Characterising the role of Cbx3/HP1γ in Normal Intestinal Homeostasis and Tumorigenesis

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Doctor of Philosophy

European Cancer Stem Cell Research Institute

Cardiff University, United Kingdom

March 2015
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This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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<td><strong>Symbols</strong></td>
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<tr>
<td>°C</td>
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<tr>
<td>µg</td>
<td>Micrograms</td>
</tr>
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<td>µl</td>
<td>Microlitres</td>
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<td>Micrometer</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
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<td>dH₂O</td>
<td>Deionised water</td>
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<tr>
<td>Dhh</td>
<td>Desert Hedgehog</td>
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<tr>
<td>DNA</td>
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<td>Deoxyribonuclease</td>
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<td>Dsh</td>
<td>Dishevelled</td>
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<td><strong>A</strong></td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>ABC</td>
<td>Avidin-Biotin Complex</td>
</tr>
<tr>
<td>Apc</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>Ascl2</td>
<td>Achaete Scutelike 2</td>
</tr>
<tr>
<td>Apc&lt;sup&gt;MIN&lt;/sup&gt;</td>
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<tr>
<td>Axin</td>
<td>Axis Inhibitor</td>
</tr>
<tr>
<td><strong>B</strong></td>
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<tr>
<td>Beta-Gal</td>
<td>Beta-Galactosidase</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>B-lymphoma Mo-MLV insertion region 1 polycomb ring-fiber oncoprotein</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2-deoxyuridine</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td></td>
</tr>
<tr>
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<td>Crypt Base Columnar cells</td>
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<tr>
<td>CBX</td>
<td>Chromobox protein homolog</td>
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<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
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<td>CK</td>
<td>Casein kinase</td>
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<tr>
<td>Cm</td>
<td>Centrimetres</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Cellular-Myelocytomatosis oncogene</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal Cancer</td>
</tr>
<tr>
<td>Cre</td>
<td>Causes recombination</td>
</tr>
<tr>
<td>CreER&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Causes recombination-Estrogen receptor fusion transgene</td>
</tr>
<tr>
<td>Cr</td>
<td>Cycle time</td>
</tr>
<tr>
<td><strong>D</strong></td>
<td></td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-diaminobenzidine</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td><strong>E</strong></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>E-cadherin</td>
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<tr>
<td>ECL</td>
<td>Electrochemiluminescence</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetra-acetic Acid</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular regulated MAP kinase</td>
</tr>
<tr>
<td><strong>F</strong></td>
<td></td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td><strong>G</strong></td>
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</tr>
<tr>
<td>GEF</td>
<td>Guanine Nucleotide Exchange Factor</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen Synthase Kinase-3</td>
</tr>
<tr>
<td>Gli</td>
<td>Glima-associated oncogene homolog</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine Triphosphatase</td>
</tr>
<tr>
<td><strong>H</strong></td>
<td></td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HDAC1</td>
<td>Histone deacetylase 1</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary Nonpolyposis Colorectal Cancer</td>
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<tr>
<td>HP1</td>
<td>Heterochromatin Protein 1</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td><strong>I</strong></td>
<td></td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ISC</td>
<td>Intestinal stem cell</td>
</tr>
<tr>
<td>J</td>
<td>Juvenile Polyposis Syndrome</td>
</tr>
<tr>
<td>K</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilograms</td>
</tr>
<tr>
<td>K-Ras</td>
<td>Kirsten Rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>KS</td>
<td>Kolmogorov-Sirnov</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>Lgr5</td>
<td>Leucine-rich repeat-containing G-protein coupled receptor 5</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>LoxP</td>
<td>Locus of crossover of Bacteriophage P1</td>
</tr>
<tr>
<td>LRP</td>
<td>Low density lipoprotein receptor related protein</td>
</tr>
<tr>
<td>M</td>
<td>Molar (moles per litre)</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen Activated Erk Kinase 1/2</td>
</tr>
<tr>
<td>mg</td>
<td>milligrams</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>miRNAs</td>
<td>Micro-RNAs</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch Repair</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
</tr>
<tr>
<td>MSH2</td>
<td>Mts E.Coli homolog of 2</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>N CID</td>
<td>Notch Receptor Intracellular Domain</td>
</tr>
<tr>
<td>NFxB</td>
<td>Nuclear Factor of kappa light polypeptide gene enhancer in B-cell</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>NRS</td>
<td>Normal Rabbit Serum</td>
</tr>
<tr>
<td>O</td>
<td>Olfactomedin 4</td>
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<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>P</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>pAb</td>
<td>Phosphor-Akt</td>
</tr>
<tr>
<td>pAkt</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBS</td>
<td>0.1% TWEEN-20 in Phosphate Buffered Saline</td>
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<tr>
<td>PBS/T</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
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<td>Post induction</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
</tr>
<tr>
<td>Ptch</td>
<td>Patched</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin homolog mutated on chromosome ten</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse-transcription PCR</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened</td>
</tr>
<tr>
<td>TA cells</td>
<td>Transit-Amplifying cells</td>
</tr>
<tr>
<td>TACE</td>
<td>Tumour Necrosis Factor Converting Enzyme</td>
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- xvii -
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<td>TBS</td>
<td>Tris Buffered Saline</td>
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<td>TBS/T</td>
<td>Tris Buffered Saline with TWEEN-20</td>
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<tr>
<td>TCF</td>
<td>T-cell factor</td>
</tr>
<tr>
<td>TCF-4</td>
<td>Transcription factor 4</td>
</tr>
<tr>
<td>Tcf/Lef</td>
<td>T-cell factor and Lymphoid enhancer factor</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-Tetramethylethlenediamine</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TTM</td>
<td>Non-fat milk powder in Tris buffered saline containing 0.1% TWEEN-20</td>
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**V**

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<td>Volts</td>
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<tr>
<td>VillinCreERT</td>
<td>Villin Cre recombinase ER transgene</td>
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<tr>
<td>v/v</td>
<td>Volume per Volume</td>
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**W**

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<td>Watts</td>
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<tr>
<td>w/v</td>
<td>Weight per Volume</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-type murine mammary tumour virus integration site family</td>
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**X**

<table>
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<th>Definition</th>
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<tr>
<td>x g</td>
<td>Times gravity</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-4-chloro-3-indolyl beta-D-galactopyranoside</td>
</tr>
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Abstract

Epigenetic modifications are being increasingly recognised to contribute to colorectal cancer formation. Emerging evidence has demonstrated that HP1γ (coded by the gene Cbx3) has an important role in carcinogenesis by regulating several mechanisms, such as heterochromatin formation, gene silencing and DNA replication and repair. The aim of this study was to characterise the role of HP1γ in normal intestinal homeostasis and cancer progression.

The Cbx3 gene was conditionally deleted in the murine small intestinal epithelium under normal intestinal homeostasis and in the context of aberrant Wnt signalling using transgenic mouse models. Furthermore, the long-term consequence of Cbx3 loss in Wnt-driven tumorigenesis was examined using a mouse model possessing a heterozygous mutation of the Apc gene.

The results presented in this thesis demonstrate that Cbx3 has subtle effects on the histone epigenome, although these do not confer any noticeable manifestation in intestinal homeostasis. Upon aberrant Wnt-activation, Cbx3 loss resulted in prolonged survival potentially via attenuation of Wnt activation at the receptor level and RNA splicing defects. Furthermore, the long-term Cbx3 deficiency accelerates both Wnt-driven tumour development and progression, and reduces survival of male mice with heterozygous loss of Apc. Additionally, marked epigenetic alternations were observed in association patterns of HP1γ and the heterochromatin markers (H3K9me3 and H4K20me3) between wild-type and Apc-deficient intestines, suggesting the multifaceted roles of HP1γ in maintaining a gene-repressive environment in heterochromatin for normal intestinal homeostasis, and providing a gene permissive environment in euchromatin for carcinogenesis.

The findings of this study demonstrate for the first time the functional versatility of HP1γ in intestinal homeostasis and tumorigenesis. Cbx3 plays a novel role in positive regulation of Wnt signaling and RNA splicing in the aberrant Wnt-activated intestine and a role in heterochromatin formation and gene silencing in long-term Wnt-activated tumorigenesis. These data contribute to the body of knowledge how colorectal cancer progresses and provide a critical foundation for further investigation of the role of HP1γ in Wnt signaling, RNA splicing, tumour progression, and its interaction with histone marks in mouse models of colorectal cancer.
Chapter One

General Introduction
1. General Introduction

1.1. The Mammalian Small Intestine

1.1.1. General Intestinal Morphology and Physiology

The mammalian digestive tract consists of the mouth and salivary glands, the stomach, the small intestine, the liver, the pancreas, the gallbladder and the large intestine. The main function of the digestive tract is to enable nutrients from consumed food and beverages to gain access to the circulatory system and the body. The gastrointestinal tract, the largest part of the digestive tract, is comprised of the stomach and the intestines. The stomach acts as a short-term storage reservoir for consumed food and serves the function of mechanical processing mixing with hydrolytic enzymes, pH change and digestion (Guyton A.C., 1996).

The mammalian intestine is divided anatomically into two major parts – the small and large intestines. The small intestine is comprised of three regions: the duodenum, jejunum and ileum. Apart from the two papillae that serve as entry points for pancreatic and bile ducts and the presence of the mucus-secreting Brunner’s glands in the submucosa of the duodenum, all three regions of the small intestine share a similar structure. The wall of the intestine is structured into four layers: serosa, muscle layers, submucosa and mucosa (as illustrated in Figure 1.1) (Crane, 1968).

The outermost layer of the intestine is represented by the serosa, which is composed of visceral peritoneum merging with connective tissue. Secondly, the muscularis externa is comprised of a thin outer layer of longitudinal muscle and a thicker inner region of circular muscle. Thirdly, the submucosal layer consists of connective tissues, blood vessels and lymphatics. Last but not least, the mucosal layer is composed of a muscularis mucosae, a lamina propria and the simple columnar epithelium. The small intestine incorporate three modifications that account for its 600-fold increased absorptive surface area. Firstly, both mucosa and submucosa are formed into plicae circulares (circular folds) perpendicular to the intestinal axis. Secondly, the surface of the mucosa is organised into finger-like projections called villi. At the base of the villi, the epithelium forms pocket-like invaginations into the lamina propria termed crypts of Lieberkuhn (intestinal crypts). Lastly, the apical
membrane of the absorptive columnar epithelium lining the surface of villi forms microscopic cytoplasmic protrusions called microvilli, forming the brush border of the small intestine and further increasing the epithelial surface area. Progressing from the duodenum towards the ileum, the thickness of the intestinal wall and the number of the circular folds decrease, so does the density and the size of the villi. In contrast, the Peyer’s patches containing lymph nodes in the ileum are larger and more numerous than those of the duodenum and jejunum (Guyton A.C., 1996).

The primary function of the small intestine is to facilitate digestion of consumed food and nutrient absorption. The majority of the water, electrolytes, vitamins and minerals are absorbed in the small intestine (primarily in the duodenum and jejunum) while the remaining is absorbed in the large intestine. The large intestine consists of four regions: the caecum, colon, rectum and anal canal. The colon is the largest part of the large intestine. The mucosa of the large intestine differs from the small intestinal mucosa through the absence of circular folds and villi. The colonic mucosa is organised into crypts similar to those found in the small intestine. However, there is a shift of cell type abundance between small and large intestine. Absorptive cells predominate in the small intestine whereas the mucus-secreting cells incorporate the majority of the large intestinal epithelium (Pitman and Blumberg, 2000).
Chapter 1: General Introduction

Figure 1.1: Schematic representation of the crypt-villus unit in the small intestine. The outermost layer of the intestine is represented by the connective tissue of the serosa. Underneath the serosa, the muscle layers surrounding the submucosa composed of connective tissue containing blood vasculature and nerves. The mucosa is composed of a muscularis mucosae, a lamina propria and the simple columnar epithelium.
1.1.2. Histology of the small intestinal epithelium

As mentioned in the previous section, the primary function of the small intestine is to facilitate nutrient absorption and therefore, the epithelium is organized into distinct crypt-villus structure to maximise the surface area for absorption (Figure 1.1). Both lysozyme-producing Paneth cells and intestinal stem cells reside within the base of crypts. The intestinal stem cells are capable of self-renewal and also give rise to the transit-amplifying (TA) cells, which function to rapidly expand epithelial renewal and initiate lineage differentiation. The differentiated cell lineages consist of absorptive enterocytes, mucin-secreting goblet cells, hormone-producing enteroendocrine cells and anti-microbial secreting Paneth cells. Apart from downward migrating Paneth cells, the differentiated cells are in continual upward migration towards the apex of the villus where they undergo apoptosis and are exfoliated within 4 to 8 days (Cheng, Merzel and Leblond, 1969; Hall et al., 1994). As a result of the continuous migration towards the villus apex, the entire intestinal epithelium is renewed every 5 days in humans and every 3 days in mice (Marshman, Booth and Potten, 2002).

The mucosa of the small intestine is lined by a simple columnar epithelium that consists primarily of enterocytes representing the absorptive lineage, with scattered goblet, enteroendocrine and Paneth cells constituting the secretory lineages (as illustrated in Figure 1.1).

The majority of the cells in the villus (80%) are represented by the only member of the absorptive lineage – enterocytes. They are positioned along the top of the crypt and over the whole of the villus and are actively renewed with an average turnover time of 3 days. The enterocytes are characterised by the presence of a tight array of microvilli on their apical membrane that constitute a brush border, which facilitate the final stages of digestion and provides a site of attachment for pathogens (Cheng and Leblond, 1974; Cheng, 1974). The apical and basal membranes of the enterocytes contain various channel proteins responsible for transporting nutrients from the intestinal lumen into the blood and lymphatic vasculature, while also transporting certain ions into the lumen (Smith, 1992).

The second most abundant cell types in the small intestinal epithelium are goblet cells, which are the most abundant cell type in the secretory lineage. Mature goblet cells
are scattered among the enterocytes from mid-crypt to the villus apex and are renewed at a similar turnover rate to that of the enterocytes (Troughton and Trier, 1969). Goblet cells are distinguished by the presence of large mucus containing granules and enlarged rough endoplasmic reticulum. The mucus secreted by the goblet cells serves as a lubricant for promoting movement and effective diffusion of gut contents, and protecting the lining of the intestine from mechanical abrasion or damage by acidic stomach contents and autodigestion by the digestive enzymes (Cheng and Leblond, 1974).

The Paneth cells represent the second most abundant member in the secretory lineage. They are located at the base of the crypts and they differ from all the other mature cell types in their downward migration, maintenance of active Wnt signalling and slower turnover time of 57 days (Ireland et al., 2005). The Paneth cells are distinguished by the presence of a large number of apical secretory granules that contain a number of substances with antimicrobial activity and therefore they are considered to play a critical role in the regulation of the intestinal microflora. Previous studies suggested that Paneth cells provide a niche for the intestinal stem cells (Sato et al., 2011).

The enteroendocrine cells represent less than 1% of the terminally differentiated cells in the intestinal epithelium and consists a family of at least 15 different cell types. These cells differ in the morphology of their secretory granules and the unifying feature of all the enteroendocrine cells is expression and secretion of a range of polypeptide hormones that regulate secretion into the gastrointestinal tract (Hocker and Wiedenmann, 1998).

In addition to the main cell types, two minor cell lineages are present in the small intestinal epithelium. Firstly, Membranous or Microfold (M) cells are located in the epithelium adjacent to the Payer’s patches. Their basolateral membrane forms invaginations occupied by the immune cells: macrophages, T and B-lymphocytes. M cells are able to internalise particles and microorganisms from the lumen by phagocytosis and transport them across the epithelial border, where they are processed by the immune cells (Neutra, Frey and Kraehenbuhl, 1996). Secondly, Tuft cells (also known as brush or fibrillovesicular cells) are scattered throughout the gastrointestinal tracts. They are characterised by a goblet-shaped body, the presence of long microvilli on the apical membrane and vesicles in the apical cytoplasm. Although their role in the small intestine is still undefined, the
abundance of the vesicles in the cytoplasm indicates that tuft cells may be involved in the secretion or absorption of certain substances (Sato, 2007).

1.1.3. Maintenance of the small intestinal homeostasis

The intestinal homeostasis has a critical function within this perpetually renewing tissue, the balance of proliferation, cell death, differentiation, migration and cell positioning must be closely regulated in order to ensure the proper maintenance and function of the intricate cellular architecture described previously. Regulation of these processes is precisely orchestrated by a number of important signalling pathways, including Wnt signalling, Notch/Delta signalling, transforming growth factor-beta (TGFβ) and bone morphogenetic protein (BMP) signalling, and Hedgehog signalling. Each of these pathways and the role they play in maintaining the intestinal homeostasis are briefly described in this section (also see Figure 1.2).
Figure 1.2: The main signalling pathways regulating homeostasis of the intestinal epithelium.

A: Wnt signalling. In the absence of Wnt ligand, β-catenin destruction complex is formed consisting of Axin, Apc and GSK3β, which binds β-catenin and mediates its phosphorylation and proteasomal degradation. In the presence of Wnt ligand, Wnt ligand binds to Frizzled receptor and LRP5/6 co-receptor results in activation of Dishevelled (Dsh) and phosphorylation of LRP5/6, which together inhibit the β-catenin destruction complex, allowing accumulation and translocation of β-catenin to the nucleus, where it docks on TCF/LEF factors and activates transcription of Wnt target gene expression.

B: Notch signalling. Binding of notch receptor cell surface ligands of the Delta/Jagged family stimulates cleavage of the Notch receptor by TACE and γ-secretase. Cleavage by γ-secretase releases the notch intracellular domain (NICD), which translocates to the nucleus and binds to transcription factor CSL to activate expression of Notch target genes.

