that irradiation-induced depletion of the Lgr5+ CBC stem cell compartment is sufficient to trigger the de-differentiation and subsequent trans-differentiation of the secretory progenitor cells, expressing Notch ligand Delta-like 1 (DLL1) on their surface, into multi-potent Lgr5+ CBC stem cells in order to participate in the subsequent regeneration of the damaged epithelium (van Es et al., 2012). Under normal conditions, the DLL1+ cells, at the +5 position are short-lived progenitors that give rise to goblet cells, enteroendocrine cells, Paneth cells and tuft cells, but can drop down into the stem cell niche and become CBC stem cells, i.e. LGR5+. In the absence of LGR5+ CBC stem cells, Paneth cells have also been shown to contribute to intestinal regeneration following radiation-induced damage. Paneth cells survive apoptosis induced by radiation, because of their intrinsic radio-resistance (Porter et al., 2002) and are able to de-differentiate to become proliferative and acquire stem-like properties (Roth et al., 2012). Indeed, it has been shown that following radiation, Paneth cells up-regulate Bmi-1 expression and are thereby able to actively contribute to the regeneration of the intestinal epithelium (Roth et al., 2012).

Although the role of Wnt in CBC stem cells was demonstrated conclusively, the Frizzled that binds the Wnt in stem cells was not defined. In our lab, experimental studies showed Fzd7 is enriched in the CBC stem cell compartment. Indeed, the Fzd7 reporter mouse (Fzd7NLS) clearly shows Fzd7 reporter activity in the CBC cells (Figure 2.1 B). In the Fzd7NLS mouse, the coding region for Fzd7 has been replaced by a nuclear localization sequence that codes for LacZ, which encodes the β-galactosidase enzyme, as mentioned above (Figure 3.1 B). Thus the mouse expresses LacZ in cells where the Fzd7 promoter is active (Flanagan et al., 2015). Enrichment of LacZ in cells where the Fzd7 promoter is active was further supported by a Frizzled gene-profiling assay. Briefly, an Agilent Array was used to assess the expression of Fzds from crypts of Lgr5CreERT2 mice. The crypts were arbitrarily sorted into five populations, with 5+ (the CBC stem cells) having the highest and 1+ having the lowest EGFP expression. Each population was compared to the 5+ fraction. This showed that Fzd2 and Fzd7 track together, the highest relative expression was seen in the CBC stem cells, with a noticeable decrease
while moving away from the crypt base, along the crypt axis. In comparison, expression of Fzd4 did the opposite and increased along the crypt axis, whereas Fzd5 and Fzd8 expression was unchanged (Flanagan et al., 2015). Independent cell sorting for GFP+ and GFP- single cells, comparing Fzd7 expression (qRT-PCR), confirmed the enrichment of Fzd7 in the CBC stem cell compartment (Flanagan et al., 2015).

Functional studies in our lab further support a role for Fzd7 in epithelial regeneration in vivo. Briefly, two days after whole body ionizing irradiation, as a means to inflict DNA damage, the crypt compartment of the mouse intestinal epithelium is lost due to widespread apoptosis of the CBC and TA cells. The loss of stem cells triggers a rapid repopulation of the crypt domains with stem cells and a regeneration of the crypts, so that by three days post radiation, large crypts with many proliferating cells are seen (Ashton et al., 2010). This rapid regeneration of the crypt compartment is impeded in the absence of Fzd7 (Flanagan et al., 2015). This is strong evidence that Fzd7 is required for efficient regeneration of the epithelium.

Lineage tracing studies to further probe the function of Fzd7 in stem cells was performed using the Lgr5CreERT2 mouse as well as the AhCre mouse. These Cre mice were crossed to floxed Fzd7 mice (Fzd7fl/fl) where the Fzd7 coding region was flanked by loxP sites, thereby generating Lgr5CreERT2;Fzd7fl/fl;LacZ and AhCre;Fzd7fl/fl;LacZ mice. The deletion of Fzd7 was either induced by tamoxifen (Lgr5CreERT2) or β-NF (AhCre). Following Fzd7 recombination, the mice no longer had blue ribbons, indicating that the stem cells, which had Fzd7 deleted were lost from the epithelium (Figure 2.1 C right panel). The epithelium looked phenotypically normal, except for the absence of “blue” stem cells at the base of the crypt. The loss of stem cells then triggered rapid repopulation. This has been demonstrated previously with several genes that are essential to stem cells such as c-Myc (Muncan et al., 2006) and Ascl2 (van der Flier et al., 2009b).
Figure 3.1 Genes required by stem cells can be determined by following the longevity of recombined cells.

(A) Cells expressing Lgr5Cre and Rosa26\textsuperscript{LSL}LacZ alleles (white cell) undergo genetic recombination following tamoxifen injection, successfully excising the LSL roadblock sequence, allowing expression of β-galactosidase.

(B) Histological analysis of LacZ activity in the intestinal epithelium from a Fzd7\textsuperscript{NLS} mouse showing nuclear expression of the reporter in the CBC cells. Schematic of the Fzd7\textsuperscript{NLS} construct. NLS – nuclear localization sequence

(C) Left panel – continual creation of genetically labelled cells following recombination of a gene not required by the stem cells.

Right panel – recombination of a gene required by the stem cells is deleterious (blue cell), thus triggering rapid repopulation by non-recombined (pink) cells.
Adapted from Barker et al., 2007

Adapted from Flanagan et al., 2015

Progeny carry “blue” genetic trace

Repopulation of stem cells by non-recombined “pink” cells
Epithelial repopulation
This indicated that Fzd7 is necessary for stem cells. Indeed analysis by qRT-PCR on crypts isolated from induced mice confirmed that Fzd7 expression was decreased after Cre-mediated recombination in the AhCre;Fzd7\(^{fl/fl}\);LacZ mice, as expected, but also showed that Lgr5 expression was concomitantly decreased at the same time. Although the loss of “blue” stem cells is striking, the rapid repopulation made it difficult to conduct molecular analysis.

Thus, the aim of this PhD project was to establish and use an in vitro culture system of three-dimensional (3D) intestinal epithelial organoids, “epithelial mini-guts”, to investigate Fzd7 function in stem cells in a defined system (Figure 3.2). Therefore, here intestinal organoid cultures were established from AhCre;Fzd7\(^{fl/fl}\) mice. Sato and colleagues (Sato et al., 2009) demonstrated that a single Lgr5 cell will self-organize to form organoids when cultured in Matrigel, a 3D laminin- and collagen-rich matrix that mimics the basal lamina, and provided with defined medium that contains the following cocktail of growth factors: R-spondin, EGF and Noggin, which represent the minimal, essential stem cell niche maintenance factors. The organoids develop defined crypt- and villus-like domains which are very similar in architecture to the small intestinal epithelium and the villus-like domains have all the cell types of the epithelium (Sato and Clevers, 2013). Thus, providing an in vitro model, suited for investigating the effect of Fzd7 deletion.

Intestinal organoids cultured in vitro provide an excellent model for studying gene function. In our lab initial attempts to culture organoids from the Lgr5Cre\(^{ERT2}\);Fzd7\(^{fl/fl}\) mice and delete Fzd7 in vitro using the Lgr5Cre were unsuccessful, because although Lgr5 is expressed in all the crypts, the transgene is expressed in a mosaic pattern and it was difficult to do analysis. Attempts to sort EGFP positive cells as the starting material for organoids was also unsuccessful, as most of the sorted cells did not survive. The alternative was to use the AhCre. Given that Fzd7 is enriched in the stem cells (Figure 3.1 B) (Flanagan et al., 2015) Cre mediated deletion of Fzd7 with AhCre would primarily affect the stem cells. That is, although all cells (bar the Paneth cells) are targeted by the AhCre and Fzd7 is deleted in them all, only the stem cells would be affected by the deletion, since they express Fzd7.
Taken together our *in vivo* data (Fzd7 expression in CBC stem cell compartment, Fzd7 requirement for regeneration after insult and loss of Fzd7 deleted “blue” stem cells at the crypt base) strongly indicated a role for Fzd7 in the CBC stem cells. Thus it was important to investigate further in a defined, isolated system. Hence isolated intestinal organoids were perfect to use to further investigate *Fzd7 in vitro*.

We have demonstrated in this study that *Fzd7* facilitates intestinal organoid culture maintenance, as organoids with *Fzd7* deleted do not regenerate. Furthermore we confirmed our previous findings that Fzd7 has a role as a Wnt receptor in intestinal CBC stem cells.

**Figure 3.2 Small intestinal epithelial organoids**

Scanning electron micrograph of the small intestine. The red box is showcasing the crypts, the villus is the much larger structure located above them.

Cartoon of the crypt. The Lgr5⁺ CBC stem cells are intercalated with Paneth cells at the crypt base. The stem cells generate the proliferating transit-amplifying cells, which occupy the remainder of the crypt.

Sketch of the Matrigel three-dimensional culture system for organoids. Intact intestinal crypts are plated into laminin-rich Matrigel bathed in medium supplemented with a cocktail of growth factors including the bone morphogenetic protein inhibitor Noggin, the Wnt agonist roof plate-specific spondin 1 (R-spondin1) and epidermal growth factor (EGF) (Sato et al., 2009). This generates self-renewing epithelial organoids organized into crypt domains surrounding a central lumen lined by a villus-like epithelium (villus domain).
3.2 Materials and Methods

Mice

The AhCre (Ireland et al., 2004), VillinCre<sup>ERT2</sup> (el Marjou et al., 2004) and Fzd7<sup>fl/fl</sup> (Flanagan et al., 2015), have all been previously described. The mice were interbred to generate compound mice with appropriate alleles. All mice were co-housed and on an inbred C57BL/6 genetic background, with appropriate littermates used as controls. All animal experiments were approved by the Animal Ethics Committee, of the Office for Research Ethics and Integrity, at The University of Melbourne.

Crypt isolation and organoid culture from small intestine tissue, adapted from Sato et al., 2011

The proximal (~10cm) end of the small intestine from mice were dissected out, cut longitudinally and washed with ice cold PBS. Then, after placing the tissue in a 10cm dish with cold PBS on ice, using a glass coverslip, the villi were scraped off. The small intestine was then cut into 5mm small pieces and transferred to a falcon tube with cold PBS (phosphate buffered saline). The pieces were washed until the supernatant was clear. Then they were incubated in a (2mM EDTA (Ethylenediaminetetraacetic acid) pH 8.0 in PBS) chelating solution for 30min on a nutator at 4°C. The pieces of intestine were then gently washed with PBS and transferred to new tubes with PBS. Using a 10mL pipette (Costar #4488), the crypts were mechanically dissociated from the surrounding tissue; this step was repeated three times, providing 4 fractions. Generally fractions 2-4 were the crypt enriched fractions and combined. The pooled fractions were washed, centrifuged (1,200rpm for 5min at 4°C) and resuspended in Advanced DMEM-F12 (Invitrogen #12643-028) containing bovine serum albumin (BSA; 0.1%), Penicillin/Streptomycin (1:100, Invitrogen #15140-122), L-Glutamine (1:100, Invitrogen #25030-081) and HEPES (10mM, Invitrogen #15630-080) (ADF). The isolated crypt suspension was filtered through a 70µm cell strainer, which was then collected and counted. The crypts were then resuspended in ice-cold Matrigel (Corning #356231) (2,000 crypts/mL) and plated at 50µL/well onto a pre-warmed (at 37°C) 24-well tissue culture plate (Costar #3526). Once the Matrigel had set, 500µl of ADF supplemented with N2 (1:100, Invitrogen #17502-048) and B27 (1:50, Invitrogen #12587-010) (ADF intestine culture medium), containing various growth factors
essential for epithelial growth: EGF (50ng/ml, Peprotech #315-09), R-Spondin (500ng/ml, R&D #3474-RS-050) and Noggin (100ng/ml, Peprotech #250-38), was added to the wells. Growth factors were added every other day, with a whole culture medium change once per week. Cultures were passaged (mechanically) and split after approximately 7-10 days.

Organoids were passaged by mechanically dispersing the organoids and Matrigel. Matrigel and organoids were scraped from the tissue culture plate and together with the medium, they were transferred to a falcon tube and diluted with fresh ADF. After dissociation using a p1000, the organoid-Matrigel mix was centrifuged at 600rpm for 2min. The supernatant was discarded, ADF was added and, with a p200, the pellet of organoids was further broken down. The wash step was repeated two more times with a p1000, as the organoids did not need to be broken down any further. The washes progressively removed the cell debris, dead cells and Matrigel. The final clean pellet was resuspended in ADF, counted and resuspended at 2,000 organoids/organoid clusters per microliter of Matrigel and plated as above in a new 24-well plate.

**β-naphthoflavone administration**

Cre-recombinase was activated *in vitro* in crypt organoid cultures by treating with 360nM β-naphthoflavone (β-NF), made up in DMSO, for 48hr at 37°C (Flanagan et al., 2015).

**4-hydroxytamoxifen administration**

Cre-recombinase was activated *in vitro* in crypt organoid cultures by treating with 500nM 4-hydroxytamoxifen (4-OHT), made up in 100% EtOH, for 48hr at 37°C (Flanagan et al., 2015).