C: TGFβ/BMP signalling. TGFβs or BMPs bind to their type I/II receptors, resulting in phosphorylation of the type I receptor. TGFβ signals through SMAD2/3 and BMP signals are transduced through SMAD1/5/8. Both result in activation of SMAD4, which translocates to the nucleus and modulates target gene expression.

D: Hedgehog signalling. In the absence of Hedgehog ligands, unbound Patched receptors inhibit the Smoothened signal transducer, allowing PKA to phosphorylate GLI2/3 transcription factor, sequestering it away from the nucleus and targeting its cleavage. Binding of Hedgehog to Patched releases repression of Smoothened, which in turn suppresses PKA, thereby allowing full-length GLI2/3 to accumulate and translocate to the nucleus, where it activates target gene expression.
1.1.3.1. The canonical Wnt pathway

The β-catenin-dependent pathway, referred to as the canonical Wnt signalling pathway, plays a key role in regulation of morphology, proliferation, motility and cell fate (Archbold et al., 2012). The canonical Wnt signalling pathway was discovered by genetic analysis in Drosophila (Rijsewijk et al., 1987), and subsequently, this signaling cascade was found to be highly conserved between Drosophila and vertebrates (Miller and Moon, 1996). Mutations and epigenetic alternations of this pathway lie at the heart of many human diseases, including colorectal cancer.

Wnt signalling in adult intestine regulates the fine balance between stem cell maintenance and differentiation. Figure 1.2A shows a schematic representation of the canonical Wnt signalling pathway. In the absence of a Wnt ligand, the tumour suppressor proteins adenomatous polyposis coli (APC) and axis inhibitor (Axin) form the scaffold of the destruction complex and bind free β-catenin enabling its phosphorylation by Casein Kinase 1 (CK1) at the Ser45 residue (Amit et al., 2002; Liu et al., 2002). Subsequently, Glycogen synthase kinase-3β (GSK3β) in the Axin complex phosphorylates β-catenin (Rubinfeld et al., 1996) which is then recognised by the E3 ubiquitin ligase β-transducing repeat-containing protein (β-TrCP), which triggers the ubiquitination of β-catenin and its subsequent degradation by proteasomes, reducing the level of cytosolic β-catenin (Hart et al., 1999).

In the presence of a Wnt ligand, the signalling pathway is activated through the binding of the Wnt ligand to the seven transmembrane serpentine receptor, Frizzled (Huang and Klein, 2004), and low density lipoprotein receptor related protein (LRP5/6) complex (Pinson et al., 2000), activating the cytoplasmic protein dishevelled (Dsh). Dsh then inhibits the activity of the Axin complex (β-catenin-APC)-GSK3β), GSK3β is no longer functional. The overall result is accumulation of cytosolic β-catenin (Tamai et al., 2004) and stabilized β-catenin will then translocate into the nucleus and bind to members of the T-cell factor (Tcf)/Lymphoid enhancing factor (Lef) family (Waterman, 2002). In a Wnt-inactive cell, Tcf proteins are associated with general transcriptional repressors, such as Groucho, and repress the expression of Tcf-responsive genes (Roose et al., 1998). On the activation of Wnt signalling, β-catenin binding of Tcf proteins displaces Groucho and leads to transcription of Wnt target genes (Daniels and Weis, 2005).
The canonical Wnt signalling pathway drives proliferation in the intestine and maintains homeostasis. A number of studies in which the Wnt signalling was inhibited at various points in the pathway, have demonstrated that loss of Wnt signalling perturbs normal intestinal structure and proliferation. Transgenic ectopic overexpression of the Wnt antagonist *Dickkopf-1* (Dkk1) in adult small intestine resulted in reduced proliferation and loss of all the secretory cell lineages, and a rapid degeneration of the crypt-villus architecture (Pinto *et al.*, 2003). Likewise, conditional homozygous deletion of β-catenin and the Wnt target gene cellular-Myelocytomatosis oncogene (*c-Myc*) in adult mouse intestine resulted in catastrophic disruption of normal intestinal homeostasis, with loss of proliferative zone and ablation of the crypts (Muncan *et al.*, 2006; Ireland *et al.*, 2004). Inhibition of Wnt signalling by deletion of Wnt transcription factor Tcf-4 in murine models resulted in loss of proliferation in embryonic intestines (Korinek *et al.*, 1998). These studies highlight the importance of Wnt signalling in controlling cell proliferation and guiding progenitor cells towards the secretory lineage.

In a similar but opposite fashion, aberrant activation of the Wnt pathway by deletion of *Apc* or by overexpression of a dominant stable form of β-catenin have resulted in dramatic increase in proliferative cells within the crypt, coupled with a complete block in cell migration and impaired apoptotic response (Sansom *et al.*, 2004; Harada *et al.*, 1999). The same studies have shown that activation of Wnt signalling by deletion of *Apc* impairs cell migration along the crypt-villus axis, resulting in the proliferative zone being poorly organised and causing mis-localisation of Paneth cell throughout the crypt rather than restricted to the crypt base. Overall, aberrant activation of the Wnt signalling resulted in a general failure to differentiate as evident by the loss of all but Paneth cell differentiation markers (Andreu *et al.*, 2005). Together, these studies demonstrate that Wnt signalling plays a role in regulating cell proliferation, cell differentiation and directing cells towards the secretory lineage.
1.1.3.2. The Notch/Delta Pathway

Notch signalling plays a range of important roles in the homeostasis of self-renewing tissues, including regulation of apoptosis, proliferation, differentiation, spatial patterning and cell fate determination (Artavanis-Tsakonas, Rand and Lake, 1999). Notch signalling is a cell-to-cell signalling pathway, indicating that signal transduction can only occur between adjacent cells. The Notch signalling cascade is initiated by binding of the Notch cell surface receptors, Notch1 and Notch2 (coexpressed on stem cells and TA cells), to one of the Delta-like or Jagged family of membrane-bound ligands (expressed by Paneth cells) on an adjacent cell (Figure 1.2B). As a result of this receptor-ligand interaction, the Notch receptor undergoes two successive proteolytic cleavage events. The first cleavage takes place in the extracellular domain and is mediated by Tumour necrosis factor (TNF)-alpha-converting enzyme (TACE). The second cleavage occurs intracellularly within the trans-membrane domain and is carried out by γ-secretase. The second cleavage releases the Notch intracellular domain (NICD). The NICD then translocates to the nucleus where, together with the Notch downstream transcription factor CSL, it activates transcription of its target gene (reviewed in (Wilson and Radtke, 2006).

In the adult intestine, a number of studies have demonstrated the importance of Notch signalling pathway in intestinal homeostasis and maintenance of the undifferentiated state of TA cells. Conditional inactivation of the essential Notch transcription factor CSL in the mouse intestine results in complete loss of all proliferative cells within the crypt and their conversion into goblet cells. An identical phenotype was achieved by blocking Notch signalling with γ-secretase inhibitors (van Es et al., 2005).

In a reverse approach, excessive activation of Notch signaling by overexpression of a constitutively active form of NICD resulted in severe deficiency of secretory cell types in the intestine, while an expansion of the proliferative compartment was observed (Fre et al., 2005). Together, these data suggests that Notch signalling is essential for maintenance of the crypt progenitors in the undifferentiated state within the crypt and for determination of the cell fate towards the absorptive lineage.
1.1.3.3. The TGF-β and BMP pathway

Transforming growth factor (TGF)-β signalling regulates a number of biological processes such as embryogenesis, wound healing, cell proliferation and differentiation (Sancho, Batlle and Clevers, 2004). Bone morphogenic proteins (BMPs) are part of the TGF-β superfamily. BMP signalling is known to play a role in development and maintenance of the intestinal epithelium. In addition, it is responsible for the regulation of the interaction between epithelial cell and the underlying mesenchyme (Ishizuya-Oka, 2005) (Figure 1.2C). Both TGF-βs and BMPs are soluble and extracellular signalling proteins. TGF-βs bind preferentially to TGF-β type II receptor, which then dimerise with type I receptor. Conversely, BMPs bind preferentially to BMP type I receptors, which then associate with the BMP type II receptor. In both cases, the type II receptors phosphorylate the cytoplasmic domain of the type I receptor which then recruits and phosphorylates members of Mothers Against Decapentaplegic homolog (SMAD) family of signal transducers. SMAD2 and 3 are preferentially phosphorylated by the TGF-β type I receptor, whereas BMP signalling the type I receptor recruits and phosphorylates SMAD1, SMAD5 or SMAD8. The SMADs that are recruited to the receptors and phosphorylated are collectively known as the Receptor associated SMADs (R-SMADs). The activated R-Smads then dissociate from the receptor and form a complex with SMAD4 (also known as co-SMAD), which then enables the translocation of the complex into the nucleus where these active SMAD complexes bind to TGF-β or BMP target genes and recruit regulatory proteins to the genes promoters to allow transcription (reviewed in Shi and Massague, 2003).

In the intestine, conditional deletion of the BMP type I receptor (BMPRI) in the epithelial cells of mouse intestine resulted in an increase in proliferation within the epithelium and development of intestinal polyps that were characterised by an expanded proliferative compartment (He et al., 2004). A further study overexpressed the BMP inhibitor Noggin in the submucosa around the crypt region of mouse intestine, thus blocking BMP signals from mesenchyme in this region, resulted in abnormal villus structure and the development of ectopic crypt structures (Haramis et al., 2004). These observations demonstrate that BMP signalling is important for interactions between overlying epithelium and underlying mesenchyme to coordinate the development of epithelial structures in the correct context.
1.1.3.4. The Hedgehog pathway

Hedgehog signalling plays an important role in a variety of biological processes, including embryonic development of the gastrointestinal tract, maintenance of tissue homeostasis, carcinogenesis and tissue repair (reviewed in van den Brink, 2007). Hedgehog signalling is governed by two transmembrane proteins called Patched and Smoothened (see Figure 1.2D). In the absence of Hedgehog proteins, the signalling pathway is inactive. Unbound cell surface receptor of Patched suppresses the activation of Smoothened. When Smoothened is inactive, protein kinase A (PKA) is able to phosphorylate the cytoplasmic transcription factor Glioblastoma-associated oncogene homolog (Gli)2/3, which serves to sequester Gli away from the nucleus, as well as to target its proteolytic cleavage and inactivation. There are three Hedgehog ligands found in vertebrates, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). These are able to bind to Patched receptors and in doing so prevent the suppression of Smoothened, which when activated prevents the phosphorylation and subsequent partial degradation of Gli2/3. This leads to accumulation of full-length Gli, which then translocate to the nucleus and activate transcription of its target gene (reviewed in van den Brink, 2007).

In the small intestine, loss of either Shh or Ihh (both are expressed throughout the epithelium) results in gross malformation of the gut epithelium (Ramalho-Santos, Melton and McMahon, 2000). Loss of Shh results in overgrown villi in the duodenum suggesting an increase in proliferation whereas loss of Ihh results in reduced proliferation in the crypt region, disruption of the intestinal mesenchymal architecture and deterioration of the extracellular matrix. In addition, loss of either Shh or Ihh results in post-natal lethality (Ramalho-Santos, Melton and McMahon, 2000). Together, these data imply Hedgehog ligands exhibit distinct roles in the small intestine. To explore the combined role of both Shh and Ihh ligands in adult intestinal homeostasis, complete abrogation of hedgehog signalling in the intestine by overexpression of the hedgehog inhibitor (Hhip) resulted in hyperproliferation of the epithelium with ectopic crypt development and abnormal branching of villi (Madison et al., 2005). Thus, the secretion of Shh and Ihh in the crypt region, acts as paracrine signals for mesenchymal stromal cells to inhibit proliferation in the villus epithelium, highlighting the importance of epithelial-mesenchymal interactions in tissue homeostasis.
1.1.4. The small intestinal stem cell

The intestinal epithelium represents the most rapidly self-renewing tissues in adult mammals. The constant renewal of the epithelium is regulated and maintained by a population of intestinal stem cells, which give rise to all the intestinal epithelial cell lineages. By definition, stem cells are able to proliferate and self-renew indefinitely, while generating phenotypically diverse progeny specific to the given tissue (Potten and Loeffler, 1990). Considering how stem cells play a critical role in intestinal epithelial homeostasis, a better understanding of intestinal stem cell function and regulation will provide insights into intestinal pathologies.

Figure 1.3: Schematic representation of the small intestinal crypt and candidate locations of the intestinal stem cell. The small intestinal stem cell locations have been proposed to be the crypt base columnar (CBC) cell population and position +4 stem cell population. Putative markers of the intestinal stem cells have been proposed for both candidate locations (boxes). Paneth cells, mesenchymal cells and stromal components are proposed to constitute the stem cell niche.
1.1.4.1. Intestinal stem cell identify, marker and location

There has been much debate over the identity and location of the intestinal stem cell within the crypt (Figure 1.3). The historic views on the stem cell position within the crypt and identification of candidate intestinal stem cell markers will be discussed in this section.

A number of early DNA labelling studies have suggested that the intestinal stem cell is located approximately 4 cell positions from the base of the crypt, directly above the Paneth cell compartment. The rational for these experiments lies in the differential kinetics of cell proliferation and migration occurring within the crypt. Labelling of the replicating intestinal cells was achieved by using tritiated thymidine ($^3$HTdR) or Bromodeoxyuridine, which are incorporated into newly synthesized DNA of replicating cells during S phase of the cell cycle. This approach will label an expanding stem cell population, as the stem cells are able to incorporate and retain the label due to their low proliferation rate. Conversely, TA cells rapidly proliferate and migrate out of the crypt and the label is rapidly diluted and lost. Thus, the slowly cycling label-retaining cells (LRCs) can be used to identify the position of the stem cell. Christopher Potten and colleagues revealed that the LRC is normally found at position +4, immediately above the Paneth cells. These cells cycled once a day on average, at half the rate of the rest of the rapidly proliferating crypt cells, and were therefore proposed as intestinal stem cells (Potten, 1974).

An alternative location for the intestinal stem cell was proposed in the same year as for the +4 position cells. This alternative position is within the slender cells intercalated between mature Paneth cells at crypt base, and is known as crypt base columnar (CBC) cells (Cheng and Leblond, 1974). This model proposes that cells below position 4 in the crypt are slowly cycling and capable to maintain an undifferentiated state. This study is further supported by a number of clonal analysis experiments that suggested the stem cell zone at positions 1-4 in the crypt base (Bjerknes and Cheng, 1981).

The ability to reliably identify and isolate intestinal stem cells would undoubtedly accelerate our understanding of stem cells roles in normal intestinal homeostasis and cancer. Many efforts have been focused on the identification of stem cell-specific molecular marker that would distinguish the stem cell from other cells in the crypt (proposed markers listed in Figure 1.3).
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One of the first intestinal stem cell markers to be proposed was the RNA-binding protein Musashi-1. Musashi-1 has been found to be highly expressed in developing and regenerating crypts, as well as in adenomas of $\text{Apc}^{\text{MIN}}$ mice (carrying a nonsense-mutation in codon 850 of $\text{Apc}$ resulting in a truncated protein), all of which potentially contains expanded stem cell populations. However, expression of Musashi-1 has been shown to be high in both +4 and CBC intestinal stem cells as well as in early progenitor cells and therefore cannot be used exclusively as an intestinal stem cell marker (Potten et al., 2003).

The microtubule-associated kinase Doublecortin and Calmodulin-kinase like 1 (DCAMKL-1) has been proposed to mark more specifically to intestinal stem cell based on its ability to stain individual cells around +4 position (May et al., 2008). However, a number of characteristics have undermined DCAMKL-1 being a specific marker for the intestinal stem cell. DCAMKL-1 positive cells were frequently found to be present among the villus cells, they were radiation-resistant and did not seem to contribute to crypt cell regeneration (May et al., 2008). Furthermore, DCAMKL-1 positive cells expressed typical tuft cell markers, such as cyclooxygenase 1 and 2, and therefore DCAMKL-1 was in fact a marker of differentiated tuft cells rather than an exclusive stem cell marker of the adult intestine (Gerbe et al., 2009).

The leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) was identified as a candidate marker of the intestinal stem cell based on a screen of Wnt-target genes (Barker et al., 2007). Localisation of Lgr5 revealed positively stained cells are confined to the crypt base and Lgr5 expression specifically marks the CBC cell population. Barker and colleagues further investigated the functional properties of Lgr5-positive cells using an inducible Cre-recombinase transgene expressed under the control of the endogenous Lgr5 promoter and the LacZ reporter allele. They demonstrated that the entire crypt-villus was populated with LacZ-expressing cells, suggesting that Lgr5-positive CBC cells are capable of generating all the cell lineages of the intestinal epithelium. This provides functional evidence that Lgr5-positive cells are intestinal stem cells as they are capable to maintain self-renewal and pluripotency for long periods of time. Three additional stem cell markers, Aschaete scute-like 2 (Ascl2), Olfactomedin-4 (Olfm4) and CD133/Prominin1 (Prom1), have been revealed based on a gene expression analysis of Lgr5-EGFP cells (van der Flier et al., 2009; van der Flier et al., 2009; Snippert et al., 2009). Ascl2 is a Wnt target gene that belongs to a member of the basic helix-loop-helix family of transcription factors; Olfm4 encodes an anti-
apoptotic factor that associated with tumour growth, cell adhesion and metastasis whereas Prom1 encodes CD133 protein, which is a cell surface marker of intestinal stem cells. Since all these genes (Ascl2, Olfm4 and CD133) have been found to be co-expressed with Lgr5 and therefore they have been established as highly specific and robust marker of Lgr5-positive CBC cells (Jubb et al., 2006; Barker et al., 2007; van der Flier et al., 2009; van der Flier et al., 2009).

Despite the fact that Barker and colleagues provided convincing evidence of the nature of CBC stem cells, a study conducted by Sangiorgi and Capecchi (2008) demonstrated that Bmi1 (the polycomb group oncogene) also marks true intestinal stem cells, but is mainly expressed in position +4 and +5 cells in the crypt, therefore adding a further layer of complexity to research aimed at identifying the key intestinal stem cell markers. The authors investigated the functional properties of Bmi1-positive cells using an inducible Cre-recombinase transgene expressed under the control of the Bmi1 promoter and the Rosa26-LacZ or Rosa26-YFP transgene, they demonstrated that Bmi1-positive cells were capable of forming long-lived cell clones that contained all of the differentiated epithelial cell linages of the epithelium. Consistent with this, ablation of the stem cell population via selective expression of diphtheria toxin in Bmi1-positive cells resulted in a complete loss of crypt structures (Sangiorgi and Capecchi, 2008). Therefore, these studies provide robust evidence that Bmi-expressing cells play a crucial role in the repopulation of the crypt-villus.

The current understanding proposes that Lgr5 specifically marks the rapidly dividing CBC cell population which is responsible for intestinal homeostasis, while Bmi1 expression mainly detects the more slowly dividing, label retaining +4 position cells, which are considered as quiescent stem cells and are activated in response to radiation-induced tissue injury or apoptotic cell death (Barker et al., 2007; Sangiorgi and Capecchi, 2008). Thus, based on the observed differences in cell cycle kinetics and locations between Lgr5-positive and Bmi-positive cells, over the recent years it has been proposed that these Lgr5 and Bmi1 label distinct populations of stem cells, with Lgr5 being a marker of active CBC stem cells and Bmi1 labeling a quiescent or slow cycling cell population (Tian et al., 2011; Yan et al., 2012).
However, the precise identity and location of the intestinal stem cell is still a matter of debate and a recent study by Buczachi and colleagues demonstrated that the quiescent stem cells gradually committed to the absorptive or secretory cell lineages and matured into Paneth and enteroendocrine cells during homeostasis. Upon intestinal injury by radiation, hydroxyurea or doxorubicin, these quiescent cells can undergo rapid proliferation and self-renewal to regenerate all of the epithelial lineages, suggesting that the quiescent cells can be recalled to the active stem cell state (Buczacki *et al.*, 2013) (reviewed in Barker, 2014). Although the intestinal stem cell field has progressed at an extraordinarily rapid pace, the precise identity and location of intestinal stem cells continues to remain under intense debate. There has not yet been a consensus regarding the ideal marker for intestinal stem cells, there is however a growing support for Lgr5, Ascl2 and Olfm4 to be the key intestinal stem cell markers.

### 1.1.4.2. Maintenance and homeostasis of the intestinal stem cell

The lack of definitive stem cell markers has hindered the progress in understanding the roles of various factors and signaling pathways involved in intestinal stem cell homeostasis. However, a number of indirect evidence has provided insights into the factors and pathways required for stem cell maintenance (Sato *et al.*, 2009). Sato and colleagues generated an *in vitro* system that allows the growth of intestinal organoids in culture, from single mouse intestinal crypt. The intestinal crypts were seeded singly in the laminin-rich Matrigel, which provides the three-dimensional growth mimicking the nature architecture of the intestine and the laminin prevent detachment induced apoptosis (anoikis) in culture (Sato *et al.*, 2009). In order to sustain the long-term growth of the organoid culture the intestinal stem cells require the presence of a number of factors: Wnt agonist R-spondin, Notch ligand Jagged, epidermal growth factor (EGF) and the BMP antagonist Noggin. These named components are sufficient to support the growth of the organoids from single Lgr5 positive cells, therefore further supporting the use of Lgr5 as a marker of the intestinal stem cell population (Sato *et al.*, 2009).
The intestinal stem cell niche, the cellular components in which the intestinal stem cells reside, is thought to provide a range of signals in establishing or maintaining stem cell homeostasis. The mesenchymal cells express a number of basal membrane components such as collagen IV, laminin and nidogen, providing mechanical support and cellular adhesion (Paulsson, 1992). Paneth cells have long been thought to provide stem cell permissive environment due to their expression of Wnt ligands Wnt3 and Wnt11, the EGF receptor ligands EGF and TGFα, the Notch ligand Dll4 and the production of protective antimicrobial factors (Sato et al., 2011a). Factors produced by the mesenchyme and Paneth cells provide an essential niche to support intestinal stem cell in vivo and in vitro culture of intestinal stem cells as outlined earlier (Bevins and Salzman, 2011; Sato et al., 2011a; Sato et al., 2011b). Notably, Lgr5 positive cells intercalated with Paneth cells at the bottom of crypts suggesting that the Paneth cells play a critical role for maintenance of the Lgr5 positive intestinal stem cells (Sato et al., 2011b). Consistent with the notion that Paneth cells are critical for the maintenance of the intestinal stem cell niche, ablation of Paneth cells via conditional knockout of Sox9 gene has resulted in crypt loss occurring with the Paneth cell turnover rate (Sato et al., 2011b). It may seem clear that Paneth cells in the crypt base are the sole niches to provide factors that regulate stem cell populations. However, it has been demonstrated that the knockout of Wnt3 from intestinal epithelium in vivo had no obvious effect on intestinal morphology and phenotype, whereas the loss of Wnt3 in intestinal organoid cultures resulted in organoid death, which is then rescued when co-cultured with mesenchymal cells (Farin, Van Es and Clevers, 2012). This indicates that other functionally important Wnts produced by extraepithelial or stromal niche cells other than the Paneth cell also contribute an essential role in the maintenance of the intestinal stem cell homeostasis.

Despite the contradictory evidence indicating that intestinal stem cells do not solely rely on the presence of Paneth cells to maintain intestinal homeostasis, recent evidence demonstrated that Paneth cells play an important role in supporting intestinal stem cell to repair epithelium after damage. Parry and colleagues generated two conditional mouse models (VillinCre and AhCre, described in section 1.3.2.2) to delete the transcription factor β-catenin within the intestinal epithelium. Rapid recovery and repopulation of the intestine
was demonstrated when the Paneth cells were retained, whereas loss of intestinal epithelial architecture was observed in the absence of Paneth cells, indicating the role of Paneth cells within the in vivo intestinal stem cell niche in aiding damage repair (Parry et al., 2013). Therefore, Paneth cells are an essential niche component that plays a crucial role in governing stem cell behavior not only during intestinal epithelial homeostasis but also during tissue repair and regeneration following injury.
1.2. Colorectal Cancer

1.2.1. Colorectal cancer in humans

1.2.1.1. Colorectal cancer statistics

“Cancer does not discriminate. It knows no nationality, no creed, no gender, no age. It affects all of us alike, not discriminating who or where you are; rich or poor alike.”

(John Kanzius 1944-2009)

It is estimated that more than one in three people will be diagnosed with some form of cancer during their lifetime. Colorectal cancer (CRC) is the third most common cancer type across both genders, after lung and breast cancer in the U.K. (excluding non-melanoma skin cancer). Over 40,700 new cases are diagnosed per year in the U.K., and the incidence of CRC has remained relatively stable for over a decade. Despite the fact that the five-year survival rates for CRC have doubled in the past 40 years, CRC is still the second most common cause of cancer death in the U.K. after lung cancer (Cancer Research UK, 2013).

1.2.1.2. Environmental risk factors

The high incidence of CRC is often attributed to unhealthy lifestyles. Studies have shown CRC risk is positively associated with high consumption of red and processed meats, and inversely associated with fishes, fresh vegetables, dietary fibres and folic acid intake (Norat et al., 2002; Park et al., 2005). Physical inactivity is also known to be a contributory factor, increasing the risk of CRC by up to 24% (Wolin, Yan and Colditz, 2011). Related to the westernised diet and lack of exercise, obesity is also known to be an important risk factor, around 13% of CRC in the U.K. are obesity-related (Murphy et al., 2000) (Cancer Research UK, 2013). Furthermore, smoking and high alcohol consumption has also linked to increase the risk of developing CRC.
1.2.1.3. Genetic risk factors and predisposition

CRCs can be divided into three categories: sporadic, familial and hereditary cases. Over 80% of the CRC cases are sporadic, which is a multistep process caused by accumulation of both acquired genetic and epigenetic mutations in multiple genes such as Adenomatous Polyposis Coli (APC), Kiresten-Ras (K-ras) and Tumour Protein 53 (TP53) (Fearon and Vogelstein, 1990). However, a significant proportion of CRC has a clear genetic background, patients with two or more first-degree (e.g. brothers and sisters) or second-degree (e.g. grandparents) relatives with CRC are considered to be at an increased risk of the disease and this is known as familial CRC. Lastly, hereditary CRC occurs in less than 10% of the CRC cases and the best-known syndromes are familial adenomatous polyposis (FAP), caused by inherited mutation in APC gene, and hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndromes, caused by mutation in one of the DNA mismatch repair (MMR) genes (Stoffel, 2010; Groden et al., 1991)

1.2.1.4. Therapeutic strategies

The course of treatment a patient undergoes is largely dependent on the severity of the tumour stage. Surgery is the cornerstone treatment for early-stage cancer that has not spread to distant sites (stage I-III), additional treatment with radiation and chemotherapy may also be used pre- or post-operation. Chemotherapy is the first treatment option for metastatic disease (stage IV) when tumour lesions are not fully resectable by surgery (NICE Guidelines, 2011). The available treatment modalities for metastatic CRC include cytotoxics (5-Flurouracil, oxaliplatin and irinotecan) and anti-epidermal growth factor agents (cetuximab and panitumumab) (Abad et al., 2008). These are used in combination or sequentially to treat CRC patients. Despite the mortality rates from colon cancer decreasing over the past 30 years, there is still a huge heterogeneity in survival rates (Cancer Research UK 2013). Basic research into intestinal tumorigenesis is critically important for improving future drug designs and development and treatment strategy.
1.2.2. Multi-step development of colorectal cancer

The development of CRC is a step-wise process in which the acquisition of multiple genetic mutations leads to transformation of normal glandular epithelium into invasive adenocarcinoma. This multi-step model was proposed in the study conducted by Fearon and Vogelstein in 1990, in which they comprehensively reviewed the genetic alterations present at various stages of the CRC and they proposed that 4 to 5 mutational steps are necessary to result in colorectal malignancy. These steps involve the gradual accumulation of oncogenes activation and tumour suppressor genes inactivation. This model has become the classical view of CRC initiation and progression and is now commonly referred to as the ‘Chromosomal instability’ (CIN) pathway. The first mutations involved in the stepwise model are in ‘gatekeeper’ tumour suppressor gene, APC, leading to formation of a benign lesion, then subsequent activation of the oncogene KRAS, allelic loss of chromosome 18q locus, and loss of expression of the tumour suppressor p53, all contributing to the progression of a benign tumour to malignant disease (Figure 1.4). However, not all CRCs follow the Fearon-Vogelstein progression pathway as other pathways of colorectal carcinogenesis have also been identified, including the ‘microsatellite instability’ pathway (Kim et al., 1999; Jass, Young and Leggett, 2002). In this report the chromosomal instability pathway will be focused, as it is known to be the most common mutational pathway of colorectal disease.
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![Figure 1.4: Colorectal Cancer is a multi-step process.](image)
The transformation of normal glandular epithelium into invasive adenocarcinoma is a multi-step process accompanied by the accumulation of genetic and epigenetic mutations.
1.2.2.1. **APC and the canonical-Wnt signalling**

The ‘gatekeeper’ tumour suppressor gene, APC, was discovered by genetic linkage analysis in FAP families (Bodmer et al., 1987; Groden et al., 1991; Joslyn et al., 1991). Mutation in the APC gene appears in aberrant crypt foci and early adenomas (Figure 1.2), suggesting that inactivation of APC occurs very early in adenoma formation (Levy et al., 1994; Hocker and Wiedenmann, 1998). As described previously, APC plays a central role in the canonical Wnt signaling pathway, a pathway that provides a key driving force behind sustained proliferation in the crypt and intestinal homeostasis. Although FAP represent a small proportion of all CRC cases, the majority of sporadic CRC show activation of the Wnt pathway and more than 80% of CRC cases are attributed to mutational inactivation of APC (Kinzler and Vogelstein, 1996). Apart from loss of APC function, mutations that stabilize β-catenin have also been observed in 10% of CRC (Morin et al., 1997).

In order to recapitulate FAP symptoms and CRC development in a controlled environment, a number of animal models targeting Wnt pathway components have been generated (reviewed in Heyer et al., 1999). The most well-known animal model, *Min (Multiple intestinal neoplasia)* mice, have been found to carry a nonsense-mutation in codon 850 of *Apc* resulting in a truncated protein. Mice heterozygous for the *APC*<sub>Min</sub> allele have a shortened lifespan and develop multiple adenomas across the gastrointestinal tract (Su et al., 1992). Notably, mouse models with *Apc* inactivation predominantly develop tumours in the small intestine, whereas the majority of *APC*-induced intestinal tumorigeneisis arise in the large intestine in human.

1.2.2.2. **Kras and the RAS-RAF-MAPK signalling**

Mutations in components of the RAS-RAF-MAPK pathway are required for the next step in progression from early adenoma to intermediate adenoma. The Rat Sarcoma viral oncogene homology (RAS) family is comprised of Harvey (H-RAS), Kirsten (K-RAS) and Neuroblastoma (N-RAS). RAS proteins are a group of small G-proteins that is commonly found to be activated in many cancers (reviewed in Bos, 1989). RAS proteins become activated when bound by GTP, which is stimulated by a number of Guanine nucleotide exchange factors (GEFs) that facilitate exchange of GDP for GTP. Once bound by GTP, the
active form of RAS is able to phosphorylate and trigger numerous downstream pathways. The most characterized pathway downstream of RAS is the RAF-MEK-ERK mitogen-activated protein kinase (MAPK) cascade. Phosphorylated MARK kinases are translocated into nucleus where they activate a range of transcription factors resulting in response that drives tumorigenesis.

In human, mutations resulting in constitutively activated K-RAS have been found to occur in approximately 50% of all CRC cases (Figure 1.4) (van Engeland et al., 2002; Sancho, Batle and Clevers, 2004). Additionally, activating mutations in B-RAF gene have been found in 20% of CRC with functional K-RAS (Rajagopalan et al., 2002). Mutations in either K-RAS or B-RAF are commonly regarded to be involved in tumour progression (Fearon and Vogelstein, 1990; Rajagopalan et al., 2002). This notion of K-RAS and B-RAF mutations as drivers of cancer progression has been supported by a number of studies. The study from Sansom showed that following loss of Apc and activation of the Wnt signaling cascade, k-Ras activation accelerated Wnt-driven tumorigenesis and conferred a more invasive phenotype compared to tumours with native k-Ras (Sansom et al., 2006). This study supports the notion that activation of RAS-RAF-MAPK pathway plays a crucial role in cancer progression.

1.2.2.3. Allelic loss of 18q and TGFβ / BMP signalling

The allelic loss of the 18q chromosomal region is the next step toward malignancy, which is found in 70% of carcinomas and 50% of late adenomas (Fearon and Vogelstein, 1990b). The candidate for involvement in colorectal cancer progression found at this locus is the tumour suppressor Deleted in Colorectal Carcinoma (DCC). The DCC gene encodes a receptor for the ligand Netrin that controls axonal growth and cellular migration (Keino-Masu et al., 1996). The role of DCC in cancers remains unclear. Studies of DCC<sup>-/-</sup> mice exhibited marked neuronal abnormalities and died shortly after birth consistent with the previously proposed role of DCC in axon guidance, however DCC<sup>+/</sup> mice survived into adulthood were found to have no predisposition for intestinal tumours. Furthermore, heterozygous DCC loss had no effect on tumour progression or multiplicity in Apc<sup>Min</sup> mice (Fazeli et al., 1997). Fazeli et al suggested that the loss of DCC in human CRC may only be a
consequence of loss of part of chromosome 18q and therefore DCC may not play a role either in initiation or in progression of intestinal disease.

Another gene, which is present in very close proximity (at 18q21.1) to the DCC-encoding gene (at 18q21.3) on chromosome 18 is Smad4 and may link to CRC (Thiagalingam et al., 1996). As previously described in section 1.1.3.3, SMAD4 is the effector protein involved in the downstream signaling of the TGF-β and BMP signaling pathway. Mutations in SMAD4 have been implicated in Juvenile polyposis syndrome (JPS) associated with increased risk of CRC. Approximately 20-25% of JPS cases were attributed to SMAD4 mutation (Howe et al., 2004; Chow and Macrae, 2005). In murine studies, Takaku et al. deleted Smad4 alone and in the context of Apc mutation (Apc is also on chromosome 18 in the mouse). Homozygous loss of Smad4 was found to be embryonic lethal, whereas Smad4 heterozygous mice exhibited little phenotypic differences compared to wild-type littermates. However, mice with heterozygous deletion of Smad4 and Apc enhanced the malignancy of tumours compared to Apc heterozygous control mice. Tumours were found to be increase in size and were more invasive, suggesting that the evidence for the role of Smad4 as a tumour suppressor in CRC is more compelling than that for DCC, especially in the aspect of cancer progression (Takaku et al., 1998).