**Imaging organoid cultures**

Differential interference contrast (DIC) images of organoid cultures were taken on a Nikon Ti-E microscope with either a 4× Plan Fluor NA 0.13 objective or a 10× Plan Fluor NA 0.3 objective. A focal stack of images was collected 10µm apart and processed through the “Best Focus” function of MetaMorph v7.7.7 (Molecular Devices) to generate the final image of individual organoids.
**Immunofluorescence staining**

Organoids were set up in Matrigel in 8-chamber slides (1 µ-Slide 8-well ibiTreat, Ibidi #80826). Following DMSO or β-NF treatment they were fixed in situ with 2% para-formaldehyde in PBS for 10min at room temperature (RT) and permeabilized with 0.5% Triton X-100 in PBS for 10min at RT to help the antibody penetrate the membrane and access the intracellular proteins (i.e Lysozyme). To block nonspecific binding of the antibodies, the organoids were incubated in 2% BSA in Dako Antibody diluent (Dako #S0809) overnight (O/N) at 4°C. During this incubation, the antibodies were pre-absorbed on Matrigel, to eliminate non-specific binding of the antibodies to the Matrigel. A 96-well plate was prepared with 30μl per well of Matrigel and incubated at 37°C to set. During the incubation, a 100μl working dilution of each antibody, both primary and secondary, in 2% BSA in DAKO Antibody diluent was made up and then added to the pre-set Matrigel, wrapped in foil to protect the secondary antibody from photobleaching and incubated O/N at 4°C. The secondary antibody was stored at 4°C until required. The primary antibodies used were mouse anti-E-cadherin (BD Transduction Labs #610181) and rabbit anti-lysozyme (ThermoScientific #RB372), the secondary antibodies were Alexa anti mouse 568 and Alexa anti rabbit 488. After the O/N incubation, the block was removed and the pre-absorbed primary antibodies were added and incubated again at 4°C O/N. The organoids were then washed free of any excess primary antibody with 2% BSA in PBS. Next, the pre-absorbed secondary antibody was added to the organoids and incubated at 4°C for 4hrs. Again the organoids were washed with 2% BSA in PBS, removing any excess secondary antibody. The nuclear counter stain DAPI (4’,6-Diamidino-2-Phenylindole) in 2% BSA in DAKO Antibody diluent was added for 5min at RT and then followed by washes in 0.2% BSA in PBS. 200μl of 0.2% BSA in PBS was then added to each chamber and stored at 4°C in the dark until imaged.

Detection of the staining for E-cadherin and Paneth cells (lysozyme) was captured on a Leica LAS AF SP5 microscope using a 63× HCX Plan Apo NA 1.4 OIL objective. A Z-stack of images was collected and processed in ImageJ v1.43u, then finalized in Adobe Photoshop CS4 v11.0.2 to generate the final images.
**RNA extraction and cDNA synthesis**

Total RNA extraction and cDNA synthesis was conducted as previously described (Vincan et al., 2007b). Briefly, following treatment, the organoids were harvested as when passaging, except the clean pellets were resuspended and homogenised in TRizol (Invitrogen #15596-018) and incubated for 30mins at room temperature. Phenol:Chloroform:Isoamylalcohol was added to the homogenised samples and mixed well. After 5mins at room temperature, the samples were centrifuged (10,000rpm) for 15mins in order to separate the upper clear aqueous phase (nucleic acid containing fraction) from the lower pink organic phase (protein containing fraction). The clear aqueous phase was removed and mixed with an equal volume of 70% Ethanol. The RNA samples were purified and DNAse treated using RNeasy mini-prep columns (Qiagen #74104) following the manufacturers’ instructions. RNA samples were then quantified using a DNA/RNA nanodrop spectrophotometer (Thermo Scientific Nanodrop 1000). cDNA was synthesized with 4µg of each RNA sample. Samples were reverse transcribed using anchored oligodT primers (Promega) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega #M1705), in a final volume of 100µL.

**RNA analysis**

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the 2x SYBR green PCR master mix (Invitrogen #4309155) and ABI PRISM 7500 Sequence Detector (Applied Biosystems) as previously described (Vincan et al., 2007b). In brief, final volumes of 20µL PCR amplification mixture (2x SYBR green I Master Mix buffer (10µL), sterile water (7µL), template cDNA (1µL) and forward and reverse primer mix (2µL)) were set up in triplicate wells of a 96-well tray (Applied Biosystems MicroAmp #4346907). The cycling conditions compromised 2min at 50°C, 10min polymerase activation at 95°C, 40 two-step cycles of 15sec at 95°C and of 1min at 60°C and a dissociation stage of 15sec at 95°C, 1min at 60°C, 15sec at 95°C and 15sec at 60°C. The housekeeping gene β-2-Microglobulin (β2M) primers were used to equalize the data; with threshold cycle (CT) values, the $2^{ΔΔCT}$ method (Vincan et al., 2007b) was used to calculate the fold change. For primer sequences see appendix Table1.
**MTT assay**

The experiments looking at regeneration were set up after treatment of the organoids. Each well was harvested, as for passaging (mentioned above), and resuspended in 150µL Matrigel. This was then dispensed to 6 replicate wells at 16µL per well of a 96-well plate (Costar #3599) (with 100µL of intestine culture medium and growth factors) and 50µL in one 24-well plate well. For the time-course experiment, organoids were set up directly in the 96-well plates (replicate plates, one per time point) and treated accordingly. They were incubated for varying times; treated same day as plating, then MTT assay was performed, one day, three days or five days after treatment. Organoids were then incubated for 4hrs with MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma #M2128) at 37°C. The intestine culture medium was removed from the organoids and replaced with lysis buffer (50% DMF, SDS, acetic acid + 2.5% 1M HCl) and incubated overnight at 37°C. The optical density (OD) was determined using the BMG Lumistar plate reader (Hansen et al., 1989).

**Genomic DNA extraction**

Genomic DNA (gDNA) was extracted from organoid pellets, washed free of cell debris and Matrigel, using standard techniques. Briefly, the organoid pellets were digested in Proteinase K lysis buffer (containing: 1M Tris-HCl pH8.5-9.0; 5M NaCl; 10% SDS; 0.5M EDTA and milliQ water with freshly added Proteinase K (final 500µg/mL; Sigma #P-4914)) overnight. The DNA was extracted using Isopropanol to precipitate the gDNA. The DNA was pelleted for 10min at 13,000rpm. The supernatant was then discarded and the pellet washed in 70% Ethanol. A final 1min spin, to help pipette off all alcohol, then the pellets were air dried briefly, followed by the addition of TE pH8.0 (Tris/EDTA: 1M Tris pH8.0; 0.5M EDTA and milliQ water) to resuspend the DNA.

**DNA detection**

Genomic DNA was subjected to PCR to detect the transgene (Fzd7flox MUT) and Cre recombinase (AhCre). To detect the recombined/deleted product (DP), PCR primers that are either side of the transgene and only work once the gene is excised, were used to amplify the product. All PCRs were run on Veriti PCR machines (for cycling conditions see appendix Table2). The products were then run on a 1.5% (w/v) agarose
gel prepared with Tris/Acetic acid/EDTA (TAE) buffer, stained with 15µg/mL ethidium bromide (EthBr) and visualized under UV trans-illumination. The DP PCR product was sequenced using standard ABI protocols, and the sequence confirmed by Prof. Nick Barker, who generated the Fzd7floxed mouse for our laboratory, to be the expected product created by recombination of the Fzd7floxed alleles.

Statistical analysis

Data are expressed as mean ± SEM, where mean represents number of mice (three or more per genotype) or number of experiments (three or more). Organoids were established from three or more hosts per group, unless otherwise stated. The statistical Student’s t test was used with Prism6 (GraphPad software) where \( P \) values of \( \leq 0.05 \) were considered significant.
3.3 Results

**Fzd7 deleted intestinal organoids do not regenerate**

As previously mentioned the *AhCre* model allows for controlled inducible Cre-mediated gene deletion in the intestinal epithelium. Organoid cultures were established from crypts isolated from the proximal small intestine of *AhCre;Fzd7*\(^{fl/fl}\) mice as well as from *AhCre;Fzd7*\(^{+/+}\) mice as controls. The isolated crypts when grown in Matrigel with medium containing growth factors, form mini-gut organoids which have the characteristics of the intact epithelium (Sato et al., 2009). Cre-mediated recombination was induced with β-naphthoflavone (β-NF); as a control, organoids were also treated with the vehicle, DMSO. In an initial pilot experiment, organoids from one mouse were set up in replicate wells of a 24-well plate for DIC imaging (Figure 3.3 A) and in replicate wells of 96-well plates for MTT assays (Figure 3.3 B). One set of plates was set up for each day (Day 0, 1, 3 and Day 5) for the MTT assay, while the same organoids were imaged over the time-course in the 24-well plate. The images taken were differential interference contrast (DIC) Z-stacks, which allow for a much crisper image. Over the time-course from Day 0 to Day 5 the deletion of *Fzd7* takes its effect on the organoids significantly by Day 3, which is noticeable in the DIC images. By Day 5 there was a distinct difference between the organoids, which were treated with DMSO, and those with β-NF. The organoids, which have had Fzd7 deleted show signs of crypt atrophy (arrow) and organoid death (#). At Day 5, the organoids from the 24-well plate were harvested and plated into a 96-well plate; they were no longer treated with DMSO or β-NF. At Day 3 they were then assessed via MTT assay. DIC images of the organoids at Day 3, before the MTT assay, demonstrated the inability of β-NF treated organoids to regenerate, however the DMSO treated organoids continue to grow and regenerate organoids. This was confirmed by MTT assay, showing considerably reduced cell viability in the organoids, after *Fzd7* deletion, suggesting that they do not regenerate after the loss of *Fzd7*; whereas the DMSO treated organoids continue expanding. This was confirmed with organoids established from an additional two mice, showing that the Fzd7 deleted cells harvested at Day 5 do not regenerate to form organoids after passaging (Figure 3.3 C, D).
To confirm loss of Fzd7 expression following gene deletion, qRT-PCRs were run on organoids from three mice harvested two days after treatment with β-NF or DMSO. The results shown in Figure 3.4-B verified the deletion of Fzd7 via Cre-recombination as Fzd7 expression was significantly reduced in the organoids treated with β-NF compared to those with DMSO. Also an associated decrease in Lgr5 expression, as was observed in the in vivo experiments (Flanagan et al., 2015), was confirmed. Furthermore, the expression level of Fzd2, whose expression was shown to track with that of Fzd7 in an Fzd gene-profiling assay, was also significantly reduced. In addition, Achaete Scute-Like 2 (Ascl2), a Wnt target gene and master regulator of the Lgr5+ CBC stem cells (Schuijers et al., 2015; van der Flier et al., 2009b), was also significantly decreased. In contrast, but further supporting the notion that we were losing the CBC stem cells, was the lack of decreased c-Myc expression. c-Myc is expressed in the stem cells, but also in the proliferating transit-amplifying compartment of the crypts (Bettess et al., 2005), which are still intact as the organoids are still intact at Day 2. Gene analysis of +4 stem cell markers m-Tert, Hopx and Lrig1 were unchanged (Figure 3.4 C), indicating that Fzd7 gene deletion in these cell types has no effect.

To further confirm recombination, PCRs (Figure 3.4 A) were performed on genomic DNA extracted from organoids harvested at the same time as those for the qRT-PCR (two days post induction). The PCR detecting the mutant Fzd7 knockin allele shows a partial excision, which is due to the recombination not affecting the Paneth cells; as already mentioned they are not targeted by the AhCre. PCR for detecting the deleted product (DP), which is the intact genomic DNA of the recombinated product after Fzd7 gene deletion, only works once the Fzd7 gene has been deleted. It therefore confirmed the recombination of Fzd7 in the organoids treated with β-NF. Faint bands present in the non- β-NF treated organoids are due to the AhCre being leaky, but not sufficient to have any effects on the organoids. PCRs for the AhCre transgene and for a region of chromosome 1, which is close to the Fzd7 locus, were also run. The first confirms that all organoids were indeed AhCre positive and the second that the quality of the DNA templates between samples was comparable.

Control experiments using AhCre;Fzd7+/+ mice (Figure 3.5 A) confirm that β-NF activation of the Cre-recombinase alone does not have a deleterious effect on the
organoids. Again using one mouse, a 24-well plate for DIC imaging and 96-well plates for MTT assays were set up. Cultures treated with either DMSO or β-NF continued to grow and regenerate new organoids when passaged. The MTT assay time-course (Figure 3.5 B) shows no decline in cell viability. Organoids passed at Day 5 and plated for MTT assay three days later (Figure 3.5 C) also show no decline in viability and that the organoids regenerate normally.

Looking to further confirm our findings, a second Cre, VillinCreERT2, was used. VillinCreERT2 is a tamoxifen-dependant recombinase, which unlike AhCre, targets all cells of the intestinal epithelium including Paneth cells (el Marjou et al., 2004). Organoid cultures were again set up in 24-well plates for DIC imaging and 96-well plates for MTT assays from one mouse for the time course experiment. Organoids were treated with 4-hydroxytamoxifen (4-OHT) to induce Fzd7 deletion and, as a control, the vehicle Ethanol (EtOH). In Figure 3.6-A five days post induction, the organoids that have had Fzd7 deleted are comparable with the AhCre;Fzd7fl/fl deleted organoids. Again, there are signs of crypt atrophy (arrows) and organoid death (#). Over the time-course from Day 0 to Day 5 of the MTT assay (Figure 3.6 B), the deletion of Fzd7 has a noticeable, albeit not significant, effect on organoid viability by Day 3. By Day 5 there was however, a significant difference between the organoids treated with DMSO and those with 4-OHT. The experiment was repeated for the Day 5 time point, and the MTT assay (Figure 3.6 C) of pooled data from the two mice, further confirms that cell viability was significantly diminished compared to that of the organoids with non-recombined Fzd7 by Day 5.

As an additional control, organoids from AhCre;Fzd5fl/fl mice (which allows for the Cre-mediated gene deletion of Fzd5) were used. Fzd5 in the mouse is located on chromosome 1 as is Fzd7 (Wang et al., 1996) and in humans they are located on chromosome 2 (Sagara et al., 1998; Saitoh et al., 2001). Fzd5 is a Wnt ligand receptor expressed in Paneth cells (van Es et al., 2005a). As with the AhCre;Fzd7fl/fl organoids, treatment was either with DMSO or β-NF. The deletion of Fzd5, was confirmed by qRT-PCR expression analysis and as seen in Figure 3.7-A, had no effect on the organoids, detectable by phenotype, visually. The MTT assay (Figure 3.7 B) however, does reveal a slight significant difference between the Fzd5 deleted and Fzd5 non-recombined
organoids. As mentioned above, AhCre does not target the Paneth cells and therefore the deletion of Fzd5 is unlikely to have an effect on them. None the less, there was a small loss in organoid numbers, approximately 17%, indicating some role for Fzd5. However in contrast, Fzd7 deleted organoids had a markedly higher loss: 60% (VillinCreERT2) and 90% (AhCre).

Thus, in this series of experiments it was demonstrated using two different intestinal Cre’s that deletion of Fzd7 leads to crypt atrophy and organoid death. Furthermore, it was verified that Fzd7 deleted organoids do not regenerate when passaged. This substantiates the requirement of Fzd7 as a Wnt receptor for the crypt base columnar (CBC) stem cells, which is not only supported by the concomitant loss of Lgr5 as well as Fzd2 and Ascl2, but also further strengthened by the inability of the Fzd7 deleted organoids to regenerate after passaging.
Figure 3.3: AhCre;Fzd7\textsuperscript{fl/fl} intestinal organoids

(A) Representative differential interference contrast (DIC) images of AhCre;Fzd7\textsuperscript{fl/fl} intestinal organoids imaged at Day 0, 3 and 5 post β-NF or DMSO treatment.

Arrows: crypt atrophy; #: organoid death. Scale bar represents 100µm.

(B) MTT cell viability assay of time-course from Day 0, 1, 3 and 5 of organoids induced with β-NF and compared to the vehicle DMSO (mean ± SEM, *p < 0.05, n = 6 replicate wells).

(C) Representative differential interference contrast (DIC) images of AhCre;Fzd7\textsuperscript{fl/fl} intestinal organoids harvested after five days treatment with either the vehicle DMSO or β-NF and then replated into a 96-well plate for MTT assay. MTT assay was performed three days after plating. Higher magnification of boxed areas is shown on the right - scale bar represents 100µm.

(D) MTT cell viability assay 3 days after passage of β-NF and vehicle (DMSO) treated organoids (mean ± SEM, *p < 0.05, n = 3 mice).
Figure 3.4: PCR and qRT-PCR gene analysis of AhCre;Fzd7$^{fl/fl}$ intestinal organoids

(A) PCRs to detect the Fzd7 mutant knockin allele (Fzd7 Mut) and recombined product after Fzd7 gene deletion (DP), AhCre transgene (AhCre) and a region of chromosome 1 close to Fzd7 locus (Chr1), in genomic DNA extracted from organoids established from three AhCre$^{+}$;Fzd7$^{fl/fl}$ mice (M1, M2, M3) at 5 days post induction (5 days PI).

(B) Gene expression levels determined by qRT-PCR and normalized to β2M. Organoids were harvested 2 days after induction with β-NF and compared to the vehicle DMSO treated organoids (mean ± SEM, *p < 0.05, n = 3 mice).

(C) Gene expression levels determined by qRT-PCR and normalized to β2M. Organoids were harvested 2 days after induction with β-NF and compared to the vehicle DMSO treated organoids (mean ± SEM, *p < 0.05, n = 3 mice).
A

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5 days PI

- Fzd7 Mut
- DP
- AhCre
- Chr 1

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C

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Figure 3.5: AhCre;Fzd7+/+ intestinal organoids

(A) Representative differential interference contrast (DIC) images of AhCre;Fzd7+/+ intestinal organoids at Day 0, 3 and 5. Organoids were treated with either vehicle (DMSO) or β-NF. Scale bar represents 100µm.

(B) MTT cell viability assay of time-course from Day 0, 3 and 5 of organoids induced with β-NF and compared to the vehicle DMSO (mean ± SEM, n = 6 replicate wells).

(C) MTT cell viability assay 3 days after passage of β-NF and vehicle (DMSO) treated organoids (mean ± SEM, n = 3 mice).
Figure 3.6: VillinCre\textsuperscript{ERT2};Fzd\textsuperscript{fl/fl} intestinal organoids

(A) Representative differential interference contrast (DIC) images of VillinCre\textsuperscript{ERT2};Fzd\textsuperscript{fl/fl} intestinal organoids at Day 5. Organoids were treated with either vehicle (ethanol - EtOH) or 4-hydroxytamoxifen (4-OHT).

Arrows: crypt atrophy; #: organoid death.

(B) MTT cell viability assay of time-course from Day 0, 3 and 5 of organoids induced with 4-OHT and compared to the vehicle EtOH (mean ± SEM, *p < 0.05, n = 6 replicate wells).

(C) MTT cell viability assay 5 days after induction with 4-OHT and compared to vehicle (EtOH) treated organoids (mean ± SEM, *p < 0.05, pooled data of organoids prepared from two mice).
A

VillinCre;Fzd7^fl/fl

EtOH

4-OHT

B

Cell viability (MTT OD)

Time (days)

EtOH

4-OHT

C

Cell viability (MTT OD)

EtOH

4-OHT
Figure 3.7: AhCre;Fzd5<sup>fl/fl</sup> intestinal organoids

(A) Representative differential interference contrast (DIC) images of AhCre;Fz<sup>d<sub>5</sub>/fl</sup> intestinal organoids at Day 0, 3 and 5. Organoids were treated with either vehicle (DMSO) or β-NF. Scale bar represents 100µm.

(B) MTT cell viability assay 3 days after passage of β-NF and vehicle (DMSO) treated organoids (mean ± SEM, *p < 0.05, n = 3 mice).
A

AhCre^+;Fzd5^fl/fl

Day 0
Day 3
Day 5

DMSO

β-NF

B

Cell viability (MTT OD)

DMSO

β-NF
Deletion of *Fzd7* results in a loss of CBC stem cells

Next, the aim was to visualize the loss of CBC stem cells. To do this crypt organoid cultures from *AhCre;Fzd7*\(^{fl/fl}\) mice were grown in 8-chamber slides. As with the previous experiments, they were either treated with β-NF to induce *Fzd7* gene deletion or with the control DMSO. Two days after induction, the organoids were fixed for immunofluorescence staining. The *Fzd7* and *Lgr5* antibodies are not reliable enough to adequately detect the loss of *Fzd7* positive and *Lgr5* positive cells. That is, no staining does not necessarily mean that the cells have been depleted. Thus, the organoids were stained with lysozyme to detect Paneth cells, E-cadherin to visualize the individual cells and DAPI for the nuclei. Fortuitously the staining for E-cadherin creates a distinct outline of the stems cells, as seen in the DMSO treated organoids (Figure 3.8 Bottom row), as the CBC express E-cadherin baso-laterally. In contrast, Paneth cells only express E-cadherin at apical junctions. In the *Fzd7* deleted organoids, the CBC cells are absent as there are no slim cells with baso-lateral staining (Figure 3.8 Top row) and only apical localized E-cadherin staining is seen (i.e. Paneth cells). Furthermore, the smallish blue nuclei of the stem cells are also not visible in-between the large Paneth cell nuclei. The observed loss of stem cells after the deletion of *Fzd7* verifies that *Fzd7* is indeed a necessary Wnt receptor for the CBC stem cells.

**Figure 3.8: Loss of Lgr5\(^+\) cells in AhCre;Fzd7\(^{fl/fl}\) induced organoids**

(Top row) Immunofluorescence analysis of intestinal organoids from AhCre;Fzd7\(^{fl/fl}\) mice treated with DMSO. Blue: nuclei - DAPI; Green: E-Cadherin; Red: Lysozyme; Scale bar represents 50µm.

(Bottom row) Immunofluorescence analysis of intestinal of organoids from AhCre;Fzd7\(^{fl/fl}\) mice treated with β-NF (induced gene deletion of Fzd7). Blue: nuclei - DAPI; Green: E-Cadherin; Red: Lysozyme; Scale bar represents 50µm.
Nuclei/E-Cadherin/Lysozyme
3.4 Discussion

The Wnt/β-catenin pathway has been indicated as the single most dominant force in controlling both homeostasis and cell fate along the crypt-villus axis in the intestinal epithelium. In this study we sought to further investigate the functionality of the Frizzled receptors during intestinal homeostasis. Focusing on Frizzled-7 (Fzd7), we verified a functional role for it as a Wnt receptor in vitro in Lgr5+ CBC stem cells. Using conditional gene deletion, we illustrated that intestinal organoid cultures either suffered from crypt atrophy or died after the loss of Fzd7.

The intestinal epithelium undergoes a remarkably rapid and continuous self-renewal. This renewal is mediated by stem cells, over which the Wnt/β-catenin pathway has a critical regulatory role (Fevr et al., 2007; Korinek et al., 1997a; Kuhnert et al., 2004; Pinto et al., 2003). The stem cells give rise to proliferative precursor cells, which go on to terminally differentiate. Wnt signalling is essential for intestinal homeostasis, the fine balance between cell proliferation and cell differentiation (Fevr et al., 2007; Pinto et al., 2003). Wnt signalling is turned “on”/found in high levels in the crypts where the stem cells and proliferating, undifferentiated precursor cells are, and “off”/found at low levels in terminally differentiated cells of the villus. This was demonstrated by studies looking at both the constitutive activation of the Wnt signalling pathway via genetic alterations of the Wnt components APC and CTNNB1 (the gene encoding β-catenin) (Sansom et al., 2004; van de Wetering et al., 2002) as well as the blockage of Wnt signals through either the deletion of TCF-4 (Korinek et al., 1998) or the over-expression of Dickkopf 1 (Dkk-1) an antagonist of Wnt signalling (Kuhnert et al., 2004).

A key component for activating Wnt signalling is the binding of Wnts to Frizzled receptors. Frizzled (Fzd) receptors, of which there are 10 in mammals, are seven-span transmembrane proteins. Of the 10 Fzds, Fzd7 is often found to be up regulated in stem cells as well as cancers from various tissues (Fernandez et al., 2014; Flanagan et al., 2015; King et al., 2012; Vincan et al., 2005; Willert et al., 2002). Furthermore, cell fractionation (Mariadason et al., 2005) and in situ mRNA expression (Gregorieff et al., 2005) studies have shown expression of Fzd7 to be confined to cells situated at the base of the crypt, where the stem cells are. Our studies in vivo demonstrated, via conditional gene deletion, that Fzd7 is responsible for transmitting the critical Wnt
signal in the intestinal CBC stem cells. Thus, revealing an importance for Fzd7 in intestinal homeostasis. Following the deletion of Fzd7 a reduction in Lgr5+ CBC stem cells as well as a simultaneous reduction in cell proliferation was observed. We also observed that one day post Fzd7 deletion, recombined stem cells were lost from the epithelium. This repopulation of the intestinal epithelium with non-recombined cells to replace the Fzd7 deficient ones reinstated homeostasis (Flanagan et al., 2015). Rapid repopulation after a deleterious insult to the CBC stem cells is not uncommon, as other genes that are essential to stem cells, also cause rapid repopulation once the targeted gene is deleted (Muncan et al., 2006; van der Flier et al., 2009b). Also, multiple studies have found that stromal cells play a significant role in supplying the stem cell niche with essential growth factors (Kabiri et al., 2014; Pinchuk et al., 2010; Shaker and Rubin, 2010) hence repopulation in vivo is very efficient. Thus, as Fzd7 deletion appears to lead to the loss of CBC stem cells, i.e. triggering rapid repopulation, it follows that Fzd7 is necessary for stem cells. At least two mechanisms have been proposed to explain the rapid repopulation. Dedifferentiation of partially committed secretory progenitor cells to regain stemness (Basak et al., 2014; Buczacki et al., 2013; van Es et al., 2012) or the triggering of mature Paneth cells to acquire stem cell-like properties in situations of damage and as such contribute to the regenerative response (Roth et al., 2012). Either mechanism is consistent with stem cell loss. In contrast to the in vivo experiments, in the current study we found that intestinal organoid cultures do not regenerate after the loss of Fzd7, indicating that “repopulation” does not occur in vitro, which made it possible to document the outcome of deleting Fzd7.