1.2.2.4. Mutation of p53 and malignancy

Fearon and Vogelstein proposed the loss of short arm of chromosome 17, which harbours the TP53 gene, encoding the tumour suppressor p53, as a final step required for cancer progression towards malignant disease (Fearon and Vogelstein, 1990b). As the first tumour suppressor gene discovered, p53 responds to cellular stresses that cause DNA damage allowing the cell to either repair the damaged DNA, or undergo apoptosis. In normal conditions p53 is rendered inactive by mouse double minute 2 homolog (MDM2), which binds to p53 and mediates p53 ubiquitination causing it to be degraded. Translational modifications of p53 (e.g. phosphorylation and acetylation) induced by cellular stress or damage to DNA cause conformational changes in p53 preventing its association with MDM2, which allow it to translocate into the nucleus and activate transcription of target genes, resulting in cell cycle arrest, stimulation of DNA repair, senescence or cell death by
apoptosis (Vousden and Lane, 2007; Helton and Chen, 2007). Loss of p53 function therefore removes its role as “guardian of the genome”, abolishing the normal regulation for the cell to carry out DNA damage repair or undergo apoptosis before proceeding to proliferate, thereby predisposing the cell to mutation accumulation and neoplastic transformation. Germline mutations of p53 are known to cause the hereditary condition, Li-Fraumeni syndrome (Li et al., 1988). Mutations of p53 are seen in approximately 50% of CRC (reviewed in Iacopetta, 2003). Consistent with the model proposed by Fearon and Vogelstein, p53 mutations have been reported in nearly 50% of carcinomas, while in less than 10% of adenomas, suggesting that the loss of p53 function is normally associated with late-stage carcinomas rather than early adenomas in human (Figure 1.4) (Purdie et al., 1991). Despite the strong association of p53 mutations with CRC in human, mouse models of p53 loss in the context of intestinal tumorigenesis have produced conflicting results. Clarke et al demonstrated that deficiency of p53 in Apc^Min mice (which are predisposed to intestinal tumour formation) did not result in an increase in tumour progression compared to p53 proficient Apc^Min mice (Clarke, Cummings and Harrison, 1995). Consistent with this, Reed et al. showed that conditional loss of p53 has very little effect on the acute activation of the Wnt pathway in the intestine, suggesting no role in early adenoma formation (Reed et al., 2008b). Petitjean et al. observed that in humans, p53 gene often acquires point mutations rendering it unable to activate gene transcription (Petitjean et al., 2007). Therefore Muller el al. compared mice bearing a mutated allele of p53 with heterozygous Apc loss and mice with heterozygous loss of p53 and Apc. They found that the number of intestinal tumours were similar in both the mice with the mutated p53 allele and mice deficient for p53. However in the mice expressing a mutant form of p53, the tumours were found to be more advanced, suggesting that some mutations in p53 cause a gain-of-function in which the mutated p53 is capable of promoting invasion, explaining the reason why the lack of gain-of-function mutation do not establish more malignant tumours in p53 deficient mice (see diagram Figure 1.4) (Muller et al., 2009).
1.3. Modeling colorectal cancer in mouse using conditional transgenesis

Mouse models have provided an important understanding into the development and progression of CRC, these models have also been useful in elucidating molecular pathways and served as important preclinical platforms to verify efficacy of potential novel therapeutic agents. Progress in transgenic technology led to the development of a new generation of models that recapitulate the initiation and progression of CRC in human.

The classic example of the mouse models to demonstrate intestinal tumorigenesis is the Min (Multiple Intestinal Neoplasia) mouse. This model was derived using a random mutagenesis approach by treating a line of mice with the mutagen ethynitrosurea (ENU), which predisposed them to the formation of intestinal tumours (Moser, Pitot and Dove, 1990a). Following the establishment of the Min mouse line, the gene mutation responsible for the phenotype was sequenced and discovered to bear a mutation in the Apc gene, at codon 850 (Su et al., 1992). As aforementioned, Apc mutation is commonly found in human FAP, therefore the $Apc^{Min}$ mouse has since become a model of human hereditary CRC predisposition syndrome FAP. This $Apc^{Min}$ mouse model has made an invaluable contribution to our understanding of the effect of environmental changes, genetic and epigenetic alterations and therapeutic agents on CRC initiation and development (Shoemaker et al., 1997). However, there are substantial differences on tumour distribution and extent of progression to advanced stages between carcinogenesis in $Apc^{Min}$ and human intestine (Heyer et al., 1999). Therefore, more sophisticated mouse models combining mutations in different genes are being constantly developed in order to model the more comprehensive aspects of tumour development and progression in human.
1.3.1. Constitutive transgenesis

Targeted transgenic technology in mice came in 1987 with the generation of targeted deletion of the hypoxanthine-guanosine phosphoribosyl transferase (HPRT) gene (Kuehn et al., 1987). Since then, numerous mouse models of constitutive gene deletion have been generated providing insights into the in vivo function of a number of genes in various conditions. Although constitutive transgenesis has been a valuable research tool allowing studies into the physiological role of numerous genes, some important limitations associated with constitutive gene loss also exist.

One of the biggest limitations of constitutive models is that the absolute requirement of many genes in the germ line for normal embryonic development. Around 15% of gene knockouts are embryonic lethal, which means the generation of an adult mouse deleted for these genes is impossible. This makes it difficult to study cancer biology, as many known tumour suppressor genes and oncogenes are vital for embryonic development. The second limitation is that constitutive knockout approach produces complete targeted gene deletion in the whole organism. This causes issues to study the effects of gene loss in a particular tissue if severe phenotypes occurring elsewhere in the organism. Finally, constitutive gene deletion is likely to affect the interaction between different cell types and also it may potentially mask the true cellular role of a gene in specific cell types due to gene redundancy or a compensatory mechanism within the organism.

In order to overcome the limitations imposed by approaches utilizing constitutive transgenesis, conditional transgenic models have been developed, thereby enabling an array of genes to be targeted in specific tissues.

1.3.2. Conditional transgenic models

The use of conditional transgenic models allows temporo-spatial control of gene deletion or expression in transgenic animals. The ability to switch gene expression ‘on’ or ‘off’ in targeted tissues at specific times allows unprecedented flexibility for exploring gene function in cancer initiation, development and progression.
A number of conditional transgenic approaches have been designed to provide the temporally ‘switchable’ mechanism that consists of two principal elements: an inducible ‘effector’ transgene and a ‘target’ transgene. Examples of these approaches used in murine models include Cre-loxP (Causes recombination (Cre) – Locus of crossover of bacteriophage P1 (loxP)), Flp-Frt (Flp-Flp recognition target) and TetO (tetracycline operator) techniques. The Cre-loxP system will be the focus of this thesis, and is described in detail below.

1.3.2.1. The Cre-loxP system

The Cre-loxP system operates as a binary mechanism. The effector component is represented by Cre recombinase, which is a 34 bp long nucleotide sequences isolated from bacteriophage P1 (Abremski and Hoess, 1984). It is a site-specific DNA recombinase which binds to DNA recognitions sequences called loxP (Hoess, Ziese and Sternberg, 1982; Sternberg and Hamilton, 1981), yielding the recombined DNA product. Depending on the loxP sites orientation, recombination results in either inversion or excision of the DNA fragment surrounding between the loxP sites. When loxP sites are inserted unidirectionally surrounding the entire gene, those genes have been targeted for recombination. When the gene is exposed to Cre recombinase, the region between loxP sites is excised or floxed out, rendering the gene completely inactive. Activation of a transgene can also be achieved using the Cre-loxP system; in this case, a transcription terminating Stop-cassette flanked by loxP sites is inserted before the gene of interest. Upon exposure to Cre recombinase, the loxP flanked Stop cassette is eliminated and the expression of the transgene of interest is resumed.

The versatility of this approach does come with some limitations. While the traditional constitutive knockout system produces complete gene deletion in the organism, the Cre-loxP approach may result in incomplete targeted gene excision. The quality of gene excision reflects not only the extent of Cre recombinase expression but also the chromosomal environment of the targeted gene. Epigenetically, floxed loci in the open and active chromatin state will be more accessible to Cre recombinase and therefore susceptible to site-specific recombination than loci in the condensed and inaccessible state. Partial gene excision produces an animal that is mosaic, although in some cases this Cre-mediated
mosaicism has been exploited and it has been believed to be one of the benefits of the system (Betz et al., 1996). On the other hand, it has been proposed that expression of Cre recombinase in mammalian cells may promote genomic instability by introducing DNA double-stranded breaks (Thyagarajan et al., 2000).

### 1.3.2.2. Post-translational regulation of Cre recombinase activity in the intestine

Post-translational regulation of Cre recombinase activity allows the combination of cell-type or tissue-specificity with inducibility under temporal control. The most common approach to this is the use of a fusion protein of Cre and a modified Estrogen receptor (ER) (el Marjou et al., 2004a; Feil et al., 1996). In general, the fusion CreER protein is sequestered in the cytoplasm preventing it from accessing loxP sites in the nucleus. However, in the presence of the ER antagonist Tamoxifen, the ER domain of the fusion protein is bound by Tamoxifen, thereby exposing the nuclear localisation signal. This facilitates the CreER fusion protein to translocate into the nucleus, where it recognises and recombines the loxP sites.

Expression of CreER fusion protein under the control of a tissue-specific promoter provides a sophisticated means of tissue-specific inducible deletion or activation of a targeted gene of interest. For example, in the intestine, the Villin promoter has been coupled to a CreER fusion protein to allow temporal control over Cre recombinase expression within the intestinal epithelium (el Marjou et al., 2004a). Another promoter also used in the intestine is AhCreER, in which control of Cre recombinase expression is regulated at two levels: by transcriptional control of the Ah promoter (activated by β-napthoflavone) and by a requirement for Tamoxifen binding. Expression of Cre recombinase from AhCreER mice was detected in the intestine, liver, pancreas, gallbladder, and stomach (Kemp et al., 2004).
1.4. Epigenetics and Cancer

“The major problem, I think, is chromatin. What determines whether a piece of DNA along the chromosome is functioning, since it’s covered with the histones? You can inherit something beyond the DNA sequence. That’s where the real excitement of genetics is now”

(Watson and Crick 2003)

Although genetic mutations have been the focus of cancer research for many years, epigenetic alterations have been increasingly recognised to also play a major role in tumorigenesis. The progression from a microadenoma to carcinomadenoma in CRC may take decades, supporting the notion that accumulated genetic and epigenetic alterations underlie the multistep developmental process of CRC (Figure 1.4). Epigenetic modifications have been proposed to contribute to CRC formation through the activation on oncogenes (gain of gene function) and the inactivation of tumour suppressor genes (loss of gene function) that regulate signalling pathways that control hallmark behaviours of cancers. Among all the epigenetic alterations in CRC, the most extensively characterised are promoter hypermethylation of MLH1, APC, RB1, VHL, MGMT, GSTP1, and BRCA1 genes, representing paradigmatic cancer-related epigenetic silencing of tumour suppressor genes (Knudson, 2001).

The term ‘epigenetics’ was first proposed by Conrad Waddington in 1939 to describe ‘the causal interactions between genes and their products, which bring the phenotype into being’ (Waddington, 1939). The current definition of epigenetics refers to the study of heritable changes in gene expression occurring without changes in primary DNA sequence (Sharma, Kelly and Jones, 2010). Three different yet highly interdependent epigenetic mechanisms have been recognised: (1) histone modification, (2) RNA interference and (3) DNA (de)methylation (Figure 1.5). In the context of this thesis, we mainly focus on histone modification.
Figure 1.5: Epigenetic modifications. Top: Histone modifications, covalent modifications of N-terminal tails of histones; Middle: RNA interference, maintenance of gene transcription in a heritable way in the form of small RNAs (microRNA); Bottom: DNA methylation, a covalent modification of the cytosine in a CpG dinucleotide (Sawan et al., 2008).
1.4.1. **Nucleosome and Post-translational Histone Modifications**

In eukaryotic cells, DNA is packaged into a compact chromatin structure. The basic chromatin unit, the nucleosome, comprises approximately 147 base pairs of super-helical DNA wrapped around an octamer of two histone H2A/H2B dimers and two histone H3/H4 dimers. The linker histone H1 binds to short linker DNA between nucleosomes. These can then be further packed into higher order chromatin structures. The crystal structure of the nucleosome core has been resolved, revealing that the N-terminal histone tails emanating from the core (Luger et al., 1997), making them subject to a variety of reversible covalent post-translational modifications which alter their interaction with DNA and other nuclear proteins.

Nucleosome density and access to the genomic DNA in eukaryotic cells is highly regulated in order for processes such as DNA damage repair, replication, transcription, and mitosis to occur at the right time and place (Sawan et al., 2008). One of the primary mechanisms regulating access to the DNA is post-translational histone modification (PTM) (Goldberg, Allis and Bernstein, 2007). The PTMs identified so far include acetylation and methylation of lysines (K) and arginines (R), phosphorylation of serines (S) and threonines (T), ubiquitination, sumoylation and biotinylation of lysines, ADP ribosylation, deimination and proline isomerisation (Kouzarides, 2007) (Figure 1.6). Histone modifications are effected by ‘writer’ enzymes: Histone acetyltransferases (HATs), histone methyltransferase (HMTs), and histone kinases; whereas Histone deacetylases (HDACs), histone demethylases (HDMs) and histone phosphatases are ‘eraser’ enzymes reversing the modification marks.

The combination of histone modifications constitutes the histone “code”, which influences chromatin condensation and the ability of various chromatin interacting proteins to recognise the presence and absence of specific PTMs, thereby inducing distinct downstream effects (Sawan and Herceg, 2010).
Figure 1.6: Schematic representation of core histone post-translational modifications (Adapted from Shechter et al., 2007).

1.4.2. The Role of Histone modification in Transcriptional control: Euchromatin and Heterochromatin

Histone modifications help to subdivide the genome into distinct domains such as euchromatin, characterised as opened and accessible state of the DNA, and heterochromatin, described as condensed and repressive state of the DNA, which is in general transcriptionally inactive (Jenuwein and Allis, 2001). There are two major subtypes of heterochromatin: constitutive and facultative. Constitutive heterochromatin describes regions that contain a high density of repetitive DNA elements and transposable elements, such as centromeres and telomeres (Grewal and Jia, 2007). Facultative heterochromatin, in contrast, refers to developmentally regulated regions where states can be converted between euchromatin and heterochromatin. This therefore can switch on or off transcription at specific times, such as during certain developmental phases of the cell cycle.
(Trojer and Reinberg, 2007). Besides the roles in gene silencing, heterochromatin also plays a role in genomic stability. Previous studies have demonstrated that animal with mutated genes that regulate heterochromatin structure increased spontaneous DNA damage in heterochromatin of somatic and meiotic cells (Peng and Karpen, 2009). Heterochromatin formation is also essential for maintaining the stability of repeat DNA sequences, presumably by suppressing inappropriate homologous recombination (Peng and Karpen, 2007). Reduced levels of heterochromatin in mice and Drosophila have also been demonstrated to have higher levels of spontaneous DNA damage, chromosomal instability, and exhibit defects in chromosomal compaction and segregation during mitosis (Yan et al., 2011).

Transcriptionally active euchromatin has been found to be associated with methylation of lysines 4, 36, and 79 on histone H3 (Gerber and Shilatifard, 2003). On the contrary, methylation of lysines 9 and 27 on histone H3 and lysine 20 of histone H4 has been strongly linked with less transcriptionally active and more condensed heterochromatin (Kouzarides, 2007).

Mutations in various factors involved in chromatin formation and maintenance have been associated to the development of disease, in particular cancer (Ko, 2008). These mutations either result in the repression of normally active genes (e.g. tumour suppressor genes) or the activation of normally silent genes (e.g. oncogenes).

Connections have been identified for various modification and at all levels of chromatin formation and maintenance: for ‘writiers’ such as H3K27 methyltransferase EZH2 which is overexpressed in metastatic prostate cancer (Varambally et al., 2002), ‘readers’ such as HP1γ which is upregulated in various tumours and is identified as a putative biomarkers for osteosarcoma and prostate cancer (Slezak et al., 2013; Saini et al., 2012), ‘erasers’ such as HDAC1 which is aberrantly recruited to promoters due to fusion with DNA-binding proteins (Lin et al., 1998), and for chromatin remodelers such as NuRD complex where its ATPase subunit is deleted in certain neuroblastomas (Bagchi et al., 2007). The multitude and variety of these connections foreshadows the enormous potential that intervention with these processes may have for the treatment of cancer. However, it is clear at this point that a deeper understanding of the epigenetic mechanisms regulating...
chromatin structure and function and the specific mechanisms of chromatin-dependent tumourigenesis is required, and much basic research will be absolutely necessary to achieve the full potential of chromatin-based therapeutics. The close relationships between chromatin and cancer make such research imperative.

Having outlined the basic principles of chromatin biology, the following section is a more detailed view of one specific non-histone component of chromatin: Heterochromatin protein 1, the chromatin effector protein that was at the centre of my PhD research.

1.4.3. Structure and Function of Heterochromatin Protein 1

Heterochromatin protein 1 (HP1) was first identified by screening a monoclonal antibody panel generated against a fraction of Drosophila non-histone chromosomal proteins associated with heterochromatin. Immunofluorescence staining of polytene chromosome revealed that HP1 localised to centric heterochromatin, telomeres and approximately 200 euchromatic regions (James and Elgin, 1986). A few years later the gene encoding HP1, SU(VAR)2-5, was found as a suppressor of position-effect variegation (PEV) (Eissenberg et al., 1990). HP1 is phylogenetically highly conserved among all eukaryotes including S. pombe, M. musculus and H. sapiens (Eissenberg et al., 1990; Singh et al., 1991; Ye and Worman, 1996). Mammalian cells contain at least three different isoforms of HP1, in these humans are named HP1α, HP1β and HP1γ (Lomberk et al., 2006). The three human HP1 proteins are encoded by the chromobox homolog (CBX) genes: CBX5 (HP1α), CBX1 (HP1β) and CBX3 (HP1γ) (Ye and Worman, 1996). The translated human HP1α, HP1β and HP1γ amino acid sequences are 191, 185 and 173 residues in length respectively, and in general, HP1α and β are localised in heterochromatin, whereas HP1γ is present in both heterochromatin and euchromatin (Minc, Courvalin and Buendia, 2000) (Table 1.1).