3D intestinal organoid cultures are the newest tool employed in stem cell research, disease modelling and regenerative medicine, as they recapitulate the epithelial architecture and retain hallmarks of the in vivo epithelium. Indeed, the above-mentioned mechanisms were studied using organoids (Basak et al., 2014; Buczacki et al., 2013; Roth et al., 2012; van Es et al., 2012). The organoids also allow for the study of cell fate determination as they recapitulate the complete stem cell differentiation hierarchy. In fact, studies focused on the Notch signalling pathway, show that inhibition in vitro (Koo et al., 2012; VanDussen et al., 2012), also causes proliferative cells to turn into goblet cells, as is exhibited in vivo (van Es et al., 2005b). Employing
the organoids in our study also allowed us to demonstrate that Fzd7 is a Wnt receptor for the Lgr5+ CBC stem cells, as inferred by our in vivo studies (Flanagan et al., 2015). Notably deletion of another Fzd gene, Fzd5, did not have the same effect as deleting Fzd7. Also, β-NF treatment alone, i.e. inducing Cre recombinase activity, did not affect the organoids.

3.5 Conclusions

The findings in this study support the previous in vivo work in our lab and together demonstrate that Fzd7 plays a critical role in Wnt/β-catenin signalling during homeostasis and is a Wnt receptor for the Lgr5+ CBC stem cells. Deleting Fzd7 in vitro in the intestinal organoid cultures enabled us to further investigate the effect of its loss on the cells when support from the surrounding tissue is absent. The death of some organoids and lack of regeneration after passaging supported our previous findings, that Fzd7 is required during homeostasis in the intestinal epithelium. Furthermore, the concomitant loss of Lgr5 and Ascl2, in addition to the inability of the organoids to repopulate following Fzd7 deletion, further confirms that Fzd7 is indeed active in the Lgr5+ CBC stem cells and that the deleterious effect it has on the organoids is detrimental and that the fail-safe mechanisms, which come into play in vivo, involving secretory progenitor or Paneth cells becoming stem cells, does not occur in vitro. Notably, it was possible to rescue the organoids, by activating the pathway downstream of Fzd7 via GSK3-β inhibition with CHIR 99021 (Bennett et al., 2002) or lithium chloride (LiCl) (Klein and Melton, 1996), indicating that there wasn’t anything inherently wrong with the organoids (Flanagan et al., 2015).

Importantly, the results from the current study presented in this chapter conclusively show that Fzd7 deletion leads to loss of CBC stem cells in the crypt. Together with the findings from other work conducted in our lab, these support our hypothesis that Fzd7 is necessary for homeostasis and regenerative processes in the intestinal epithelium.
Chapter 4

Frizzled-7 function in intestinal adenomas and cancer stem cells
4.1 Introduction

A hallmark of CRC is the aberrant activation of the Wnt/β-catenin signalling pathway (also referred to as canonical Wnt signalling). In brief, upon Wnt signalling activation, via binding of a Wnt ligand to its receptor complex composed of Frizzled (Fzd) and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), a series of events is triggered that disrupts a cytoplasmic multi-component destruction complex. This destruction complex, compromised of adenomatous polyposis coli (APC), Axin, glycogen synthase kinase-3 alpha/beta (GSK3) and casein kinase-1 (CK1), is required for the targeted degradation of the transcriptional activator β-catenin. Inhibition of the destruction complex allows for the stabilization, accumulation and nuclear translocation of β-catenin, where it engages the N terminus of DNA-binding proteins of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, regulating the transcription of Wnt/β-catenin target genes (e.g. c-Myc, Cyclin D1 (CCDN1), CD44, Sox9) (Clevers and Nusse, 2012). Aberrant activation of the Wnt/β-catenin signalling pathway is initiated by mutations in the APC gene, whose inactivation induces stabilization of β-catenin and a constitutively active Wnt/β-catenin pathway (Kinzler et al., 1991b; Kinzler and Vogelstein, 1996). A consequence of hyperactive gene transcription is for example c-Myc, whose product is vital in driving malignancy in both human and mouse (Albuquerque et al., 2011; Fearon, 2011).

In 80-90% of CRCs, inactivating mutations of the tumour-suppressor gene, APC, a negative regulator of Wnt/β-catenin signalling, are present and proposed as the initiating step of CRC, which is often called the ‘classical’ route (Morin et al., 1997a). The inactivating mutations generally cluster in one region of the APC gene, called the mutation cluster region (MCR), between codons 1286 and 1513. These mutations lead to expression of a truncated APC protein that lacks the C-terminal half (Miyoshi et al., 1992). Inherited loss of APC function, due to germline mutations in the APC gene, is the cause of familial adenomatous polyposis (FAP), a human autosomal dominant syndrome, in which patients develop numerous colorectal polyps (Groden et al., 1991; Rustgi, 2007), which, if not removed, can progress to colorectal carcinomas following concomitant activating mutations in KRAS and inactivating mutations in TP53 (Korinek et al., 1997a; Morin et al., 1997a). FAP patients inherit one mutated APC allele and the
second allele is spontaneously inactivated due to somatic mutations of the *APC* gene, which leads to the formation of masses of adenomas. Thus in adenoma tissues of FAP patients both alleles of *APC* are mutated yielding a truncated APC protein. However FAP patients only account for less than 1% of all CRCs, with the majority of CRCs being sporadic. In the remaining CRC patients with truncated APC protein, *APC* is mutated spontaneously, with both alleles made inactive. Therefore both inherited and sporadic CRCs have both alleles mutated and yield truncated APC proteins. Of the remaining 10-20% of CRCs with intact *APC* genes, many are associated with either oncogenic mutations of CTNNB1 (gene that codes for β-catenin) (Morin et al., 1997a), mutations of the negative Wnt/β-catenin signalling regulator *Axin2* (Liu et al., 2000) or with mutations of DNA mismatch repair (MMR) genes (Scherer et al., 2005; Wei et al., 2002).

As the Wnt/β-catenin signalling pathway is constitutively active via genetic mutation, it was thought that there would be no additional role for the upstream components of the pathway (i.e. at the plasma membrane). Contrary to the belief that pathway modulation upstream of the destruction complex is no longer possible or necessary, it has been shown that extracellular secreted Wnt antagonists (e.g. sFRP and Dkk) are frequently epigenetically silenced via promoter hypermethylation (Aguilera et al., 2006; Caldwell et al., 2004; Suzuki et al., 2004). It has been established, that aberrant hypermethylation of gene promoters takes place during the transition from normal epithelium to adenoma and can occur before *APC* mutation (Suzuki et al., 2004), which implies a role for Wnt and Fzd at adenoma initiation, and continue to accumulate during the transition from adenoma to carcinoma. Additionally, inhibition of GSK3, can upregulate TCF/β-catenin, despite mutation to *APC* (Vincan et al., 2007b). Furthermore, the restoration of sFRP function in CRC cells, even in the presence of downstream Wnt pathway mutations, attenuates Wnt signalling (Caldwell et al., 2004; Suzuki et al., 2004). Moreover, other naturally occurring inhibitors, Wnt inhibitory factor-1 (WIF1) (Taniguchi et al., 2005) and Dickkopf-1 (DKK-1) (Aguilera et al., 2006) are epigenetically inactivated in CRCs. This continued deregulation of Wnt antagonists, which act by inhibiting the binding of Wnt to the receptor complex Fzd and LRPS/6, suggests a role for Wnt(s) and Fzd(s). Indeed, Wnt ligands (Dimitriadis et al., 2001) and
Fzd receptors are commonly upregulated in CRCs and considered to be a contributing factor (Ueno et al., 2013; Vincan et al., 2007b). Thus research into the functional relevance of Fzd receptors has also been of interest.

There are 10 mammalian Frizzled family members, which are grouped into subclasses based on shared sequence and homology: Fzd1, Fzd2 and Fzd7; Fzd3 and Fzd6; Fzd5 and Fzd8; and Fzd4 with Fzd9 and Fzd10 (Sagara et al., 1998; Yu et al., 2012). It was originally thought that Fzds only function in either the canonical pathway (Wnt/β-catenin) or the non-canonical pathway (PCP and Wnt/Ca^2+), however it is now generally accepted that Fzd signalling (canonical or non-canonical) is primarily determined by the spatial and temporal ligand-receptor expression profile. Commonly over-expressed Fzd receptors in CRCs with elevated Wnt activity are Fzd3, Fzd6 and Fzd7 (Ueno et al., 2008; Vincan et al., 2007b). Others and we have shown that Fzd7 is commonly upregulated in colon, gastric and hepatocellular carcinomas and is also associated with poor prognosis and survival (Merle et al., 2004; Merle et al., 2005; Schmuck et al., 2011; Vincan et al., 2007b; Zhao et al., 2014). Downregulating Fzd7 was shown to reduce cell viability, cell migration and cell invasion within various cancer types both *in vivo* and *in vitro* (Merle et al., 2004; Merle et al., 2005; Ueno et al., 2009; Ueno et al., 2008; Vincan et al., 2007b). In addition, Fzd7 is one of the few Fzds capable of transmitting both canonical and non-canonical Wnt signals. Both of which, have been demonstrated to play critical roles during tumourigenesis and tumour progression (Asad et al., 2014; Ueno et al., 2009; Vincan et al., 2007b).

As discussed in the previous Chapter, the Wnt/β-catenin signalling pathway plays a critical role in intestinal homeostasis and as demonstrated above, it is one of the most affected pathways in CRCs and plays a pivotal role in their initiation, which occurs due to the disruption of epithelial differentiation and stem cell homeostasis. The formation of adenomatous polyps and benign adenomas, as a result of *APC* mutations, is a consequence of the afore-mentioned nuclear localization of β-catenin and the ensuing abnormal expansion of the transformed stem cell compartment. In a role similar to that of the CBC stem cells in the normal intestinal epithelium, cancer stem cells (CSC) are suggested to give rise to progenitor cells that populate the majority of the tumour (Gupta et al., 2009). Two models describing the pathogenesis of CRC have been
proposed: the ‘top-down’ and the ‘bottom-up’ morphogenesis (Preston et al., 2003; Schwitalla et al., 2013; Shih et al., 2001). Wnt signalling is considered an important regulator in both models. The ‘top-down’ model is based on the discovery, via tissue autoradiograph following intravenous administration of radioactive (tritiated) thymidine to a FAP patient, of very immature adenomatous polyps at the top of colonic crypts, far removed from the base where the stem cell niche is located (Cole and McKalen, 1963). The ‘top-down’ model proposes, that hyperactivation of the Wnt/β-catenin signalling pathway drives the more differentiated epithelial cells into re-acquiring stem cell-like properties, thereby forming new, dis-regulated crypt-like structures that later develop into adenomas. Using transgenic mouse models it has been shown that elevated nuclear factor-κB (NF-κB) signalling enhances Wnt/β-catenin signalling activation, thereby inducing dedifferentiation of epithelial cells that acquire tumour-initiating cell properties (Schwitalla et al., 2013).

On the other hand, the ‘bottom-up’ model was proposed based on immunohistochemical studies of early sporadic colorectal adenomas, from patients with either FAP or sporadic CRC, showing adenomatous lesions near the crypt base. The adenomatous lesions showed increased proliferative activity together with nuclear β-catenin while the corresponding surface epithelium maintained β-catenin at the sub-membrane adherens junctions (Preston et al., 2003). Thus suggesting that the stem cells reside at the base of the crypt, from where they expand and migrate upwards and constitute the tumour-initiating cells. Further supporting the ‘bottom-up’ model are the recent observations that CRCs potentially derive from stem cells expressing Lgr5 or Bmi1 (Barker et al., 2009; Sangiorgi and Capecchi, 2008; Schwitalla et al., 2013), the same genes shown to mark CBC stem cells (Barker et al., 2007; Sangiorgi and Capecchi, 2008). Using mouse models, it was demonstrated that the conditional deletion of APC in Lgr5⁺ CBC stem cells induced their rapid transformation into micro-adenomas, indicating that increased Wnt/β-catenin signalling in the Lgr5⁺ stem cell compartment may trigger a tumour-initiating process (Barker et al., 2009) (Figure 3.1). Similarly, generation of adenomas in the duodenum was shown, following the conditional activation of β-catenin in Bmi1⁺ label-retaining cells (Sangiorgi and Capecchi, 2008).
These studies suggest that the excessive Wnt/β-catenin pathway activation in the stem cell compartment is an essential step in neoplastic transformation.

Mouse models of colorectal cancer provide an excellent in vivo system to recapitulate the human disease and are powerful tools in helping to understand its biology and test therapies. In this study the APC\textsuperscript{min} mouse was employed (Figure 4.1). The multiple intestinal neoplasia (Min) mouse was identified in 1990 during a mutagenesis project in which germline mutations were induced following treatment with ethylnitrosourea (ENU) (Moser et al., 1990). It was later established that the APC gene had a truncating mutation at codon 850, which was responsible for the phenotype, which resembles that of human FAP (Su et al., 1992). During adulthood, spontaneous somatic mutations to the second APC allele occur and the mice develop intestinal adenomas (Moser et al., 1990). Notably, a major difference between the mouse model and the human disease is that FAP patients primarily develop colonic lesions, whereas the mice develop polyps in the small intestine. The reason for this is unclear and most likely to be multifaceted. The anatomy of the mouse and human large intestine are quite different; the mouse colon is much shorter, rather smooth and has no division, whereas in the human it is clearly divided into ascending, transverse and descending colon (Nguyen et al., 2015). Furthermore, the composition of microbiota varies significantly as 85% of bacteria found in the mouse gut are not present in human (Ley et al., 2005). Additionally, the mouse jejunum, ileum and cecum are derived from the midgut and the colon is generated by the hindgut (Davidson et al., 2015). In contrast, the small intestine, cecum, ascending colon and the proximal two-thirds of the transverse colon in the human is derived from the midgut and only the latter part (the distal one-third) of the transverse and descending colon is from the hindgut (Drake et al., 2014). Another notable difference is that human FAP, if it goes untreated, can progress to invasive carcinomas; however this is rarely seen in mice and no incidence of metastasis has been reported (Boivin et al., 2003). Notably, oncogenic activation of β-catenin also yields adenomas in the small intestine rather than the colon, in mice (Harada et al., 1999). Thus, the genetic mutations that occur in human CRC, when introduced into mice, consistently yield intestinal adenomas. Therefore, the APC\textsuperscript{min} mouse has proven to be an invaluable tool in understanding human CRC initiation.
In this study, the latest “state-of-the-art” adenoma organoid system was established. Adenomas from APC\textsuperscript{min} mice were harvested and cultured as 3D adenoma organoids, thus allowing for \textit{in vitro} studies, which provide an excellent model for studying gene function.

A role for Fzd7 as a Wnt receptor in intestinal CBC stem cells was confirmed in the previous Chapter; however a role for Fzds in cancer stem cells has not been investigated. Here we first demonstrated that both Wnts and Fzds are potentially required for proliferation of adenoma organoids. We then showed that by blocking Fzd7 receptors, adenoma organoid growth is impeded. Furthermore a role for Fzd7 as a Wnt receptor in cancer stem cells is proposed.

\textbf{Figure 4.1 Mouse models of colon cancer}

(Top row) APC\textsuperscript{min} mice harbour a truncating mutation in one \textit{APC} allele. After birth, the wild type allele is lost by somatic mutation, resulting in numerous polyps (arrowhead).

(Bottom row) \textit{APC}^{fl/fl} mice have flox sites flanking exon 14 of the \textit{APC} gene, allowing timed truncation. Stem cell specific truncation with Lgr5-EGFP-Cre results in numerous polyps. Lgr5\textsuperscript{+} (EGFP\textsuperscript{+}) cells persist in the polyps (arrows).
**APC**<sup>min</sup> mouse: *in-vivo* model of colon cancer

APC<sup>min</sup><br>
- APC<sup>850</sup> truncated allele<br>
- somatic loss of WT allele

Numerous polyps (adenomas)

APC<sup>580S/fl;LGR5Cre</sup> mouse: inducible *in-vivo* model of colon cancer

APC<sup>580S/floxed</sup><br>
- APC<sup>580S</sup> floxed allele<br>
- tamoxifen induction

**Figure**: Diagram illustrating the processes in the APC<sup>min</sup> and APC<sup>580S/fl;LGR5Cre</sup> mouse models of colon cancer.
4.2 Materials and Methods

Mice

The AhCre (Ireland et al., 2004), Lgr5^{EGFP-ires-CreERT2} (Barker et al., 2007), Fzd7^{fl/fl} (Flanagan et al., 2015) and APC^{min} (Moser et al., 1990), have all been previously described. The mice were interbred to generate compound mice with appropriate alleles. All mice were co-housed and on an inbred C57BL/6 genetic background, with appropriate littermates used as controls. All animal experiments were approved by the Animal Ethics Committee, of the Office for Research Ethics and Integrity, at The University of Melbourne.

Adenoma organoid culture from APC^{min} adenomas, adapted from Sato et al., 2011

Fragments containing adenomas were disected out from the proximal (~10cm) end of the small intestine and washed with ice cold PBS. The fragments were then incubated in a (2mM EDTA pH 8.0, 0.5mM DTT in PBS) chelating solution for 60min on ice. After two washes in chelation buffer to remove the epithelial cells from the fragments, the adenomas were then incubated in digestion buffer (75U/mL collagenase (CLS-4 Worthington, #LS004188) and 125μg/mL dispase (GIBCO, #17105-041) in DMEM with 2.5% FCS and 1% PenStrep) for 30min at 37°C and vortexed ever 10min. Following the incubation, 10mL PBS was added and the fragments were pipetted up and down firmly ensuring detachment of the adenoma digest from the remaining intestinal fragments. The supernatant with the adenoma cells was transferred to a new tube and pelleted down for 3min at 200g. The pellet was washed in PBS, spun down again (3min at 200g) and then resuspended in ice-cold Matrigel (Corning #356231) (500 cells/mL) and plated at 50μL per well onto a pre-warmed (at 37°C) 24-well tissue culture plate (Costar #3526). Once the Matrigel had set, 500μL of ADF supplemented with N2 (1:100, Invitrogen #17502-048) and B27 (1:50, Invitrogen #12587-010) (ADF intestine culture medium), containing the growth factor EGF (50ng/ml, Peprotech #315-09) was added to the wells. EGF was added every other day, with a whole culture medium change once per week. Cultures were passaged (mechanically) and split after approximately 7-10 days.
Adenoma organoids were passaged by mechanical disruption: Matrigel and organoids were scraped from the tissue culture plate and together with the medium, they were transferred to a falcon tube and diluted with fresh ADF. After dissociation using a p1000, the adenoma organoid-Matrigel mix was centrifuged at 600rpm/76rcf for 2min. The supernatant was discarded, ADF was added and the wash step was repeated two more times. The washes progressively removed the cell debris, dead cells and Matrigel. The final clean pellet was resuspended in ADF, counted and resuspended at 500 adenoma organoids per microliter of Matrigel and plated as above in a new 24-well plate.

**β-naphthoflavone administration**

Cre-recombinase was activated in vitro in crypt organoid cultures by treating with 360nM β-naphthoflavone (β-NF), made up in DMSO, for 48hr at 37°C (Flanagan et al., 2015).

**Tamoxifen administration**

Cre-recombinase was activated in vitro in crypt organoid cultures by treating with 500nM 4-hydroxytamoxifen (4-OHT), made up in 100% EtOH, for 48hr at 37°C (Flanagan et al., 2015).

**Imaging organoid cultures**

Differential interference contrast (DIC) images of organoid cultures were taken on a Nikon Ti-E microscope with a 2× Pan Apo Lambda NA 0.1 objective, a 4× Plan Fluor NA 0.13 objective or a 10× Plan Fluor NA 0.3 objective. A focal stack of images was collected 10µm apart and processed through the “Best Focus” function of MetaMorph v7.7.7 (Molecular Devices) to generate the final image of individual organoids.

**RNA extraction and cDNA synthesis**

Total RNA extraction and cDNA synthesis was performed as previously described (Vincan et al., 2007b). Briefly, following treatment, the organoids were handled as when passaging, except the clean pellets were resuspended and homogenised in TRIzol (Invitrogen #15596-018) and incubated for 30mins at room temperature. Phenol:Chloroform:isoamylalcohol was added to the homogenised samples and mixed.
well. After 5mins at room temperature, the samples were centrifuged (10,000rpm) for 15mins to separate the upper clear aqueous phase (nucleic acid containing fraction) from the lower pink organic phase (protein containing fraction). The clear aqueous phase was removed and mixed with an equal volume of 70% Ethanol. The RNA samples were purified and DNAse treated using RNeasy mini-prep columns (Qiagen #74104) following the manufacturers’ instructions. RNA samples were then quantified using a DNA/RNA nanodrop spectrophotometer (Thermo Scientific Nanodrop 1000). cDNA was synthesized with 4µg of each RNA sample. Samples were reverse transcribed using anchored oligodT primers (Promega) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega #M1705), in a final volume of 100µL.

**RNA analysis**

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the 2x SYBR green PCR master mix (Invitrogen #4309155) and ABI PRISM 7500 Sequence Detector (Applied Biosystems) as previously described (Vincan et al., 2007b). In brief, final volumes of 20µl PCR amplification mixture (2x SYBR green I Master Mix buffer (10µL), sterile water (7µL), template cDNA (1µL) and forward and reverse primer mix (2µL)) were set up in triplicate wells of a 96-well tray (Applied Biosystems MicroAmp #4346907). The cycling conditions compromised 2min at 50°C, 10min polymerase activation at 95°C, 40 two-step cycles of 15sec at 95°C and of 1min at 60°C and a dissociation stage of 15sec at 95°C, 1min at 60°C, 15sec at 95°C and 15sec at 60°C. The housekeeping gene β-2-Microglobulin (β2M) primers were used to equalize the data; with threshold cycle (CT) values, the 2^-ΔΔCT method (Vincan et al., 2007b) was used to calculate the fold change. For primer sequences see appendix Table1.

**MTT assay**

After treatment, the adenoma organoids were harvested, as for passaging (mentioned above), and resuspended in 150µL Matrigel. This was then dispensed to 6 replicate wells at 16µL per well of a 96-well plate (Costar #3599) (with 100µL of adenoma culture medium and EGF) and 50µL in one 24-well plate well. Adenoma organoids were then incubated for 4hrs with MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma #M2128)
at 37°C. The adenoma culture medium was removed from the organoids and replaced with lysis buffer (50% DMF, SDS, acetic acid + 2.5% 1M HCl) and incubated overnight at 37°C. The optical density (OD) was determined using the BMG Lumistar plate reader (Hansen et al., 1989).

**Genomic DNA extraction**

Genomic DNA (gDNA) was extracted from adenoma organoid pellets, washed free of cell debris and Matrigel, using standard techniques. Briefly, the organoid pellets were digested in Proteinase K lysis buffer (containing: 1M Tris-HCl pH8.5-9.0; 5M NaCl; 10% SDS; 0.5M EDTA and milliQ water with freshly added Proteinase K (final 500µg/mL; Sigma #P-4914)) overnight. The DNA was extracted using Isopropanol to precipitate the gDNA. The DNA was pelleted for 10min at 13,000rpm. The supernatant was then discarded and the pellet washed in 70% Ethanol. A final 1min spin, to help pipette off all alcohol, then the pellets were air dried briefly, followed by the addition of TE pH8.0 (Tris/EDTA: 1M Tris pH8.0; 0.5M EDTA and milliQ water) to resuspend the DNA.

**DNA detection**

Genomic DNA was subjected to PCR to detect the transgene (Fzd7floxed MUT) and Cre recombinase (AhCre or Lgr5CreERT2). To detect the recombined/deleted product (DP), PCR primers that are either side of the transgene and only work once the gene is excised, were used to amplify the product. All PCRs were run on Veriti PCR machines (for cycling conditions see appendix Table2). The products were then run on a 1.5% (w/v) agarose gel prepared with Tris/Acetic acid/EDTA (TAE) buffer, stained with 15µg/mL ethidium bromide (EthBr) and visualized under UV trans-illumination. The DP PCR product was sequenced using standard ABI protocols, and the sequence confirmed by Prof. Nick Barker (Singapore), who generated the Fzd7floxed mouse for our laboratory, to be the expected product created by recombination of the Fzd7floxed alleles.

**Statistical analysis**

Data are expressed as mean ± SEM, where mean represents number of mice (three or more per genotype) or number of experiments (three or more). Adenoma organoids were established from three or more hosts per group, unless otherwise stated. The
statistical Student’s t test was used with Prism6 (GraphPad software) where $P$ values of $\leq 0.05$ were considered significant.
4.3 Results

**APC\textsuperscript{min} adenomas display high Wnt/β-catenin signalling**

It has been well documented that Frizzleds (Fzd) are over-expressed in colorectal cancer (CRC) cell lines (Ueno et al., 2008; Vincan et al., 2007b). Notably, Fzd7 is the predominant Fzd gene in the CRC cell lines tested. All the CRC cell lines harbour APC or β-catenin mutations and thus have active Wnt signalling. Fzd7 was shown to be a Wnt/β-catenin target gene (Vincan et al., 2010; Willert et al., 2002). As detailed in Chapter 2, Fzd7 is a critical stem cell Wnt receptor; and CRC starts in the stem cells. Thus it is important to determine if Fzd7 functions in adenomas. The aim here was to determine the expression profile of Fzd7 and the other two Fzds in the Fzd7 subclass, namely Fzd1 and Fzd2.