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Gene</th>
<th>Location(s)</th>
<th>Knock-out</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP1α</td>
<td>CBX5</td>
<td>Heterochromatin</td>
<td>Same as wildtype</td>
</tr>
<tr>
<td>HP1β</td>
<td>CBX1</td>
<td>Heterochromatin</td>
<td>Lethal when born</td>
</tr>
<tr>
<td>HP1γ</td>
<td>CBX3</td>
<td>Heterochromatin and Euchromatin</td>
<td>Lack of germ cells</td>
</tr>
</tbody>
</table>

Table 1.1: The localisation of HP1 family (Brown et al., 2010a).
All HP1 proteins identified in eukaryotes share a well-conserved architecture consisting of an amino-terminal chromodomain (CD) and a carboxy-terminal chromoshadow domain (CSD) linked by a flexible hinge region (Aasland and Stewart, 1995; Kellum, 2003). The CD is a chromatin-binding module that specifically recognises and binds to heterochromatin (Ball et al., 1997). The CSD is primarily involved in homo- and/or heterodimerisation and interaction with other proteins (Thiru et al., 2004) (Figure 1.7).

### 1.4.3.1. The chromo domain

The amino-terminal chromodomain (chromatin organisation modifier) is identified in MOF (Males Absent on the First), HP1, Pc (Polycomb) and CHD1 (chromo-ATPase / helicase-DNA-binding protein) (Pearce, Singh and Gaunt, 1992). The functions of the CDs are diverse between different proteins. For instance, MOF associates with the X-chromosome via the interaction of its CD with roX2 RNA that is enriched on the male X-chromosome.
(Akhtar, Zink and Becker, 2000). On the other hand, the CD of HP1, Pc and CHD1 interact with methylated lysine residues on histone tails in heterochromatin, while the most studied CD is that of HP1, which has been demonstrated to interact specifically with the di- and tri-methylated lysine 9 of histone H3 (H3K9m2 and H3K9me3) (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002). Previous studies support that HP1 and H3K9me2 co-localise extensively, but not exclusively on polytene chromosomes (Li, Yi and Makova, 2002). In addition, mutations in a conserved valine 26 to methionine in the CD in Drosophila abolished the ability of HP1 to bind to the H3K9me2. Consequently, this resulted in delocalization of HP1 from the chromosome, leading to disruption of gene silencing and late larval lethality (Jacobs et al., 2001; James et al., 1989).

The interaction with the methylated H3K9 is not the only binding partner of the HP1 CD. Mouse HP1β was demonstrated to interact with the nuclear envelope proteins through the CD, and the interaction with lamina-associated polypeptide 2β (LAP2β) and lamin B receptor (LBR) was mapped to the CD, suggesting HP1 may play a role in nuclear envelope reassembly and architecture (Kourmouli et al., 2000). Furthermore, the CD of mammalian HP1γ binds to the retinoblastoma protein (Rb) as demonstrated by yeast-two-hybrid analysis and immunoprecipitation (Williams and Grafi, 2000). Therefore, the HP1γ is thought to target to the CyclinE promoter to silence gene expression (Nielsen et al., 2001a; Nielsen et al., 2001b)

### 1.4.3.2. The hinge region

The hinge region is highly flexible and allows the CD and the CSD to move independently to each other. The hinge of HP1 interacts with RNA and histone H1 in Drosophila (Nielsen et al., 2001a) and mouse, and histone deacetyltransferase (HDACs) in humans (Zhang, McKinsey and Olson, 2002). Interaction between the hinge of HP1 with H1 and HDACs may correlate with the formation of higher order chromatin structures, gene repression and silencing. In addition, phosphorylation of the hinge region is dynamic through the cell cycle and is believed to play a role in HP1-mediated gene silencing (Zhao, Heyduk and Eissenberg, 2001). Furthermore, phosphorylation of serine 83 in HP1γ confers an interaction with Ku70 (involved in DNA double stranded break repair and telomere
maintenance) and disrupts silencing when tethered by GALA-DNA binding domain suggesting an important role of dynamic phosphorylation and dephosphorylation in HP1 binding and gene silencing (Lomberk, Wallrath and Urrutia, 2006).

1.4.3.3. The chromo shadow domain

The Carboxy-terminal CSD is structurally similar to the CD and it is reported to be responsible for homodimerization of HP1 through an alpha-helical region (Brasher et al., 2000; Jacobs et al., 2001). Homodimerisation also creates a hydrophobic platform for interaction with a variety of nuclear proteins such as the pentapeptide motif PxVxL-containing proteins (Cowieson et al., 2000). Many proteins interact with the HP1 family through the PxVxL, including KRAB-associated protein (Kap1) and chromatin assembly factor 1 subunit p150 (CAF1) involved in gene silencing. These PxVxL-containing proteins possess a diverse range of functions including chromatin remodeling, DNA replication and transcription regulation.

On the other hand, there are also several nuclear proteins that interact with the HP1 CSD through non-PxVxL motifs. For example, Brg1 (ATPase subunit in SW1/SNF chromatin remodeling complexes) has been shown to interact with mammalian HP1y by co-immunoprecipitation (Nielsen et al., 2002a). In addition, the histone methyltransferase SU(VAR)3-9 associates with HP1 both in vitro and in vivo through a CSD dimer. Ku70 interacts with HP1α through a 28 amino acid leucine-serine (LS) repeat of Ku70 and the CSD of HP1α. Therefore, the CSD of HP1 allows interaction with different pentapeptide-motif proteins as well as non-PxVxL containing proteins ranging from chromatin modifiers, transcriptional activators and transcriptional repressors (Thiru et al, 2004).
1.4.4. Localisation and function of HP1 family

In recent years, great progress has been made in our understanding of the general principles how chromatin is organised and functions within the cell. However, there are still many functional questions in HP1 biology that have not been understood. For example, a full understanding of the different roles of the three HP1 isoforms, of the regulation of HP1’s diverse molecular interactions, or of the exact molecular mechanism(s) of HP1-mediated gene silencing or activation, splicing, DNA damage repair and telomere maintenance will still require plenty of research.

It has been established that each HP1 isoform exhibits distinct localization patterns on chromatin. HP1α and β are generally localised in heterochromatin, whereas HP1γ is present in both heterochromatin and euchromatin (Minc, Courvalin and Buendia, 2000). The localisation of HP1 appears to be dynamic and cell cycle dependent (Dialynas et al., 2007). The main mechanism localising HP1 within centric heterochromatin is via its CD interaction with H3K9me3. Studies have demonstrated that this HP1-methylated-H3K9 binding can be abolished by either phosphorylation of H3S10 or phosphorylation of H3S10 associated with acetylation of H3K14 (Hirota et al., 2005; Fischle et al., 2005; Mateescu et al., 2004). It has been reported that HP1α specifically binds to the H3Y41 region via its CSD and phosphorylation of H2Y41 by JAK2 leads to dissociation of HP1α from chromatin (Dawson et al., 2009). The versatile and dynamic localisation behaviour of HP1 remains unclear and further complexities occur in relation to the length of time that HP1 binds to different regions in different contexts. Alternative mechanisms defining the sub-nuclear localisation of HP1 comprised of post-translational modification of the HP1 protein itself, interactions between the CSD and other transcription factors, and direct binding of HP1 to DNA and RNA transcripts (Badugu, Shareef and Kellum, 2003; Piacentini et al., 2003).

The most studied function of HP1 is in the heterochromatin formation. The underlying basis for formation of heterochromatin and gene silencing revolves around interactions between the SU(VAR)3-9, HP1 and the H3K9me3 modification. Trimethylation of Histone H3K9 is mediated by SU(VAR)3-9, the CD of HP1 is a binding module for H3K9me3 while SUR(VAR)3-9 itself interacts with HP1 via the CSD. Once formed, this complex then allows the spreading of heterochromatic states and mediates silencing and proper
chromatin compaction (reviewed in Fanti and Pimpinelli, 2008). Evidence implicates a pivotal role for HP1 proteins in the assembly and spread of heterochromatin containing H3K9 methylation. In the study, Canzio and colleagues demonstrated that the S. pombe HP1 homolog, Swi6, recognises the H3K9me3 site at the interface of two CDs. This binding causes Swi6 to tetramerise on a nucleosome, generating two CD sticky ends, which can bridge nearby methylated nucleosomes, enhancing silencing and heterochromatin spread in vivo (Canzio et al., 2011).

In addition to being required for heterochromatin formation and gene silencing, HP1 proteins are reported to play roles in the regulation of gene expression at multiple euchromatic regions. Evidence has accumulated to implicate HP1 proteins as a positive regulator of gene expression at a subset of euchromatic loci. In Drosophila, high-resolution genome-wide mapping experiments have revealed HP1 is associated with transcriptionally active chromatin (de Wit, Greil and van Steensel, 2007). Detailed mapping studies of HP1 have demonstrated its enrichment at developmentally regulated genes and at heat-shock-induced chromosomal puff loci, which are structures with intense gene activity (Piacentini et al., 2003; Cryderman et al., 2005). Furthermore, Drosophila HP1γ homolog, HP1c, has been shown to interact with Facilitates Chromatin Transcription (FACT), which is a histone chaperone complex. It was demonstrated that HP1c guides the recruitment of FACT to active genes and links FACT to active forms of RNA polymerase II. The recruitment of FACT into heat-shock loci is impaired in the absence of HP1c and subsequently causes a defect in heat-shock gene expression (Kwon et al., 2010). In murine cells, HP1γ was observed to localise to the coding regions of several genes (Vakoc et al., 2005a). In addition, HP1γ has been implicated in alternative splicing for a subset of genes (Saint-Andre et al., 2011). Recent published data has indicated a role for HP1γ in aiding in efficient co-transcriptional RNA processing through recruitment of the splicing machinery. Depletion of Cbx3 diminished the recruitment of splicing factors such as SNRNP70 and SRSF1 to gene bodies, resulting in RNA processing defect and subsequently leading to an increase in unspliced transcripts of its target genes (Smallwood et al., 2012). Collectively, these studies implicate a role of HP1 proteins in positive gene expression and RNA splicing.

Furthermore, emerging evidence indicates that HP1 proteins play roles in the DNA damage response (DDR). A number of studies have established that the compact
architecture of heterochromatin is structurally inhibitory to DDR. Decreasing the levels of HP1 results in relaxation of the chromatin structure, thereby allowing a chromatin dynamic that facilitates bypassing the requirement for ATM signalling and enhances the efficiency of DNA repair (Ayoub et al., 2008). All three isoforms of HP1 have been shown to be recruited to UV-induced DNA lesions in both human and mouse cells (Dinant and Luijsterburg, 2009), and *C. elegans* possessing HP1 knockout are more susceptible to UV-induced DNA damage (Luijsterburg and van Attikum, 2011). It is the CD of HP1 that appears to be the most important component for this function (Dinant and Luijsterburg, 2009).

Although the majority of information from the literature studied the members of HP1 family as a whole this could be misleading, as there is little redundancy among the HP1 family. Knockout of HP1β, but not HP1α, was associated with genome instability in mice while depletion of HP1γ, but not HP1α or β, led to mitotic defects in human tissue culture cells (Aucott et al., 2008). In addition, dysfunction of HP1α and HP1β but not HP1γ plays a crucial role during the process of tumorigenesis (Sharma et al., 2003a) and the down-regulation of HP1α but not HP1β and HP1γ is implicated in invasive and metastatic phenotype of breast cancer (Kirschmann et al., 2000). Thus, there are substantial functional differences among HP1 variants. Furthermore, in many cases, the same HP1 variant is implicated in multiple functions.

### 1.4.5. Heterochromatin protein 1 gamma and Cancer

In the past few years, several studies have implicated genetic alteration of HP1γ in the development of several types of human cancers. HP1γ is frequently up-regulated in many cancer cell lines, including colon, cervix, stomach, lung and myxoid liposarcoma (Takanashi et al., 2009). Enhanced expression of HP1γ was detected in peripheral blood leukocytes of patients with advanced leukaemia compared to those from normal donors (Popova et al., 2006). Similar increases in HP1γ protein level were observed in seminoma tumours, which are germ cell cancers of the testes (Bartkova et al., 2011). Additionally, expression level of *Cbx3*, the gene that encodes for HP1γ, was markedly increased in lung cancer tissues compared to normal lung tissues, suggesting that *Cbx3* may be a promising candidate gene in lung adenocarcinoma (Han et al., 2014). Silencing of *Cbx3* in selected
cancer cell lines causes the inhibition of growth. Consistent with this, the deletion mutant of HP1γ lacks tumorigenesis activity in a mouse xenograft model (Sharma et al., 2003). Recent evidence has shown that HP1γ is absent in differentiated cells of various normal human tissues, suggesting that the loss of HP1γ is required for cell differentiation to occur (Takanashi et al., 2009). In HP1γΔ mutant embryos, the number of primordial germ cells (PGCs) was reduced dramatically and this could be caused by inhibition of G1/S phase transition, suggesting that HP1γ plays an important role in maintaining enough germ cells by regulating the PGC cell cycle (Abe et al., 2011). Cbx3 has been documented to negatively regulate adipocyte differentiation from preadipocyte cells and is required for male germ cell survival and spermatogenesis (Brown et al., 2010). HP1γ has been implicated to be associated with Suv4-20h1 and Suv4-20h2 (Schotta et al., 2004) and directly affects the methylation level of histone H4K20 and consequently regulates gene expression epigenetically. Molecular interaction between HP1γ and histone H4K20 is expected to be a key mechanism of cell differentiation. In addition, HP1γ is reported to have a role in alternative splicing through recruitment of the splicing machinery and defective mRNA splicing contribute to cancer development (Saint-Andre et al., 2011). A summary of the proposed functions of HP1γ is outlined in Figure 1.8. The studies mentioned here demonstrate that HP1γ in cancer is often up-regulated and absence of HP1γ is required for cells to differentiate, together these may suggest that the expression of HP1γ may contribute to cancer progression.

The involvement of HP1γ in human cancer underlines the importance of learning more about this important chromatin protein. A better understanding of the multiple roles that HP1γ plays in the process of cancer development will not only enormously contribute to our understanding of chromatin but may also lead to insights that could one day be translated for the benefit of human patients in the clinic.
Figure 1.8: A summary of the proposed functions of HP1γ.
1.4.6. Preliminary results of HP1 gamma studies

Prior to my PhD project, preliminary work conducted by Dr. Karen Reed and Dr. John Jenkins (Liverpool University) examining Cbx3 expression by qRT-PCR analysis using samples from human colorectal cancer patients, demonstrated a significant increase in Cbx3 expression (3.03 medium fold change) compared to normal tissues (Figure 1.9).

![Figure 1.9: Cbx3 specific qRT-PCR expression analysis.](image)

qRT-PCR expression analysis using tumour samples from 18 CRC patients compared to matched normal tissues, shows a significant increase in Cbx3 expression tumour samples (Medium fold change 3.03, Wilcoxon Signed Rank Test: Significant with P = 0.0001). Data generated by Dr. Karen Reed and Dr. John Jenkins.

Additionally, a microarray expression analysis and differential iTRAQ-QSTAR protein profiling was carried out and identified Cbx3 / HP1γ to be up-regulated in the mouse model of colorectal cancer (Hammoudi et al., 2013). Cbx3-specific qRT-PCR expression revealed a significant increase in Cbx3 expression (2.5 medium fold change) following the conditional loss of Apc in intestinal tissues. Consistent with this result, HP1γ-specific Western blot analysis demonstrated an increase in the level of HP1γ protein following the conditional loss of Apc in intestinal tissues (Figure 1.10).

Furthermore, the Cbx3 gene is over-expressed and ranked within the top 10% of gene changes in all but one of the colorectal cancer data sets on the online cancer microarray database, Oncomine. Collectively, these data highlight an interesting increased
trend in HP1γ levels in colorectal cancer, suggesting that HP1γ may be an effective target for gene therapy against human colorectal cancer.

**Figure 1.10:** Over-expression of Cbx3/HP1γ following the conditional loss of Apc in intestinal tissues of mice models.