To assess the relative expression level of the Fzd7 subclass of the Fzd gene family members 1, 2 & 7 and Wnt target genes in APC\textsuperscript{min} adenomas compared to normal small intestinal epithelium, RNA was extracted and examined by qRT-PCR. Adenomas were harvested from the proximal end of the small intestine of aged APC\textsuperscript{min} mice (≥150 days) as well as normal proximal small intestinal epithelium of aged C57BL/6 mice (≥150 days), as the APC\textsuperscript{min} mice are on a C57BL/6 background. Compared to the normal epithelium, the APC\textsuperscript{min} adenomas displayed significantly higher expression levels of the three Frizzleds tested. Fzd7 expression was increased >150 fold, while Fzd2 and Fzd1 were increased >100 and 15 fold respectively (Figure 4.2 A). In addition, the levels of intestinal stem cell markers (Lgr5, Ascl2 and Sox9) and the well-characterised Wnt target gene transcripts c-Myc, Cyclin D1 (CCND1), Cyclin D2 (CCND2) (cell proliferation), and CD44 (differentiation), which have all been shown to be highly expressed in CRCs with truncated APC (Cole et al., 2010; Lu et al., 2008; Sansom et al., 2004; Ziskin et al., 2013), displayed very high expression levels compared to the normal epithelium (Figure 4.2 B). Thus Wnt signalling is very high in the adenoma tissue compared to the normal epithelium and all three Fzds in the Fzd7 subclass are highly expressed.

To investigate the function of Fzd7 in adenoma growth, adenoma organoids were established in vitro from the APC\textsuperscript{min} adenomas. This allowed easy access to the adenoma tissue compared to in vivo. Adenoma organoid cultures were established
from cells isolated from adenomas, which were harvested from the proximal small intestine of $APC^{\text{min}}$ mice. The isolated cells when grown in Matrigel with medium containing the growth factor EGF (epidermal growth factor), form spheroid shaped organoids, which differ from the normal small intestinal ‘mini-gut’ organoids (Figure 4.2 C).

**Figure 4.2: $APC^{\text{min}}$ adenomas display high Wnt/β-catenin signalling**

(A) Frizzled gene expression levels determined by qRT-PCR and normalized to β2M. Adenomas were harvested from aged mice (≥ 150 days) and compared to the normal small intestinal epithelium of C57BL/6 aged mice (≥ 150 days) (mean ± SEM, *p < 0.05, n = ≥ 3 mice).

(B) Intestinal stem cell markers and Wnt target genes, gene expression levels determined by qRT-PCR and normalized to β2M. Adenomas were harvested from aged mice (≥ 150 days) and compared to the normal small intestinal epithelium of C57BL/6 aged mice (≥ 150 days) (mean ± SEM, *p < 0.05, n = ≥ 3 mice).

(C) Representative differential interference contrast (DIC) images of C57BL/6 intestinal organoids (left) and $APC^{\text{min}}$ adenoma organoids (right).

Scale bar represents 100µm.
A

![Graph A]

B

![Graph B]

C

Normal small intestinal organoids

APC<sup>min</sup> adenoma organoids
Blocking Wnt secretion via Porc inhibitors affects cell viability

Employing the Porc inhibitor IWP2, which blocks all Wnt secretion, we looked to see whether disrupting the Wnt/β-catenin signalling pathway upstream of the already constitutively active pathway would have any effects on the adenoma organoids. Initial experiments on adenoma organoid cultures from four mice, suggested that by treating the adenoma organoids with IWP2 or the vehicle DMSO as a control, a significant decrease in cell viability took place, although visually no changes in cell morphology were noted (data not shown). The adenoma organoids were thus treated and co-treated with the CHIR99021 compound to establish whether it was possible to restore cell viability to levels comparable to the control. Adenoma organoids were set up in replicate wells of a 24-well plate for DIC imaging (Figure 4.3 A) and treated with the vehicle DMSO, the Porc inhibitor (IWP2), the GSK3β inhibitor CHIR99021 or both IWP2 and CHIR99021. Concentrations for both IWP2 and CHIR were 5µM. Over a five-day period, the adenoma organoids were treated on Days 0, 2 and 4 and imaged on Day 0 before treatment, Day 3 and 5. As with the initial experiment, no visual changes between the four treatments were noticeable. On Day 5 the organoids were harvested for mRNA and MTT assay. For the MTT assay, the adenoma organoids were plated into a 96-well plate and then MTT was performed. Again the MTT assay shows a significant decline in cell viability after treatment with IWP2 and this was significantly increased, i.e. rescued, when CHIR99021 was added to activate the pathway below the receptor (Figure 4.3 B). Interestingly CHIR99021 alone also decreased in cell viability compared to the vehicle, indicating that too much β-catenin/Tcf signalling leads to decreased growth, which is known (Kim et al., 2000; Meniel et al., 2013; Murphy et al., 2008). Collectively, these results imply a small but reproducible effect on Wnt signalling despite an already active β-catenin/Tcf pathway.
Figure 4.3: Blocking Wnt secretion via PORC inhibitors affects cell viability

(A) Representative differential interference contrast (DIC) images of APC\textsubscript{min} adenoma organoids at Day 0 and 5. Organoids were treated with either vehicle (DMSO), IWP2, CHIR99021 or co-treated with IWP2 and CHIR99021.

Scale bar represents 100µm.

(B) MTT cell viability assay from Day 5 of organoids treated with either IWP2 or CHIR99021 and compared with the vehicle (DMSO) and co-treatment with IWP2 and CHIR99021 (mean ± SEM, *p < 0.05, n = 6 replicate wells).
A

Day 0 Day 5

DMSO

IWP2

CHIR

IWP2+CHIR

B

Cell viability (MTT OD)

DMSO IWP2 CHIR IWP2+CHIR

* *
Using Fzd7 antibodies to block Fzd7 receptors

The Fzd7 antibody OMP-18R5, also known as Vantictumab, is one of the furthest developed biological agents to inhibit Wnt binding to Fzd receptors. It was initially isolated based on its ability to bind to Fzd7. Further investigation found, that it binds 5 of the 10 Fzd receptors (Fzd1, Fzd2, Fzd5, Fzd7 and Fzd8). It blocks Wnt/β-catenin signalling induced by multiple Wnt family members and is active as a single-agent across a range of human tumours, CRCs included (Gurney et al., 2012).

Adenoma organoids were initially treated with 50nM of Fzd7Ab OMP-18R5 (Gurney et al., 2012), which had no effect on the organoids (data not shown). Other experiments in the lab also using the antibody, demonstrated that by increasing the concentration to 100nM, gastric tumour growth was inhibited (Flanagan et al., unpublished). The higher concentration was thus implemented as well as the use of recombinant Wnt3a, to overwhelm the antibody and rescue the organoids from the effects of the antibody. Wnt3 is a known Wnt ligand necessary for Wnt/β-catenin signalling in the intestine and when removed, Wnt signalling activity can be reinstated by the addition of recombinant Wnt3a or Wnt3a conditioned medium (Farin et al., 2012; Sato et al., 2011). A second Fzd7Ab from our collaborator Stephane Angers’ lab (S.A.), which also binds to the same five Fzds, was used. Adenoma organoids were set up in replicate wells of a 24-well plate for DIC imaging (Figure 4.4 A and Figure 4.5 A) and treated with the control (IgG), OMP-18R5, OMP-18R5 with Wnt3a, Fzd7Ab S.A. or Fzd7Ab S.A. with Wnt3a. Concentration for both Fzd7 antibodies and the recombinant Wnt3a was 100nM. Over a five-day period, the adenoma organoids were treated on Days 0, 2 and 4 and imaged on Day 0 before treatment, Day 3 and 5 and then harvested for mRNA. Focusing on the OMP-18R5 antibody first, differences between the three treatments (IgG, OMP-18R5, OMP-18R5+Wnt3a) are visible. Both the IgG and OMP-18R5+Wnt3a treated adenoma organoids look unaffected, however the OMP-18R5 treated organoids appear not to have grown to the same extent as the control and Wnt3a added cultures, and some organoid death was observed. The mRNA levels of both the intestinal stem cell markers and Wnt target genes were examined by qRT-PCR (Figure 4.4 B). All of the genes show significant decrease in expression following treatment with OMP-18R5 and an increase in expression when Wnt3a is added, albeit not quite
to the level of the control IgG. This suggests, that by blocking Fzd7 receptors, some Wnt/β-catenin signalling is inhibited resulting in decreased growth and organoid death. Results using another Fzd7Ab from S.Angers, show significant decreases in mRNA expression levels of the same intestinal stem cell markers and Wnt target genes (Figure 4.5 B), however not as low, ≥ 60% (Fzd7Ab S.A.) compared to ≤ 55% (OMP-18R5), as those from the OMP-18R5 treated adenoma organoids. Furthermore, the co-treatment of Fzd7Ab S.A. with Wnt3a seems to track with the Fzd7Ab S.A. treated organoids and not increase like the organoids co-treated with OMP-18R5 and Wnt3a did. The DIC images reveal no difference in organoid size and there is no evidence of organoid death.

Thus, the two antibodies decrease Wnt/β-catenin signalling, albeit to different degrees. Addition of excess Wnt restores adenoma organoid growth with the Oncomed antibody, but not the S. Angers antibody. This implies that the decrease in signalling with the S. Angers antibody might be due to toxicity rather than actual inhibition of signalling. Furthermore, these results support previous studies that have suggested a role for the Fzd7 receptor in the Wnt/β-catenin signalling pathway in CRC despite an already active pathway via the mutation of APC and confirm that adenoma organoid cultures are a useful model for investigating Fzd7 function further. Moreover, they show Wnt3a has a potential role as the Wnt ligand interacting with Fzd7 to further activate the pathway, which verifies the Co-immunoprecipitation results from Chapter 2 demonstrating an interaction between Wnt3 and Fzd7. These findings are further supported by studies, which have shown a functional interaction between Wnt3 and Fzd7 in hepatocellular carcinoma cells (Kim et al., 2008) and in non-transformed hepatic cells (Nambotin et al., 2012).
Figure 4.4: Using Fzd7 antibody OMP-18R5 to block Fzd7 receptors

(A) Representative differential interference contrast (DIC) images of APC\textsuperscript{min} adenoma organoids at Day 0, 3 and 5. Organoids were treated with either the control IgG, Fzd7Ab OMP-18R5 or co-treated with Fzd7Ab OMP-18R5 and recombinant Wnt3a.

#: organoid death. Scale bar represents 100µm.

(B) Intestinal stem cell markers and Wnt target genes, gene expression levels determined by qRT-PCR and normalized to β2M. Adenoma organoids were harvested on Day 5 after treatment with the Fzd7Ab OMP-18R5 and compared to the IgG treated and Fzd7Ab OMP-18R5 and recombinant Wnt3a co-treated cultures (mean ± SEM, *p < 0.05, n = 3 mice).
Figure 4.5: Using a Fzd7 antibody from S. Angers (S.A.) to block Fzd7 receptors

(A) Representative differential interference contrast (DIC) images of APC<sup>min</sup> adenoma organoids at Day 0, 3 and 5. Organoids were treated with either the control IgG, Fzd7Ab S.A. or co-treated with Fzd7Ab S.A. and recombinant Wnt3a. Scale bar represents 100µm.

(B) Intestinal stem cell markers and Wnt target gene, gene expression levels determined by qRT-PCR and normalized to β2M. Adenoma organoids were harvested on Day 5 after treatment with the Fzd7Ab S.A. and compared to the IgG treated and Fzd7Ab S.A. and recombinant Wnt3a co-treated cultures (mean ± SEM, *p < 0.05, n = 3 mice).
A

<table>
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<th>Day 3</th>
<th>Day 5</th>
</tr>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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<tr>
<td>Fzd7Ab S.A. + Wnt3a</td>
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<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B

- IgG
- Fzd7Ab (S.Angers)
- Fzd7Ab (S. Angers)+Wnt3a

Bar chart showing fold change of various genes: Lgr5, Sox9, Ascl2, c-Myc, Cyclin D1, Cyclin D2, CD44.
Deleting Fzd7 in adenoma organoid stem cells

As the previous Chapter revealed, Fzd7 plays a role as a Wnt receptor for the crypt base columnar stem cells. Having established, from both studies conducted by other labs and our lab that Fzd7 has a role in CRC and having demonstrated that it functions in the adenoma organoid cultures, the next question asked was whether Fzd7 plays a role in LGR5+ cancer stem cells, which have been shown to fuel adenoma growth (Barker et al., 2009; Schepers et al., 2012).