LEFT: Cbx3-specific qRT-PCR expression analysis in normal and Apc-deficient intestinal tissues from mice model. Cbx3 is significantly increased in intestinal tissues of Apc-deficient models compared to wildtype (Medium fold change 2.5-fold over-expression). RIGHT: HP1γ-specific Western Blot Analysis in wildtype and Apc-deficient intestinal tissues from mice model. Data generated by Dr. Karen Reed.
1.5. Project aims

A growing body of evidence indicates that Cbx3 functions as a driver in cancer cell growth. This is supported by our preliminary observations that over-expression of HP1γ (coded by the gene Cbx3) was detected in tumour samples of colorectal cancer patients compared to matched normal tissues. In concordance, marked increase in HP1γ level was observed in mouse models of colorectal cancer. Thus accumulating evidence strongly implicates HP1γ in cancer development. The principle aim of this study is to characterise the effects of homozygous Cbx3 deletion on normal intestinal homeostasis and acute Wnt-activated intestines, and the long-term implications of Cbx3 loss on Wnt-driven tumorigenesis to assess if Cbx3 loss would attenuate Wnt-driven cancer progression in mouse models of colorectal cancer. Project plans are summarised in Figure 1.11. It is envisaged that more can be learned about the role of HP1γ in colorectal cancer progression and this research will contribute to our knowledge of the phenotypic and mechanistic basis of colorectal cancer progression, and could potentially pave the way for more effective treatments to be developed.
### Chapter 1: General Introduction

<table>
<thead>
<tr>
<th>Strategy</th>
<th>To study the immediate intestinal phenotypes following Cbx3 loss</th>
<th>To study the effect of Cbx3 loss in the Wnt-activated small intestines</th>
<th>To study the delayed phenotypes, including adenoma formation in APC&lt;sup&gt;Cre&lt;/sup&gt;+, in conjunction with the loss of Cbx3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype</strong></td>
<td>Cre&lt;sup&gt;+&lt;/sup&gt; LacZ&lt;sup&gt;+&lt;/sup&gt; Cbx3&lt;sup&gt;+/−&lt;/sup&gt; APC&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Cre&lt;sup&gt;+&lt;/sup&gt; LacZ&lt;sup&gt;+&lt;/sup&gt; Cbx3&lt;sup&gt;−/−&lt;/sup&gt; APC&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Cre&lt;sup&gt;+&lt;/sup&gt; LacZ&lt;sup&gt;+&lt;/sup&gt; Cbx3&lt;sup&gt;−/−&lt;/sup&gt; APC&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Induction</td>
<td>4 daily intraperitoneal injections of 80mg/kg Tamoxifen at 8 weeks of age</td>
<td></td>
<td></td>
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<tr>
<td><strong>Endpoints</strong></td>
<td></td>
<td>Day 4 Various time points</td>
<td>Various time points Age to Day 500</td>
</tr>
<tr>
<td>Day 4</td>
<td>Day 7</td>
<td>Day 22</td>
<td>Day 366</td>
</tr>
<tr>
<td><strong>Phenotype Characterisations</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>A.</strong> Histological and immunohistochemical status:</td>
<td></td>
<td></td>
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<tr>
<td><strong>B.</strong> Differentiation status:</td>
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<td><strong>C.</strong> Migration rates along the crypt-villus axis:</td>
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<tr>
<td><strong>D.</strong> QRT-PCR and immunohistochmistry expression analysis for:</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>A panel of intestinal stem cell markers and Wnt, Noth, TGF-β, PI3K signaling target genes.</td>
</tr>
<tr>
<td><strong>Mechanistic studies</strong></td>
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<tr>
<td><strong>A.</strong> Transient knock down of Cbx3 using siRNA and Topflash assay.</td>
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<td></td>
<td></td>
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<tr>
<td><strong>B.</strong> Co-localisation studies using immunofluorescence.</td>
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<tr>
<td><strong>C.</strong> RNA-sequencing</td>
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</tr>
</tbody>
</table>

**Figure 1.11: Summary of project plan.**
Chapter Two

Materials and Methods
2. Materials and Methods

2.1. Generation of Experimental Mouse Models

The Cbx3 transgenic mice with the loxP flanked Cbx3 alleles (Cbx3^{fl/fl}) (Brown et al., 2010) were obtained from Prim B. Singh (Research Center Borstel, Germany) and crossed with VillinCreER\(^{+}\)APC^{fl/fl} mice (Shibata et al., 1997) to investigate their compound effects on intestinal homeostasis and tumorigenesis (Figure 2.1). The Cre-LoxP (Cause Recombination Locus Of X-over P1) recombinase system allows recombination of DNA in the intestine at a specified time point and therefore was employed for this study. The VillinCreER transgene (el Marjou et al., 2004) was induced through the intraperitoneal (IP) injection of Tamoxifen once daily, for four consecutive days, at a dose of 80 mg/kg dissolved in corn oil (Sigma). The extent of Cbx3 deletion was confirmed using the Rosa26-floxed-stop-LacZ surrogate reporter and by Western blot and immunohistochemistry. All experimental procedures were conducted in accordance with the UK Animals Act 1986 and current UK Home Office regulations under valid personal (PIL: 30/9727) and project licenses (PLL: 30/2737).

![Graphical representation of Cre-LoxP targeted alleles.](image)

(A) Two loxP sites are placed either side of exon 14 of the Apc gene. (B) Two loxP sites are placed in the Cbx3 gene so that they flank exons 2 and 3. In this inducible Cre recombinase system, intraperitoneal injection of Tamoxifen will cause excision of the floxed sites.
2.1.1. Animal Husbandry

2.1.1.1. Colony maintenance

Mice were housed in a standard facility and had access to the standard diet (Special Diets Service UK, RM3(E)) and fresh drinking water.

2.1.1.2. Breeding

All mice were maintained on an outbred background. Adult animals (6 weeks and older) of known genotype were generally bred in trios (one male with two females). After birth, pups were left with their mothers until capable of feeding independently then they were weaned, usually at four weeks of age.

2.1.1.3. Identification and ear biopsies

At weaning age, mice were sexed and separated. Ear clipping was carried out for identification purposes. I would like to acknowledge Matt Zverev and Elaine Taylor for their technical assistance with ear clipping. The resulting ear pinna was used for DNA extraction and genotyping (see below).

2.1.2. Experimental Procedures

2.1.2.1. Injection of Tamoxifen

Expression of Cre recombinase from the VillinCre transgene was induced using Tamoxifen (Sigma) delivered in corn oil (Sigma). Powdered Tamoxifen was added to corn oil in an amber bottle to give a concentration of 10 mg Tamoxifen per 1 ml corn oil. The solution was heated to 80°C with regular shaking to ensure complete dissolution of Tamoxifen. Once completely dissolved, the Tamoxifen solution was aliquoted and stored at -20°C until needed. Prior to injection, aliquots of Tamoxifen were defrosted, reheated to approximately 80°C to re-dissolve the Tamoxifen and kept at this temperature until immediately before injection to prevent precipitation. Animals were then injected IP at a dose of 80 mg Tamoxifen per kg body weight once a day for 4 days (Figure 2.2).
2.1.2.2. Injection of 5′-Bromo-2-deoxyuridine

Where indicated, animals were administered 0.25 ml of 10 mg/ml 5′-Bromo-2-deoxyuridine (BrdU, Biosciences) IP either 2 or 24 hours prior to harvesting in order to label cells in S-phase of the cell cycle.

2.1.3. PCR Genotyping

PCR genotyping was performed on all transgenic animals prior to experimental use by PCR using DNA extracted from ear biopsies at weaning age (four weeks). I wish to acknowledge Mark Bishop for all his assistance with genotyping. All PCR genotyping primers were designed using the Primer3 web-based software at http://bioinfo.ut.ee/primer3/ unless provided by earlier publications. Sequences were verified using BLAST engine against Ensembl database at http://www.ensembl.org/Multi/blastview and synthesised by Sigma Genosys.

2.1.3.1. DNA purification

Ear biopsy samples were stored at -20°C until analysis to prevent degradation. For DNA isolation 250 μl of Cell Lysis Solution (Gentra) and 5 μl of 20 mg/ml Proteinase K (Roche) were added to the tissue and digested overnight at 37°C with vigorous agitation. The next day, the digested samples were cooled to room temperature and 100 μl of Protein Precipitation Solution (Gentra) added. Tubes were inverted to mix and centrifuged at 13000 rpm in a microcentrifuge for 10 min to pellet all protein and insoluble material. The supernatant was removed into a clean tube containing 250 μl isopropanol and the mixture
was centrifuged at 13000 rpm for 15 min. The supernatant was carefully discarded, and the DNA pellet allowed to air-dry at room temperature for 1 hour. The dried DNA pellet was then dissolved in 250 μl of PCR-grade water (Sigma) at 37°C with agitation.

2.1.3.2. **Generic protocol for PCR genotyping**

All PCR reactions were performed in thin-wall 96-well plates or in thin-wall 0.2 ml strip tubes (Greiner Bio-One) and run on either PTC-100 Peltier (MJ Research), Technne Flexigene (Krackeler Scientific) or GS1 (G-Storm) thermal cycler. Pipetting of all DNA samples and reagents was carried out using filtered pipette tips to avoid aerosol contamination of samples. 2.5 μl of crude genomic DNA extract was loaded into wells using a multi-channel pipette. A master mix for all samples was then prepared containing all other reaction components (distilled water, GoTaq 5X PCR buffer (Promega), 25mM Magnesium Chloride (Promega), 25 mM dNTPs (Bioline), Primers (Sigma-Genosys) and either GoTaq (Promega) or DreamTaq (Fermentas) DNA polymerase). 47.5 μl of the appropriate master mix were added to each well to make the reaction volume up to 50 μl. 96-well plates were sealed with aluminium foil tape and strip tubes with appropriate caps (Greiner Bio-One). Samples were then gently tapped on the bench to remove air bubbles and ensure the mixture was at the bottom of the well. Reactions were then run using cycle conditions outlined in Table 2.1. The primer sequences used for genotyping of the particular transgenes and their corresponding product sizes are provided in Table 2.2.

2.1.3.3. **Visualisation of PCR products**

Upon completion of PCR reactions the products were visualised by agarose gel electrophoresis. To each sample, 5 μl DNA loading dye (50% Glycerol (Sigma), 50% distilled water, 0.1% (w/v) Bromophenol Blue (Sigma)) was added. All samples were then loaded with an appropriate marker (e.g. 100 bp ladder (Promega)) onto 2% agarose gel (4 g agarose (Eurogentech), 200 ml 1X Tris-Borate-EDTA (TBE) buffer (Sigma), 10 μl of 10 mg/ml ethidium bromide (Sigma) or 10 μl Safeview (NBS Biologicals)). Gels were run in 1X TBE buffer at 120 V for approximately 30 minutes. Products were then visualised in UV light using GelDoc apparatus (BioRad).
## Chapter 2: Materials and Methods

<table>
<thead>
<tr>
<th>PCR Reaction Components:</th>
<th>Cre/LacZ</th>
<th>ApcLoxP</th>
<th>Cbx3LoxP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA extract</td>
<td>2.5 μl</td>
<td>2.5 μl</td>
<td>2.5 μl</td>
</tr>
<tr>
<td><strong>Master Mix:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR-grade Water (Sigma)</td>
<td>31.5 μl</td>
<td>31.7 μl</td>
<td>31.7 μl</td>
</tr>
<tr>
<td>Go Taq PCR Buffer (5X, Promega)</td>
<td>10 μl</td>
<td>10 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>Magnesium Chloride (25 mM, Promega)</td>
<td>5 μl</td>
<td>5 μl</td>
<td>5 μl</td>
</tr>
<tr>
<td>dNTPs (25 mM, Bioline)</td>
<td>0.4 μl</td>
<td>0.4 μl</td>
<td>0.4 μl</td>
</tr>
<tr>
<td>Forward Primer (100 mM, Sigma Genosys)</td>
<td>0.2 μl</td>
<td>0.1 μl</td>
<td>0.1 μl</td>
</tr>
<tr>
<td>Reverse Primer (100 mM, Sigma Genosys)</td>
<td>0.2 μl</td>
<td>0.1 μl</td>
<td>0.1 μl</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>0.2 μl</td>
<td>0.2 μl</td>
<td>0.2 μl</td>
</tr>
<tr>
<td>Brand of Taq DNA Polymerase</td>
<td>Go Taq</td>
<td>Dream Taq</td>
<td>Go Taq</td>
</tr>
<tr>
<td><strong>Total Reaction Volume:</strong></td>
<td>50 μl</td>
<td>50 μl</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

**Cycling conditions (Time; Temperature):**

<table>
<thead>
<tr>
<th></th>
<th>Cre/LacZ</th>
<th>ApcLoxP</th>
<th>Cbx3LoxP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>3 min; 94°C</td>
<td>3 min; 94°C</td>
<td>4 min; 94°C</td>
</tr>
<tr>
<td>Cycle number</td>
<td>30</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Step 1: Denaturation</td>
<td>30 sec; 95°C</td>
<td>30 sec; 95°C</td>
<td>1 min; 94°C</td>
</tr>
<tr>
<td>Step 2: Annealing</td>
<td>30 sec; 55°C</td>
<td>30 sec; 60°C</td>
<td>1 min; 60°C</td>
</tr>
<tr>
<td>Step 3: Elongation</td>
<td>1 min; 72°C</td>
<td>1 min; 72°C</td>
<td>1 min; 72°C</td>
</tr>
<tr>
<td>Final Extension</td>
<td>5 min; 72°C</td>
<td>5 min; 72°C</td>
<td>5 min; 72°C</td>
</tr>
<tr>
<td>Hold</td>
<td>15 °C</td>
<td>15 °C</td>
<td>15 °C</td>
</tr>
</tbody>
</table>

*Table 2.1 PCR Genotyping reaction conditions*
### Table 2.2 Primer Sequences used for PCR Genotyping

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer Sequence (5’ to 3’)</th>
<th>Reverse Primer Sequence (5’ to 3’)</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre</td>
<td>TGA CCG TAC ACC AAA ATT TG</td>
<td>ATT GCC CCT GTT TCA CTA TC</td>
<td>100 bp</td>
</tr>
<tr>
<td>LacZ</td>
<td>CTG GCG TTA CCC AAC TTA AT</td>
<td>ATA ACT GCC GTC ACT CCA AC</td>
<td>500 bp</td>
</tr>
<tr>
<td>ApcLoxP</td>
<td>GTT CTG TAT CAT GGA AAG ATA GGT GGT C</td>
<td>CAC TCA AAA CGC TTT TGA GGG TTG ATT C</td>
<td>WT: 226 bp; Targeted: 314 bp</td>
</tr>
<tr>
<td>Cbx3LoxP</td>
<td>AGT CCC AGT ACT GAG AGT TC</td>
<td>CTC TAC CTC CTG AGT ACT AG</td>
<td>WT: 200 bp   Targeted: 400 bp</td>
</tr>
</tbody>
</table>

**Chapter 2: Materials and Methods**
2.2. Tissue harvesting and processing

2.2.1. Tissue Harvesting

Animals were sacrificed using a schedule 1 approved method of culling (cervical dislocation or carbon dioxide) and were dissected using a micro-dissection kit. The fur was sprayed with 70% ethanol and an incision made along the mid-line of the abdomen through both skin and peritoneal wall. Tissue samples were collected after animal sacrifice and placed in appropriate fixatives as quickly as possible to avoid RNA and protein degradation. The small intestine (SI) and colon were flushed with ice-cold 1X phosphate-buffered solution (PBS; Invitrogen). Selected regions of intestine were either bundled using surgical microtape (Micropore) or ‘swiss-rolled’ (intestine was opened longitudinally, rolled from end to end using forceps and secured with a 23G syringe needle (BD Microlance)). Epithelial cell extracts were collected from 10 cm session of small intestinal tissue in ice-cold Weiser solution (section 2.2.6). Fresh frozen tissues of liver and small intestine were preserved in dry ice before long-term storage at -80°C freezer and the tissues were used for RNA (Section 2.8.1) and protein (section 2.7.1) isolation. The large intestine was flushed with ice-cold 1X PBS and ‘swiss-rolled’.

2.2.2. Tissue Fixation

Once the tissue samples were collected, they were fixed in ice-cold 10% neutral buffered formalin (Sigma) and incubated for 12-24 hours at 4°C. Following fixation, samples were either processed straight away or transferred into ice cold 70% ethanol and stored at 4°C until processing.

Fixation method used for intestinal wholemounts and β-galactosidase staining is outlined in section 2.2.5.
2.2.3.  Tissue processing and sectioning

2.2.3.1.  Tissue dehydration

Tissue was processed using an automatic processor (Leica TP1050). Tissue was dehydrated in increasing concentrations of ethanol (70% for 1 hour, 95% for 1 hour, 100% 2 x 1 hour 30 min, 100% for 2 hours), Xylene 2 x 1 hour and paraffin 1 x 1 hour and 2 x 2 hours. After dehydration, tissue was embedded in paraffin wax.

2.2.3.2.  Tissue sectioning

Paraffin blocks were sectioned on a microtome (Leica RM2135) at a thickness of 5 μm. Sections were then floated onto slides coated with poly-L-lysine (PLL) (Polysine, Thermo Fisher) and baked at 58°C for 24 hours. I would like to thank Derek Scarborough and Mark Isaac for their histology services on tissue dehydration and sectioning.

2.2.4.  Tissue preservation for RNA, DNA or protein extraction

2.2.4.1.  For RNA extraction

Intestinal tissues or epithelial extracts were placed in screw-cap tubes containing 1.4 mm ceramic beads (Lysing Matrix D tubes, MP biomedical) and 1 ml of Trizol reagent (Invitrogen). Samples were then either stored at 4°C (for several weeks) or -20°C (for several months).

2.2.4.2.  For DNA extraction

Small samples were removed from tissues intended for DNA extraction and were placed in 1.5 ml eppendorf tube and stored at -20°C until DNA extraction (usually within 2 weeks).

2.2.4.3.  For Protein extraction

Tissue samples or intestinal epithelial extracts were transferred into 1.5 ml eppendorf tube and quickly immersed in liquid nitrogen or dry ice to snap freezes tissue.
Chapter 2: Materials and Methods

Tissues were removed from liquid nitrogen or dry ice at convenience and stored at -80°C until needed.

2.2.5. Analysis of beta-galactosidase activity

2.2.5.1. Intestinal tissue wholemount

Mice bearing the Rosa26/LacZ reporter transgene were used to assess levels of recombination in small and large intestinal tissues. Defined regions of the intestine were removed and flushed with ice-cold 1 X PBS (Invitrogen) and ice-cold X-Gal fixative solution (2% formaldehyde (Sigma), 0.1% glutaraldehyde (Sigma) in 1 X PBS). Intestines were placed on a set paraffin wax in 15 cm plastic Petri dish (paraffin wax (Sigma), with 10% mineral oil (Sigma)). The intestines were cut opened longitudinally and pinned epithelial side facing up using entomological pins (Watkins & Doncaster).

2.2.5.2. Fixation and demucification of the intestinal wholemounts

Intestinal wholemounts were fixed in X-Gal fixative for at least 1 hour at room temperature. Plates were then washed with 1 X PBS before adding demucifying solution (10% glycerol (Sigma), 0.01 M Tris-HCl (pH 8.2), 20% ethanol (Fisher Scientific), 0.9% NaCl, 0.003% (w/v) Dithiothreitol (DTT, Sigma)). Intestines were incubated with demucifying solution with agitation for 1 hour. The plate then was flooded with 1 X PBS and any adhering mucin was washed off the tissue by pipetting the 1 X PBS with force onto the intestine using a Pasteur pipette, and examined under a dissection microscope to ensure complete removal of mucins. This procedure was repeated using fresh 1 X PBS until intestines were clean.

2.2.5.3. Staining with X-Gal substrate

The wholemounts were stained using X-Gal staining solution: 1 mM MgCl₂ (Sigma), 3 mM potassium ferricyanide (Sigma), 3 mM potassium ferrocyanide (Sigma) in 1 X PBS. The solution was prepared in advance and stored in the dark at 4°C and X-Gal solution (5% in DMF, Promega) was added to a concentration of 0.02% immediately prior to staining. The intestines were incubated with the staining solution overnight or until the sufficient level of staining was achieved in the dark with agitation. Once stained, tissues were washed with 1 X PBS and fixed with formalin to avoid further staining. Stained wholemounts were illuminated with Leica CLS50X light source and visually analysed under Olympus SZX12 low-
magnification stereomicroscope. Pictures were taken with Olympus C-4040ZOOM 4.1 Megapixel digital camera. When required, formalin-fixed intestines were 'swiss-rolled' and processed for histology as described in section 2.2.3.

2.2.6. Isolation of intestinal epithelium

2.2.6.1. Isolation of intestinal epithelial cells using Weiser solution

A method for enrichment of intestinal epithelial cells was adapted from (Flint, Cove and Evans, 1991). An approximately 10 cm of the small intestine was flushed with ice-cold 1 X PBS (Invitrogen) opened longitudinally and placed into a 50 ml Falcon tube (Greiner Bio-One) containing 15 ml of freshly prepared ice-cold modified Weiser epithelial extraction buffer (5.6 mM disodium hydrogen orthophosphate (Sigma), 8 mM potassium dihydrogen orthophosphate (Sigma), 96 mM sodium chloride (Sigma), 1.5 mM potassium chloride (Sigma), 27 mM tri-Sodium citrate (Sigma), 0.5 mM dithiothreitol (DTT, Sigma), 1.5% sucrose (Sigma), 1% D-sorbitol (Sigma), 6.07 mM EDTA (Sigma), 4 mM EGTA (Sigma), pH 7.3). The intestine was washed by gentle shaking in three changes of ice-cold Weiser solution. The tube was then attached to the vortex mixer and shaken at the lowest setting for 15 min, generating the first ‘fraction’ of epithelial cell extract that were transferred into a fresh tube. Fresh 15 ml of Weiser solution was added to the tube containing the intestine and the shaking steps were repeated twice. Three 15 ml fractions were joined together and the intestine was discarded.

2.2.6.2. Processing of crude epithelial cell suspensions

Epithelial cell suspensions in the 45 ml Weiser buffer were centrifuge at 1500-2000 rpm for 5 min. The supernatant was aspirated and the resulting pellet was resuspended in 20 m of ice-cold 1 X PBS and centrifuged. The wash in 1 X PBS was repeated twice, the pellet was finally resuspended in 3 ml of 1 X PBS and aliquoted into three 1.5 ml eppendorf tubes. The tubes were subsequently centrifuged for 1 minute and the pellet was stored at -80°C until needed.
Chapter 2: Materials and Methods

2.3. Histological analysis

2.3.1. Haematoxylin and Eosin (H&E) staining of tissue sections

Tissue sections were de-waxed and rehydrated as described in section 2.4. Sections were then stained for 5 min in Mayer’s Haemalum (R.A. Lamb) followed by washing in running tap water for 5 min. Sections were then stained in 1% aqueous Eosin (R.A. Lamb) for 5 mins followed by two 15 second washes in water. Stained sections were then dehydrated and mounted as described in section 2.4.

2.3.2. Quantitative histological analysis of H&E-stained tissue sections

Quantification of histological sections was performed on an Olympus BX41 light microscope fitted with a Colorview III (5 megapixel, Soft Imaging Systems) camera aided with AnalySIS software (Version 3.2, Build 831, Soft Imaging Systems) or Moticam 5000 camera (5 megapixel, Motic Instruments) aided with Motic Images Advanced Software (Version 3.2, Motic China Group). Quantitative and statistical analysis of histological parameters was performed as described in section 2.10.

2.3.2.1. Scoring of crypt length

Crypt length was scored by counting the total number of epithelial cells from the base to the crypt-villus junction. This was performed for 50 half-crypts per section and average of 50 crypts were scored per mouse.

2.3.2.2. Scoring of villus length

Villus length was scored by counting the total number of epithelial cells from the crypt bottom to the villus tip. Scoring was carried out for 50 half-villi per section. The average villus length was calculated as average total crypt-villus length minus average crypt length for each mouse.
2.3.2.3. **Scoring of Mitotic index**

Mitotic cells were identified on H&E stained sections based on their morphological appearance. Number of mitotic figures was scored in 50 half-crypts per section. Mitotic index was calculated as average number of mitotic figures per half-crypt for each mouse.

2.3.2.4. **Scoring of Apoptotic index**

Apoptotic cells were identified on H&E stained sections based on their distinctive morphological appearance. The number of apoptotic bodies was counted in 50 half-crypts per slide. The apoptotic index was calculated as average number of apoptotic bodies per half-crypt for each mouse. Where applicable, the position of apoptotic bodies in the crypt was scored to examine the distribution of apoptosis within the crypt. The positional analysis was conducted as described in section 2.10.

2.3.3. **Use of special stains for quantification of intestinal histological traits**

2.3.3.1. **Grimelius staining**

The Grimelius staining method was used to identify acgyrophilic enteroendocrine cells. Prior to staining, all glassware used for the staining was rinsed with ultrapure double-distilled (dd) H$_2$O. Tissue sections were de-waxed and rehydrated as described in section 2.4. 1% (w/v) silver nitrate (Sigma) was dissolved in Acetate buffer (0.02 M acetic acid (Fisher Scientific), 0.02 M sodium acetate (Sigma) in ddH$_2$O) and preheated to 65°C in a coplin jar in a water bath. Once slides were immersed in this solution, the coplin jar was sealed, wrapped in tin foil and incubated at 65°C for 3 hours. Meanwhile, reducing solution (0.04 M sodium sulphite (Fisher Scientific), 0.1 M hydroquinone (Sigma) in ddH$_2$O) was freshly prepared and preheated to 45°C. The slides were then transferred from sliver solution into reducing solution where they were incubated for 5 minutes or until the tissue developed yellow colour. Stained slides were then dehydrated and mounted as described in section 2.4.

Stained sections were examined and the number of enteroendocrine cells was scored in 50 half crypt-vils axes per section. The average number of enteroendocrine cells
per half crypt-villus axis was calculated for each mouse and analysed as described in section 2.10.

2.3.3.2. Staining with Alcian Blue

Alcian Blue staining for mucins was used to identify mucin-secreting Goblet cells. Tissue sections were de-waxed and rehydrated as described in section 2.4. Slides were then immersed in Alcian Blue staining solution (1% (w/v) Alcian Blue (Sigma) in 3% (v/v) acetic acid (Fisher Scientific)) for 30 seconds followed by washing in running water for 5 minutes. Tissue sections were then counterstained with 0.1% nuclear fast red (Sigma) for 5 minutes. Sections were washed well in running tap water for 5 minutes and then dehydrated and mounted as described in section 2.4.

Stained sections were analysed and the number of goblet cells were counted in 50 half crypt-villus axes per section. The average number of goblet cells per half crypt-villus axis was calculated for each mouse and analysed as described in section 2.10.

2.3.3.3. Alkaline phosphatase staining

Staining for brush border-specific Alkaline phosphatase was used as a marker of enterocytes. Slides were de-waxed and rehydrated as described in section 2.4. Fast red substrate (DAKO), prepared as described in the manufacturer’s instructions, was applied to slides for 20 minutes at room temperature. Slides were rinsed in dH₂O and counterstained in Mayer’s Haemalum for 30 seconds followed by a 5 minutes wash in running water. As Fast Red staining is not stable in solvents, slides were mounted using glycerol (Sigma).

The intensity and thickness of the brush border staining were visually analysed using light microscopy.

2.3.3.4. Lysozyme staining

Immunohistochemistry with an anti-lysozyme antibody (see section 2.4) was employed to identify Paneth cells. Both Paneth cell number and position were scored. The number of Paneth cell was counted as the number of lysozyme-positive cells in 50 half-crypts per section. The average number of Paneth cells per half-crypt was calculated for
each mouse and analysed as described in section 2.10. Paneth cell position was scored from
the base of the crypt and the positional analysis was carried out as described in section 2.10.

2.3.3.5. Ki67 staining

Immunohistochemistry with an anti-Ki67 antibody (see section 2.4) was used to
identify proliferating cells to confirm the morphology-based Mitotic index score. Both Ki67
cell number and position was scored to assess the size and distribution of proliferative
compartment within the crypt. The number of Ki67-positive cells was scored as the number
of proliferating cells in 50 half-crypts per section. The average number of Ki67-positive cells
per half-crypt was calculated for each mouse and analysed as described in section 2.10.
Position of Ki67-positive cell were scored from the base of the crypt and the positional
analysis was performed as described in section 2.10.

2.3.3.6. Cleaved Caspase-3 staining

Immunohistochemistry with an anti-cleaved Caspase-3 antibody (see section 2.4) was used to
identify programmed cell death as a supplement to the Apoptotic index. Both
the number and position of Caspase-3 positive cells were scored to assess the quantity of
cell death and distribution of apoptosis within the crypt. The average number of apoptotic
cells per half-crypt was calculated for each mouse and analysed, and the positional analysis
was carried out as described in section 2.10.

2.3.3.7. BrdU staining

Intestinal tissue samples harvested from mice injected with BrdU at 2 or 24 hours
before dissection were stained with an anti-BrdU antibody as described in section 2.4. Both
the number and position of BrdU positive cells at 2 and 24 hours post-labelling were used to
assess proliferation activity and cell migration respectively. The number of BrdU positive
cells was counted in 50 half-crypts per section and the average number of BrdU positive
cells per half-crypt was calculated for each mouse and analysed as described in section 2.10.
The position of BrdU labelled cells was scored from the base of the crypt and cell positional
analysis was carried out as described in section 2.10.
2.4. Immunohistochemistry (IHC)

2.4.1. Generic immunohistochemistry protocol

All IHC was performed following the generic protocol described below with specific conditions and modifications for particular antibodies outlined in Table 2.3. All incubation steps were carried out at room temperature (approximately 18-22°C) in a humidified chamber to prevent tissue sections drying out.

2.4.1.1. De-waxing and rehydration of tissue sections

Tissue sections were de-waxed/deparaffinised by washing for 2 x 5 min in Xylene (Fisher Scientific) followed by rehydration washes in decreasing concentrations of ethanol (Fisher Scientific): 2 x 3 min washes in 100% and 1 x 3 min wash in 95% and 1 x 3 min wash in 70% ethanol. Tissue sections were then washed in dH₂O.

2.4.1.2. Antigen retrieval

Antigen retrieval was achieved by heat treatment in a boiling water bath or microwave in either 1 X citrate buffer (LabVision) or a custom citrate buffer (10 mM Sodium Citrate (Sigma), pH 6.0). For retrieval in a boiling water bath, slides were immersed in preheated buffer in a Coplin jar (R.A. Lamb) and incubated at 99°C for the time specified in Table 2.3. For microwave antigen retrieval, adequate buffer was preheated in a plastic container or a plastic pressure cooker for 5 min in a domestic microwave at high power (850W). Slides held in a plastic slide rack were then immersed in preheated buffer and further heated according to conditions specified in Table 2.3. Slides were allowed to cool in antigen retrieval buffer at room temperature for at least 30 min followed by 5 min wash in dH₂O. Slides were then washed in washing buffer (either 1 X PBS (Invitrogen) or 1 X TBS (Sigma) in dH₂O with 0.1% (v/v) TWEEN-20 (Sigma) as specified in Table 2.3).

2.4.1.3. Blocking of endogenous peroxidases

Endogenous peroxidase activity was blocked by incubating tissue sections with hydrogen peroxide. Either 30% hydrogen peroxide (Sigma) diluted in dH₂O to appropriate concentration or a commercial peroxidase blocking solution (Envision+ Kit (Dako)) was used. The appropriate hydrogen peroxide concentrations and incubation times for specific
antibodies are detailed in Table 2.3. Tissue sections were circled with water-resistant wax pen (DAKO), placed in a slide chamber and peroxidase-blocking solution was applied to cover the tissue. Following incubation, slides were briefly rinsed in dH2O and washed 1 X 5 min in washing buffer.

2.4.1.4. Blocking of non-specific antibody binding

Non-specific binding of antibody was blocked by treating tissue sections either with normal serum (DAKO) or BSA (Sigma) diluted in wash buffer as described in Table 2.3. Typically, the serum used for blocking was derived from the species in which the secondary antibody was raised (i.e. if the secondary antibody was raised in goat, normal goat serum was used to block non-specific binding). Normal serum was applied and slides incubated for the times indicated in Table 2.3. Following incubation, blocking solution was removed and primary antibody was applied without washing of slides.

2.4.1.5. Primary antibody incubation

Primary antibody was appropriately diluted before use in serum block solution. The dilutions and incubation times used with specific antibodies are outlined in Table 2.3. After incubation, primary antibody was removed from the section, the slides were washed 3 X 5 min in washing buffer.

2.4.1.6. Secondary antibody incubation

Tissue sections were incubated with secondary antibody following the optimised protocols outlined in Table 2.3. When signal amplification step was required by the protocol, an appropriate biotinylated secondary antibody (Dako) was diluted 1:200 in serum block. Otherwise, an appropriate Horseradish peroxidase (HRP)-conjugated secondary antibody (Envision+ Kit (Dako)) was used undiluted. Appropriate secondary antibodies and incubation times for specific primary antibodies are indicated in Table 2.3. After incubation, slides were washed 3 X 5 min in wash buffer.

2.4.1.7. Signal amplification

When the protocol required the incorporation of a signal amplification step, the Avidin-Biotin Complex reagent (Vectastain ABC kit, Vector labs) was prepared according to
manufacturer’s instructions 30 min before used and stored at room temperature. The ABC reagent was then applied to slides for 30 min at room temperature, followed by 3 X 5 min washes in washing buffer.

2.4.1.8. **Signal detection using 3,3’-diaminobenzidine (DAB)**

The 3,3’-diaminobenzidine (DAB) method was used for colourimetric detection of signal. The (DAB) reagents (Envision+ kit (DAKO)) were prepared according to the manufacture’s protocol before incubation (2 drops of DAB chromogen mixed with 1 ml of DAB substrate). Tissue sections were incubated with DAB reagents until adequate brown staining was visible under a microscope. Slides were then washed 1 X 5 min in dH₂O.