As discussed in the introduction to this Chapter, Barker et al (Barker et al., 2009) and Schepers et al (Schepers et al., 2012) demonstrated that APC mutant Lgr5+ stem cells form adenomas. As these LGR5+ stem cells are enriched in Fzd7, we employed the same stem cell specific Lgr5EGFP-Ires-CreERT2 (Lgr5CreERT2) knockin mice to investigate Fzd7 function in adenomas. The Lgr5CreERT2 mice were crossed with APCmin and Fzd7fl/fl mice, allowing for Cre-mediated recombination (= Fzd7 deletion) in the adenoma organoid cultures. Initial experiments were preformed with adenoma organoid cultures established from cells isolated from adenomas located in the proximal small intestine of the Lgr5CreERT2;Fzd7fl/fl;APCmin mice. Cre-mediated recombination of Fzd7 was induced with 500nM 4-hydroxytamoxifen (4-OHT); as a control, organoids were treated with the vehicle, Ethanol (EtOH). Adenoma organoids were set up in replicate wells of a 24-well plate for DIC imaging (Figure 4.6-A) on Day 0 before treatment, Day 3 and 5. The adenoma organoids were then harvested on Day 5 for total RNA extraction and qRT-PCR, gDNA extraction and MTT assay. The Fzd7 deleted adenoma organoids compared to the controls showed no difference in appearance at Day 5 and the MTT assay confirmed that there was no decline in cell viability (Figure 4.6-B). In addition, qRT-PCRs (Figure 4.6-D) looking at mRNA expression levels to confirm gene deletion of Fzd7 show a noticeable, but not significant reduction in expression. The mRNA levels of the stem cell marker Lgr5 are comparable with the control cultures as well as the expression levels of the Wnt target genes that were assessed. To definitively confirm recombination in the adenoma organoids, since the decrease in mRNA expression of Fzd7 was not significant, PCRs (Figure 4.6-C) were performed on genomic DNA from organoids harvested at the same time as those for the qRT-PCR (five days post induction). The PCR detecting the mutant Fzd7 knockin allele shows a partial excision,
which is due to the recombination being confined to the LGR5\(^+\) cells. PCRs for detecting the deleted product (DP), which is the intact genomic DNA of the recombined product after Fzd7 gene deletion, work only once the Fzd7 gene has been deleted. Detection of the deleted product at 25 cycles is usually more than adequate to detect recombination; however, as there are few Lgr5\(^+\) cells, only low levels of recombination were detected. Thus the PCR was run for an additional 10 cycles (35 cycles in total) and the recombination of Fzd7 in the adenoma organoids treated with 4-OHT was then clearly visible. PCRs for the Lgr5Cre\(^{ERT2}\) transgene and for a region of chromosome 1 which is close to the Fzd7 locus were also run. The first confirms that all organoids were indeed Lgr5Cre\(^{ERT2}\) positive and the second that the quality of the DNA templates between samples was comparable.

Since initial attempts to study the effects of deleting Fzd7 in the Lgr5Cre\(^{ERT2}\);Fzd7\(^{fl/fl}\);APC\(^{min}\) adenoma organoids did not provide any conclusive data, the alternative was to again employ the AhCre, as was done in the previous Chapter. Thus AhCre;Fzd7\(^{fl/fl}\);APC\(^{min}\) mice were utilized. Adenoma organoids harvested from AhCre\(^{+}\);Fzd7\(^{fl/fl}\);APC\(^{min}\) mice were as previously, set up in replicate wells of a 24-well plate for DIC imaging (Figure 4.7-A) on Day 0 before treatment, Day 3 and 5. The adenoma organoids were then harvested on Day 5 for total RNA extraction and qRT-PCR, gDNA extraction and MTT assay. At Day 3 the Fzd7 deleted adenoma organoids do not look as healthy as the DMSO treated ones and it would have been expected that by Day 5 they would be dying or dead. However, this is not the case, instead by Day 5, the organoids have recovered and are again comparable to the control organoids. Indeed, cell viability of the Fzd7 deleted adenoma organoids is equal to that of the DMSO treated ones (Figure 4.7-B). In addition, qRT-PCRs (Figure 4.7-D) looking at mRNA expression levels to confirm gene deletion of Fzd7 instead revealed a significant increase in Fzd7 expression levels as well as an increase in c-Myc which is associated with cell proliferation (Bettess et al., 2005; Sansom et al., 2004) and Sox9, a known stem cell marker (Formeister et al., 2009; Ramalingam et al., 2012) and cell proliferation regulator (Bastide et al., 2007). Again, to confirm recombination in the adenoma organoids, since an increase and not a decrease in mRNA expression of Fzd7 was shown, PCRs (Figure 4.7-C) were performed on genomic DNA from organoids.
harvested at the same time as those for the qRT-PCR (five days post induction). The PCR detecting the mutant Fzd7 knockin allele shows a partial excision, which is due to the recombination not affecting the Paneth cell lineage, as they are not targeted by the AhCre (Ireland et al., 2004). PCRs for detecting the deleted product (DP) show three faint bands after 25 cycles, which confirmed the recombination of Fzd7 in the adenoma organoids treated with β-NF, however at lower levels relative to the recombination in the intestinal organoids (Figure 2.4-A). Following 10 more cycles (35 cycles in total), the bands confirming recombination were more visible. The faint bands detected in the DMSO treated organoids are due to the AhCre being leaky and by running the PCR for 35 cycles, any small amounts of DNA are amplified and as such appear. PCRs for the AhCre transgene and for a region of chromosome 1, confirm that all organoids were indeed AhCre positive and the quality of the DNA templates between samples was comparable.

Control experiments using adenomas from AhCre⁺;Fzd7flo/flo;APCmin mice, since at the time no AhCre⁺;Fzd7⁺/⁺;APCmin mice were available (Figure 4.8-A), confirm that β-NF does not have a deleterious effect on the organoids. Adenoma organoids were set up in replicate wells of a 24-well plate for DIC imaging and treated with either DMSO or β-NF. Both cultures appeared to be doing the same by Day 5 and the MTT assay (Figure 4.8-B) showed no decline in cell viability between the two. The qRT-PCR results (Figure 4.8-C) confirm that mRNA expression levels are comparable.

These observations, suggest, that upon the initial deletion of Fzd7, the adenoma organoids are affected, most likely via cell proliferation inhibition, but rapidly recover. Thus potentially implying a role for Fzd7 in adenoma organoid growth and a strong drive to restore Fzd7 expression to maintain growth.
Figure 4.6: Lgr5Cre<sup>ERT2</sup>;Fzd7<sup>fl/fl</sup>;APC<sup>min</sup> adenoma organoids

(A) Representative differential interference contrast (DIC) images of Lgr5Cre<sup>ERT2</sup>;Fzd7<sup>fl/fl</sup>;APC<sup>min</sup> adenoma organoids at Day 0 and 5. Organoids were treated with either the control EtOH or 4-OHT. Scale bar represents 100µm.

(B) MTT cell viability assay 5 days after induction with 4-OHT and compared to vehicle (EtOH) treated adenoma organoids (mean ± SEM, n = 6 replicate wells).

(C) PCRs to detect the Fzd7 mutant knockin allele (Fzd7 Mut) and recombined product after Fzd7 gene deletion (DP), Lgr5Cre<sup>ERT2</sup> transgene (Lgr5Cre<sup>ERT2</sup>) and a region of chromosome 1 close to Fzd7 locus (Chr1), in genomic DNA extracted from adenoma organoids established from three Lgr5Cre<sup>ERT2</sup>;Fzd7<sup>fl/fl</sup>;APC<sup>min</sup> mice (M1, M2, M3) at 5 days post induction (5 days PI).

(D) Intestinal stem cell markers and Wnt target genes, gene expression levels determined by qRT-PCR and normalized to β2M. Adenoma organoids were harvested on Day 5 after treatment with 4-OHT and compared to the EtOH treated cultures (mean ± SEM, n = 3 mice).
A.

Lgr5Cre\textsuperscript{ERT2+}; Fzd7\textsuperscript{fl/fl}; APC\textsuperscript{min}

Day 0

Day 5

Ethanol

Tamoxifen

B.

Cell viability (MTT OD)

Ethanol

4-OHT

C.

4-OHT

Fzd7 Mut 25 cycles

DP 25 cycles

DP 35 cycles

Lgr5Cre\textsuperscript{ERT2}

Chr 1

5 days PI

D.

Fold Change

Ethanol

4-OHT

Fzd7

Lgr5

Fzd1

Fzd2

Ascl2

Sox9

c-Myc

Cyclin D1

Cyclin D2

CD44
Figure 4.7: AhCre\(^{+}\);Fzd7\(^{fl/fl}\);APC\(^{min}\) adenoma organoids

(A) Representative differential interference contrast (DIC) images of AhCre\(^{+}\);Fzd7\(^{fl/fl}\);APC\(^{min}\) adenoma organoids at Day 0, 3 and 5. Organoids were treated with either the control DMSO or β-NF.

Red arrows: Revived organoids. Scale bar represents 100µm.

(B) MTT cell viability assay 5 days after induction with β-NF and compared to vehicle (DMSO) treated adenoma organoids (mean ± SEM, n = 6 replicate wells).

(C) PCRs to detect the Fzd7 mutant knockin allele (Fzd7 Mut) and recombined product after Fzd7 gene deletion (DP), AhCre transgene (AhCre) and a region of chromosome 1 close to Fzd7 locus (Chr1), in genomic DNA extracted from adenoma organoids established from three AhCre\(^{+}\);Fzd7\(^{fl/fl}\);APC\(^{min}\) mice (M1, M2, M3) at 5 days post induction (5 days PI).

(D) Intestinal stem cell markers and Wnt target genes, gene expression levels determined by qRT-PCR and normalized to β2M. Adenoma organoids were harvested on Day 5 after treatment with β-NF and compared to the DMSO treated cultures (mean ± SEM, \(p < 0.05\), n = 3 mice).
AhCre\(^+\);Fzd7\(^{fl/fl}\);APC\(^{min}\)

**A**

Day 0 | Day 3 | Day 5
--- | --- | ---
DMSO | | |
\[\text{images showing cell cultures under DMSO condition}\
\]
\[\text{images showing cell cultures under \(\beta\)-NF condition}\
\]

**B**

<table>
<thead>
<tr>
<th></th>
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**C**

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<td>+</td>
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<td>Fzd7 Mut 25 cycles</td>
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<tr>
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<tr>
<td>AhCre</td>
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<td></td>
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<tr>
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5 days PI

**D**

<table>
<thead>
<tr>
<th></th>
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<tr>
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<td>CD44</td>
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Fold Change

|          | | |
|----------| | |

* indicates statistical significance.
Figure 4.8: AhCre;Fzd7^{fl/fl};APC^{min} adenoma organoids

(A) Representative differential interference contrast (DIC) images of AhCre⁻;Fzd7^{fl/fl};APC^{min} adenoma organoids at Day 0, 3 and 5. Organoids were treated with either the control DMSO or β-NF.

Scale bar represents 100µm.

(B) MTT cell viability assay 5 days after induction with β-NF and compared to vehicle (DMSO) treated adenoma organoids (mean ± SEM, n = 6 replicate wells).

(C) Intestinal stem cell markers and Wnt target genes, gene expression levels determined by qRT-PCR and normalized to β2M. Adenoma organoids were harvested on Day 5 after treatment with β-NF and compared to the DMSO treated cultures (mean ± SEM, n = 3 mice).
A

AhCre\textsuperscript{+}; Fzd7\textsuperscript{fl/fl}; APC\textsuperscript{min}

Day 0   Day 3   Day 5

DMSO

\beta\text{-NF}

B

Cell viability (MTT OD)

DMSO
\beta\text{-NF}

C

Fold Change

Fzd7  Lgr5  Fzd1  Fzd2  Sox9  Ascl2

DMSO
\beta\text{-NF}
4.4 Discussion

Aberrant regulation of Wnt signalling has emerged as one of the most affected pathways in intestinal cancers. Indeed, hyperactivation of β-catenin-TCF/LEF regulated gene transcription is a hallmark of colorectal cancer (CRC) development. Wnt signalling has emerged during evolution as a highly conserved signalling pathway that regulates a wide array of cellular processes during development and continued through to adulthood. Thus it is of little surprise that due to a deregulated Wnt pathway, the same processes are compromised, leading to the initiation and subsequent development of CRC carcinomas (Polakis, 2012). Our understanding of Wnt signalling in cancers has primarily arisen from the study of CRCs and its embedded role in initiation and progression (Kinzler and Vogelstein, 1996; Korinek et al., 1998; Morin et al., 1997b; Polakis, 1997; Sansom et al., 2004; Su et al., 1992). Indeed, the over-activation of the Wnt signalling pathway, caused by mutations to some of its intracellular components, are found in nearly all CRCs, both sporadic and familial (Kinzler et al., 1991a). Worth particular mention is the tumour suppressor gene APC, which is found to be mutated in approximately 80-90% of CRCs. However in addition to these APC mutations, the over-expression of certain upstream signalling components (e.g. Frizzleds) has also demonstrated that Wnt activity can be regulated upstream, irrespective of the downstream pathway-activating mutations (Caldwell et al., 2004; Suzuki et al., 2004; Ueno et al., 2009; Vincan et al., 2007b; Vincan et al., 2005). Thus providing a novel avenue to investigate the therapeutic potential of treating CRCs via manipulation of upstream signalling components, such as the cell surface receptors, the Frizzleds.

The results presented in the current study, using adenoma organoids from APC\textsuperscript{min} mice, verify previous findings that the Wnt receptor Fzd7 plays a role in CRCs. Furthermore, it verified that the APC\textsuperscript{min} adenoma organoids are a useful model for continued studies into the potential role of Fzd7 as a Wnt receptor for the cancer stem cells in CRC. Thus, using conditional gene deletion, initial experiments suggest that Fzd7 is a potential Wnt receptor for the CSCs. This was corroborated by the demonstration that an anti-Fzd7 antibody can decrease adenoma growth. Importantly, the results presented here also indicate a strong drive to restore Fzd7\textsuperscript{+} cells in the
adenoma organoids. This aspect will need further investigation, but is beyond the scope of this PhD. These findings offer the basis on which to continue researching the role of Fzd7 in CSCs.

Based on the discovery that extracellular inhibitors (e.g. sFRPs, DKK, WIF) of Wnt/β-catenin signalling are epigenetically silenced, it has been proposed that their loss of function points to there being a potential role for Wnts and Fzds in CRC despite the constitutively active pathway. The over-expression of Wnts (Dimitriadis et al., 2001) and Fzds (Ueno et al., 2008; Vincan et al., 2007b) in CRCs compared to normal epithelium, further supports this. Studies have shown a role for Wnt2 in CRC cell proliferation, as the ablation of Wnt2 inhibits CRC cell proliferation and the neutralization of Wnt2 secretion, suppresses CRC cell proliferation (Jung et al., 2015). In addition, a potential role for Wnt11 in CRC progression was also demonstrated via the use of Wnt11 transfectants, which showed an increase in proliferation and migration/invasion activities (Nishioka et al., 2013). In CRC tissues and cell lines, Fzd7 has been shown to be one of three highly expressed Fzds (Fzd3, Fzd6, Fzd7) (Sagara et al., 1998; Ueno et al., 2008; Vincan, 2004; Vincan et al., 2007b). Experiments, looking at the functional role of endogenous Fzd7 in a CRC cell line that harbours inactivating mutations in both APC alleles, demonstrated that by employing a dominant-negative receptor ectodomain of Fzd7 (Fzd7ΔC), tumour growth could be blocked (Vincan et al., 2005). The Fzd7ΔC was employed, as ectopic expression of the N-terminal extracellular domain of Fzd receptors (the receptor ectodomain) is known to modulate endogenous Fzd function, including Fzd7 (Djiane et al., 2000; Garcia-Castro et al., 2002; Tanaka et al., 1998). Similarly, studies in hepatocellular carcinomas, which have CTNNB1 gene (encodes β-catenin) mutations, thus an aberrantly active Wnt signalling pathway and yet sFRPs are also hypermethylated, showed that expression of a dominant-negative Fzd7 receptor mutants inhibits wild-type β-catenin accumulation and motility (Merle et al., 2004). Furthermore, in CRC cell lines, the down-regulation of Fzd7 with small-interfering RNA (siRNA) resulted in decreased in vitro invasion activity (Ueno et al., 2008). Thus, a role for upstream components of the Wnt signalling pathway in cancers is becoming more widely accepted in the field (Virshup, 2015) as solid evidence is continually accumulating.
As it has been shown that Fzd7 can dimerize with several Fzd family members (Kaykas et al., 2004) and activate different branches of Wnt/Fzd mediated signalling, in a context dependant manner (Djiane et al., 2000; Medina et al., 2000; Sumanas et al., 2000; Winklbauer et al., 2001), this suggests that Fzd7 would be a reasonable candidate in contributing to the processes that govern tumour growth. As mentioned above, using a dominant-negative receptor ectodomain of Fzd7, tumour growth was blocked in vivo. In the current study, this was investigated further in vitro, by using adenoma organoids established from APC\textsuperscript{min} adenomas. The APC\textsuperscript{min} (multiple intestinal neoplasia (MIN)) mouse model is the most commonly used mouse model of CRC. It has been employed in CRC research for over 25 years, for a broad range of studies; foremost have been chemoprevention studies and functional testing of genes with the potential to modify tumourigenesis as well as treatment studies of established tumours.

The expression profile of Fzd7 and the Wnt target genes c-Myc, CCND1, CCND2 and CD44 in the adenoma organoids, were confirmed to imitate the over-expression of these genes in human CRC. Thus, suggesting their levels of biological relevance and therefore recommending the adenoma organoids, as an in vitro model, for continued investigation into the role of Fzd7 in CRC growth.

Recently small-molecule modulators of the Wnt/β-catenin pathway that inhibit all Wnt secretion have been identified. The inhibitors of Wnt production (IWP compounds) are also capable of inhibiting cellular response (Chen et al., 2009). The IWP compound targets the acyltransferase Porcupine (Porc), a member of the membrane-bound O-acyltransferase (MBOAT) family and inactivates them. Porc is required by Wnts for their signalling capabilities and secretion, as it adds an essential palmitoyl group to the Wnt proteins (Takada et al., 2006). There are four IWP compounds, which all share the same core structure. Studies using the IWP2 compound on human CRC organoids showed that growth of a single CRC organoid culture with a mutation in the negative Wnt feedback regulator, RNF43, could be inhibited (van de Wetering et al., 2015). Of the organoid cultures with APC or CTNNB1 mutations, loss in cell viability was minimal. However, treating the APC\textsuperscript{min} adenoma organoids with the Porc inhibitor, IWP2, we demonstrated that the inhibition of Wnt secretion affects cell viability. This is not due
to toxicity as the co-treatment of IWP2 with the GSK3 inhibitor, CHIR99021, increases cell viability and thus restores the phenotype. Therefore, despite an already active Wnt/β-catenin pathway, Wnt activity is affected by blocking Wnt secretion thereby hindering Wnts from binding to Fzds.

Another method of inhibiting Wnt/β-catenin signalling upstream of the pathway, is by using antibodies. The monoclonal antibody OMP-18R5, also known as Vantictumab, is currently in clinical trials for pancreatic cancer and non-small cell lung cancer. Initially identified by binding to Fzd7, it has since been shown to interact with five of the 10 Fzd receptors (Fzd1, Fzd2, Fzd5, Fzd7, Fzd8) through a conserved epitope within the extracellular domain. It was also demonstrated, that it blocks Wnt signalling induced by multiple Wnt family members (Gurney et al., 2012). Moreover, they revealed that by blocking the Wnt pathway, growth of several types of human tumours, including colon (with mutations in APC or CTNNB1), breast, pancreatic and lung tumours, was blocked in human tumour xenografts in mice. In this study, we demonstrated that by blocking the Fzd7 receptors with the antibody, adenoma organoid death occurred and Wnt/β-catenin signalling was decreased. Furthermore, upon the addition of recombinant Wnt3a, adenoma organoid growth was restored.

Wnt/β-catenin signalling is essential for homeostasis and the maintenance of intestinal stem cells (Fevr et al., 2007). As demonstrated in the previous chapter (Ch.), Fzd7 plays an important role as a Wnt receptor in homeostasis and is vital for the crypt base columnar (CBC) Lgr5\(^+\) stem cells. Studies investigating the stem cells in CRC, i.e. cancer stem cells (CSC), have shown that Lgr5, a known marker of the intestinal stem cells (Barker et al., 2007), marks the APC mutant Lgr5\(^+\) CSCs as well (Barker et al., 2009; Schepers et al., 2012). In this study we demonstrated that in adenoma organoid cultures established from adenomas of AhCre\(^+\);Fzd7\(^{fl/fl}\);APC\(^{min}\) mice, we could successfully conditionally delete Fzd7 in the adenoma organoids, which initially looked to have a negative effect on organoid health. However the organoids made a comeback and significantly elevated mRNA expression levels for Fzd7 and c-Myc suggest, that following the deletion of Fzd7, there was a strong drive to restore c-Myc expression, which is supported by the increase in c-Myc, a known driver of cell proliferation (Bettess et al., 2005; Sansom et al., 2007). The increase in Sox9, a known
stem cell marker (Formeister et al., 2009; Ramalingam et al., 2012), cell proliferation regulator (Bastide et al., 2007) and required for Paneth cell differentiation (Bastide et al., 2007; Blache et al., 2004), which is commonly over-expressed in CRC (Lu et al., 2008; Matheu et al., 2012) is striking. It has been shown, that Bmi1 is a potential Sox9 target in primary mouse cells and transformed cells, hence repressing the tumour suppressors p16 and p19ARF, leading to cell cycle progression and bypassing of apoptosis (Matheu et al., 2012). An increase in Bmi1 with a subsequent decrease in p16 was observed in Sox9 over-expressing human CRC cells (Matheu et al., 2012). In the normal intestinal epithelium, Bmi1-expressing stem cells, which reside above the crypt base at the +4 position, could compensate for the loss of the Lgr5+ CBC stem cells (Tian et al., 2011). Potentially, what we are seeing is a role for Sox9-expressing cells to compensate for the loss of the Lgr5+ CSCs, which are potentially affected by the deletion of Fzd7, and that Sox9, through the targeting of Bmi1, is restoring cell proliferation. This would need to be studied further, but is unfortunately outside the scope of this PhD. Additionally, another avenue in which the role of Fzd7 in the Lgr5+ CSCs and CRC initiation could be investigated, is by employing the same model used for lineage tracing, i.e. the Lgr5;Fzd7fl/fl;APCfl/fl;LacZ and AhCre;Fzd7fl/fl;APCfl/fl;LacZ mice in vivo. This too was outside the scope of this PhD, due to multiple issues with mice.

4.5 Conclusion

The results from the current study presented in this chapter show, that the adenoma organoid cultures are an accessible model to research adenoma growth and maintenance in vitro. Additionally, further evidence supporting Wnts and Fzd7 as modulators of the Wnt/β-catenin pathway in colorectal cancer is provided. Lastly, Fzd7 is potentially a Wnt receptor of the Lgr5+ cancer stem cells.

Together with the findings from other work conducted in our lab and by others, these further support our hypothesis that Fzd7 has a functional role in colorectal cancer and as such, the potential for it as a therapeutic target is increasing.
Chapter 5

Final discussion and future directions
5.1 Final discussion and future directions

The gastrointestinal tract’s (GIT) main purpose is to digest food and absorb the nutrients for use by the cells of the body. Along with selectively absorbing the nutrients, the intestinal epithelium that lines the organs of the GIT, is also tasked with simultaneously excluding luminal pathogens and pro-inflammatory molecules from host tissues. Thus, the epithelium of the small intestine has established mechanisms in place to continually monitor the health of the epithelium and as such undergoes continual renewal to clear the epithelium of any cells that have potentially undergone damage. To preserve this consistent creation and loss of cells, the turnover and renewal of cells is driven by the undifferentiated self-renewing stem cells, which give rise to all the cell types of the intestinal epithelium. Control of homeostasis in the epithelium is vital, since loss of homeostatic control can lead to neoplastic transformation of cells, which can under the right conditions progress to cancer.

Fundamental to both homeostasis and cancer is the Wnt signalling pathway, which is an evolutionarily conserved gene program. It is a key player during development and adulthood and can lead to cancer if abnormally activated. The research conducted in this thesis has been focused on a specific “player” in the Wnt signalling pathway, namely Fzd7. Intense investigation to exploit the functions of Fzd7 in stem cells and cancer ensued.

The results presented in this PhD thesis have been discussed in the context of current and past literature in the relevant chapters. Here is an overview and perspective, i.e. impact of current work.

Our laboratory has identified a key role for Fzd7 in intestinal stem cells which we recently published (Flanagan et al., 2015), and includes work from this thesis. We demonstrated that Fzd7 transmits the stem cell Wnt signal and that loss of Fzd7 by gene deletion, compromises stem cell function. These findings have since been corroborated. Genentech have made peptides that block the interaction of Wnt with Fzd7 (Hannoush, 2016). They demonstrate the same impaired stem cell function when Fzd7 is blocked with the peptide as we have shown genetically. These peptides are just the first generation; more development is now in process to identify the amino acid residues that dictate the Wnt pathway that Fzd7 activates and to block specific Wnt-
Fzd7 interactions. These advances offer hope for better, selective targeting of Wnt signalling in stem cells and cancer via Fzd7. The data presented in this thesis has contributed to this advance.

Recent evidence from studies in mouse breast cancer models (Ghajar and Bissell, 2016) show that dissemination of tumour cells to the secondary organ can occur very early. Furthermore, because the disseminated cells go to the secondary site very early, i.e. when the primary tumour is just starting to grow, the primary and secondary tumours evolve and accumulate genetic mutations and modifications independently and have a different set of genetic mutations. Thus, genome profiling of the primary tumour at the time of diagnosis, i.e. long after cells have disseminated, may have little predictive value. The field is moving along very fast indeed. Importantly, based on research in our lab, the dormant cells in CRC express Fzd7. Thus Fzd7 might offer the opportunity to target the dormant cells before they can re-establish tumours at the secondary site. As most patients die because of their metastases, elimination of disseminated tumour cells would have a major impact on patient survival.
References


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intestinal stem-cell homeostasis by negative regulation of ErbB signalling. Nature Cell Biology 14, 401-408.


### Human qRT-PCR primer sequences

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### Mouse qRT-PCR primer sequences

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