2.4.1.9. **Counterstaining, dehydration and slide mounting**

Nuclei were counter-stained in Mayer’s Haemalum (R.A. Lamb) for 30 seconds and washed in tap water for 2 minutes. Slides were dehydrated by immersion in a gradient of ethanol (1 X 3 min 70%, 1 X 3 min 95%, 2 x 3 min 100%) and then in 2 x 5 min washes in Xylene. Sections were mounted in DPX-mountant (R.A. Lamb), covered with 24 x 50mm slips and left to air-dry in a fume hood.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Manufacturer</th>
<th>Antigen retrieval</th>
<th>Non-specific signal block</th>
<th>Wash buffer</th>
<th>Primary antibody conditions</th>
<th>Secondary antibody conditions</th>
<th>Signal amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td>Becton-Dickinson</td>
<td>20 min, 100°C water bath, citrate buffer</td>
<td>peroxidase block (Dako)</td>
<td>3 X 5 min PBS/T</td>
<td>1:200 in 1% BSA, 1 h at RT</td>
<td>Envision + HRP-conjugated anti-mouse (DAKO), 30 min at RT</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>1.5% H₂O₂, 15 min, RT</td>
<td>1.5% H₂O₂, 15 min, RT</td>
<td>3 X 5 min PBS/T</td>
<td>1:200 in 1% BSA, 1 h at RT</td>
<td>1:100 in 10% NGS, 1 h at RT</td>
<td>Envision + HRP-conjugated anti-mouse (DAKO), 30 min at RT</td>
<td>N/A</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Neomarkers</td>
<td>20 min, 100°C water bath, citrate buffer</td>
<td>1.5% H₂O₂, 15 min, RT</td>
<td>3 X 5 min TBS/T</td>
<td>1:300 in 1% BSA, 2 h at RT</td>
<td>Envision + HRP-conjugated anti-mouse (DAKO), 1 h at RT</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>RB-372</td>
<td>(Thermo)</td>
<td>1.5% H₂O₂, 30 min, RT</td>
<td>3 X 5 min TBS/T</td>
<td>1:400 in 10% NGS, 0/N at 4°C</td>
<td>Envision + HRP-conjugated anti-Rabbit (DAKO), 30 min at RT</td>
<td>N/A</td>
</tr>
<tr>
<td>Beta-Catenin</td>
<td>Transduction Labs</td>
<td>20 min, 100°C water bath, citrate buffer</td>
<td>2% H₂O₂, 10 min, RT</td>
<td>3 X 5 min PBS</td>
<td>1:20 in 20% NRS, 0/N at 4°C</td>
<td>Envision + HRP-conjugated anti-Rabbit (DAKO), 30 min at RT</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>310154</td>
<td>(Thermo)</td>
<td>10% NGS, 30 min, RT</td>
<td>3 X 5 min PBS</td>
<td>1:20 in 20% NRS, 0/N at 4°C</td>
<td>Biotinylated anti-mouse (DAKO), 30 min at RT</td>
<td>N/A</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>R&amp;D systems</td>
<td>30 min, 100°C water bath, citrate buffer</td>
<td>0.5% H₂O₂, 20 min, RT</td>
<td>3 X 5 min TBS/T</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>ABC kit (Vector Labs)</td>
<td>N/A</td>
</tr>
<tr>
<td>Ki67</td>
<td>Vector labs</td>
<td>30 min, 100°C water bath, citrate buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AF835</td>
<td>(Thermo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VPK452</td>
<td>(Thermo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: NRS = normal rabbit serum, NGS = normal goat serum, BSA = bovine serum albumin, RT = room temperature, O/N = overnight, PBS/T = PBS with tween-20, TBS/T = tris buffered saline with tween-20, PBS = phosphate buffered saline.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>HP1y</th>
<th>HP1y (phosphor S83)</th>
<th>Akt (phosphor S473)</th>
<th>Pten</th>
<th>Smad2/3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Millipore 05-690</td>
<td>Abcam Ab45270</td>
<td>Cell Signalling 3787</td>
<td>Cell Signalling 9559</td>
<td>Santa Cruz SC11769</td>
</tr>
<tr>
<td>Antigen retrieval</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>20 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
</tr>
<tr>
<td>Non-specific signal block</td>
<td>0.5% H₂O₂, 20 min, RT; 10% NRS, 30 min, RT</td>
<td>Peroxidase block (Dako), 5 min, RT; 10% NGS, 30 min, RT</td>
<td>3% H₂O₂, 20 min, RT; 5% NGS, 45 min, RT</td>
<td>3% H₂O₂, 20 min, RT; 5% NGS, 30 min, RT</td>
<td>3% H₂O₂, 20 min, RT; 5% NRS, 35 min, RT</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>3 X 5 min PBS</td>
<td>3 X 5 min TBS/T</td>
<td>3 X 5 min TBS/T</td>
<td>3 X 5 min TBS/T</td>
<td>3 X 5 min PBS</td>
</tr>
<tr>
<td>Primary antibody conditions</td>
<td>1:1000 in 10% NRS, O/N at 4°C</td>
<td>1:500 in 10% NGS, O/N at 4°C</td>
<td>1:50 in 5% NGS, O/N at 4°C</td>
<td>1:100 in 5% NGS, O/N at 4°C</td>
<td>1:500 in 10% NRS, O/N at 4°C</td>
</tr>
<tr>
<td>Secondary antibody conditions</td>
<td>Envision + HRP-conjugated anti-mouse (DAKO), 30 min at RT</td>
<td>Envision + HRP-conjugated anti-rabbit (DAKO), 30 min at RT</td>
<td>Biotinylated anti-rabbit (DAKO), 30 min at RT</td>
<td>Biotinylated anti-rabbit (DAKO), 30 min at RT</td>
<td>Biotinylated anti-mouse (DAKO), 1:200 in 20% NRS, 30 min at RT</td>
</tr>
<tr>
<td>Signal amplification</td>
<td>N/A</td>
<td>N/A</td>
<td>ABC kit (Vector Labs)</td>
<td>ABC kit (Vector Labs)</td>
<td>ABC kit (Vector Labs)</td>
</tr>
</tbody>
</table>

2.5. **Immunofluorescence**

2.5.1. **Deparaffinising and rehydration of tissue sections**

Tissue sections were deparaffinised for 2 x 5 minutes in Xylene (Fisher Scientific) followed by rehydration washes in decreasing concentrations of ethanol (Fisher Scientific): 2 x 3 minutes washes in 100% and 1 x 3 minutes wash in 95% and 1 x 3 minutes wash in 70% ethanol. Tissue sections were then washed in dH₂O.

2.5.2. **Antigen retrieval**

Antigen retrieval was achieved by heat treatment in a boiling water bath in citrate buffer (Thermo). Slides were immersed in preheated buffer in a Coplin jar (R.A. Lamb) and incubated at 100°C for 30 minutes as outlined in Table 2.4. Slides were then allowed to cool in antigen retrieval buffer at room temperature for at least 30 minutes followed by 5 minutes wash in dH₂O. Slides were then washed in 1x PBS (Invitrogen) for 5 minutes.

2.5.3. **Blocking of endogenous peroxidases**

Endogenous peroxidase activity was blocked by incubating tissue sections with hydrogen peroxide. A commercial peroxidase blocking solution (Envision+ Kit (Dako)) was applied on each slide for 5 minutes at room temperature as outlined in Table 2.4. Following incubation, slides were briefly rinsed in dH₂O and washed in 1x PBS washing buffer for 5 minutes.

2.5.4. **Blocking of non-specific antibody binding**

Non-specific binding of antibody was blocked by treating tissue sections with normal serum (DAKO) diluted in wash buffer as described in Table 2.4. Normal serum was applied and slides incubated for the times indicated in Table 2.4. Following incubation, blocking solution was removed and primary antibody was applied without washing of slides.

2.5.5. **Primary antibody incubation**

Primary antibody was appropriately diluted before use in serum block solution. The dilutions and incubation times used with specific antibodies are outlined in Table 2.4.
incubation, primary antibody was removed from the sections, the slides were washed 3 x 5 minutes in washing buffer.

2.5.6. Secondary antibody incubation

Tissue sections were incubated with fluorescent secondary antibodies in a moist and dark chamber for 60 minutes. Appropriate secondary antibodies and incubation times for specific primary antibodies are indicated in Table 2.4. After incubation, slides were washed 3 x 5 minutes in wash buffer.

2.5.7. Nuclear staining

DAPI (4’, 6-Diamidino-2-Phenylindole, Dilactate (DAKO)) solution was applied on each slide and sections were subsequently covered with 24 x 50mm slips. Slides were stored in the dark at -20°C.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Manufacturer</th>
<th>Antigen retrieval</th>
<th>Non-specific signal block</th>
<th>Wash buffer</th>
<th>Primary antibody conditions</th>
<th>Secondary antibody conditions</th>
<th>Nuclear stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP1γ</td>
<td>Millipore 05-690</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>peroxidase block (Dako), 5min, RT; 10% NGS, 30 min, RT</td>
<td>3 X 5 min PBS</td>
<td>1:1000 in 10% NGS, O/N at 4°C</td>
<td>1:400 Anti-mouse 594 (Invitrogen), 60 min at RT</td>
<td>DAPI (Dako)</td>
</tr>
<tr>
<td>HP1α</td>
<td>Abcam Ab77256</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>peroxidase block (Dako), 5min, RT; 10% NMS, 30 min, RT</td>
<td>3 X 5 min PBS</td>
<td>1:500 in 10% NGS, O/N at 4°C</td>
<td>1:400 Anti-goat 488 or 594 (Invitrogen), 60 min at RT</td>
<td>DAPI (Dako)</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Abcam Ab8580</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>peroxidase block (Dako), 5min, RT; 10% NGS, 30 min, RT</td>
<td>3 X 5 min PBS</td>
<td>1:500 in 10% NGS, O/N at 4°C</td>
<td>1:400 Anti-rabbit 488 or 594 (Invitrogen), 60 min at RT</td>
<td>DAPI (Dako)</td>
</tr>
<tr>
<td>H3K9me3</td>
<td>Abcam Ab8898</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>peroxidase block (Dako), 5min, RT; 10% NGS, 30 min, RT</td>
<td>3 X 5 min PBS</td>
<td>1:500 in 10% NGS, O/N at 4°C</td>
<td>1:400 Anti-rabbit 488 or 594 (Invitrogen), 60 min at RT</td>
<td>DAPI (Dako)</td>
</tr>
<tr>
<td>H4K20me3</td>
<td>Active Motif 39180</td>
<td>30 min, 100°C water bath, citrate buffer (Thermo)</td>
<td>peroxidase block (Dako), 5min, RT; 10% NGS, 30 min, RT</td>
<td>3 X 5 min PBS</td>
<td>1:500 in 10% NGS, O/N at 4°C</td>
<td>1:400 Anti-rabbit 488 or 594 (Invitrogen), 60 min at RT</td>
<td>DAPI (Dako)</td>
</tr>
</tbody>
</table>

2.6. *In situ* hybridisation

*In situ* hybridisation was performed to assess the expression of the intestinal stem cell marker *Olfm4*. This technique uses digoxigenin (DIG) labelled RNA probes (riboprobes) complementarily bind to *Olfm4* mRNA sequence. Probe binding can be detected using an anti-digoxigenin alkaline phosphatase-conjugated antibody by the development of a purple stain. The probe for *Olfm4*, cloned into a pBluescript II SK+ vector, was provided by Hans Clever’s group (Hubrecht Institute, Netherlands). All solutions were made using RNase-free water (Sigma) or DEPC treated water. All glassware used in the protocol was heat-treated by baking at 200°C overnight and sterile filtered pipette tips were used throughout.

2.6.1. Probe synthesis

The *Olfm4* probe was synthesised by linearization of plasmid DNA for the transcription of DIG-labelled riboprobes using T7 promoter. 30 μg of plasmid DNA was linearised for probe preparation using NotI restriction enzyme (NEB) according to manufacturer’s instructions. The resultant digestion product was analysed on an agarose gel to confirm complete linearisation. The *Olfm4* digest was approximately 700 bps after digest. Linearised DNA was subsequently purified using DNA purification columns (Qiagen). The DNA was quantified using a NanoDrop machine (Thermo Scientific) and adjusted to a concentration of 1 μg/μl using 10 mM Tris (pH 8.0) as required. The linearised plasmid DNA was used as a template for the transcription of DIG-labelled riboprobes using T7 RNA polymerase (Roche). The components of the DIG-labelling reaction are listed in Table 2.5.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase Free H2O</td>
<td>12</td>
</tr>
<tr>
<td>Transcription Buffer (10x) (Roche)</td>
<td>2</td>
</tr>
<tr>
<td>DIG RNA Labelling mix (Roche)</td>
<td>2</td>
</tr>
<tr>
<td>RNasin (Promega)</td>
<td>1</td>
</tr>
<tr>
<td>T7 RNA Polymerase (Roche)</td>
<td>2</td>
</tr>
<tr>
<td>DNA (1 μg/μl)</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.5: The constituents required for DIG labelling
The labelling reaction was performed for 2 hours at 37°C, followed by DNA template digestion by incubation with 20 units of D Nasel (Ambion) for 15 minutes at 37°C. Riboprobes were then purified using the RNeasy Mini kit (Qiagen) according to manufacturer’s protocols. Probes were eluted with 100 μl of Ultrapure RNase-free H2O and stored at -80°C in 10 μl aliquots until use.

2.6.2. Probe hybridisation

Sections were dewaxed in 2 x 10 minutes changes of xylene, and rehydrated by 1 minute washes in ethanol of decreasing concentration: 2 x 100%, 95%, 75%, 50% and 30%. Endogenous alkaline phosphatase activity was blocked in 6% hydrogen peroxide (in 1x PBS) at room temperature for 30 minutes. Slides were then washed 2 x 5 minutes in 1x PBS and digested in Proteinase K (Roche) solution for 5 minutes (200 μg/ml proteinase K, 50 mM Tris, 5 mM EDTA in dH2O), washed for 5 minutes in 1x PBS, post-fixed in 4% paraformaldehyde for 5 minutes followed by 2 minutes wash in DEPC treated H2O. To minimise non-specific probe binding, slides were treated in acetic anhydride solution (0.01 M acetic anhydride (Sigma) in 0.1 M triethanolamine hydrochloride (Sigma)) for 10 minutes at room temperature while stirring, washed once in 1x PBS and once in 1x saline then dehydrated through 1 minutes washes in 30%, 50%, 75%, 95% and 2 x 100% ethanol solutions. Slides were allowed to air-dry for 30 minutes. The Olfm4 probe was diluted in 1 in 100 in hybridisation buffer (5 x SSC, 50% formamide, 5% SDS, 1 mg/ml heparin, 1 mg/ml calf liver tRNA (Merck) in dH2O), which was heated at 80°C for 3 minutes. 100 μl of this was then applied to each slide, which was covered in parafilm to prevent probe mixture evaporation, and incubated overnight in a dark and moist chamber at 65°C overnight.

2.6.3. Post-hybridisation treatment

Slides were removed from the chamber and incubated in 5x SSC buffer at 65°C for 15 minutes then washed 2 x 30 minutes in 50% formamide, 5x SSC and 1% SDS (Sigma) for 30 minutes at 65°C. Post hybridisation washes were followed by 2 x 10 minutes washes in 0.5 M NaCl (Sigma), 10 mM Tris pH 7.5 (Sigma), 0.1% Tween-20 (Sigma) at room temperature. Sections were then incubated for 45 minutes in 0.5 M NaCl, 10 mM Tris pH 7.5, 0.1% Tween-20 with 25 μg RNase A (Qiagen) at 37°C. Sections were washed 2 x in 50% formamide, 5x SSC for 30 minutes at 65°C and pre-blocked in 10% heat-inactivated sheep
serum (DAKO) in PBS/T for 2-3 hours. Anti-DIG alkaline phosphatase conjugated antibody (Roche) was pre-absorbed in 1% heat-inactivated sheep serum in PBS/T containing 5 mg/ml mouse intestinal powder for 3 hours at 4°C and diluted to a final concentration of 1:2000 in 1% sheep serum in PBS/T. Slides were overlaid with 100 μl of antibody solution and were then covered in parafilm and incubated in a dark and moist chamber at 4°C overnight.

2.6.4. Signal detection

Slides were washed 3 x in PBS/T for 5 minutes, followed by 3 x 30 minutes wash in PBS/T. Slides were then preconditioned to inhibit endogenous alkaline phosphatase by washing 3 x in NTMT buffer (0.1 M NaCl, 0.1 M Tris pH 9.5, 0.05 M MgCl₂ (Sigma), 0.1% Tween, 2 mM Levamisole (Sigma)). The BM Purple substrate (Roche) was added to the slides and incubated in the dark at room temperature until desired level of signal was developed. Slides were then washed in PBS/T for 10 minutes and counterstained with Eosin (Sigma) for up to 10 seconds. Excess Eosin was washed off under running tap water and slides were dipped in xylene prior to air-drying and mounting using DPX mounting media (R.A. Lamb).

2.7. Quantitative Protein Analysis

2.7.1. Protein extraction from tissue samples

Epithelial cell extract samples and whole tissue samples (prepared as described in section 2.2.6 and 2.2.4, respectively) in eppendorf tubes were removed from storage at -80°C and placed on dry ice. 200 μl of cell lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 0.5% (v/v) NP-40 (Sigma)) containing protease inhibitors (Complete mini protease inhibitor cocktail tablets (Roche)) and phosphatase inhibitors (25 mM sodium β-glycerophosphate (Calbiochem), 100 mM sodium fluoride (Sigma), 20 nM Calyculin A from Discodermia calyx (Sigma), 10 mM sodium pyrophosphate (Sigma)) were added. Cells were sheared by passing through a 23-gauge needle using a syringe at least 20 times. Tubes were then centrifuged in a refrigerated centrifuge (Legend Micro 17R, Thermo Scientific) at 9500 x g for 10 min at 4°C to remove residual insoluble material. The supernatant was then aliquoted into clean tubes and stored at -80°C until needed.
2.7.2. Quantification of protein concentration

The relative amount of total protein in each protein extract was determined using the Pierce BCA (Bicinchoninic acid) kit (Thermo Scientific). Serial dilutions of BSA (from 5 μg/ml to 35 μg/ml) in 1 X PBS (Invitrogen) were prepared along with serial dilutions of protein extract samples (1:100, 1:200, 1:400 and 1: 800 in 1 X PBS) and blank solutions containing 1 X PBS. All solutions were loaded in duplicates onto a 96-well plate. BCA kit reagents A and B were mixed in 50:1 ratio according to the manufactory guidelines. 100 μl of the mix was added to each well and mixed gently by pipetting. The plate was then incubated at 37°C for 1 hour or at room temperature overnight. After incubation, the plate was read at 590 nm on an appropriate colourimeter or a microplate reader (BioTek). A standard curve was generated using serial dilutions of BSA and respective protein concentrations in extracted samples were calculated. The standard curve was then used to extrapolate the relative concentration of protein in each experimental sample.

2.7.3. Western-blotting analysis

2.7.3.1. Preparation of protein samples

Samples were removed from storage at -80°C, defrosted on ice and appropriate amount of protein extract containing 30 μg of protein was transferred into a clean eppendorf tube. Samples were made up to 25 μl using Laemmli buffer (see Table 2.7 for composition). Samples were denatured by heating to 95°C for 5-10 min and then quenched on ice just prior to loading.

2.7.3.2. Casting of polyacrylamide gels

SDS-PAGE gels consist of resolving and stacking phases. Solutions for resolving (10%) and stacking (5%) gels were prepared according to conditions laid out in Table 2.6, but without addition of TEMED. SDS-PAGE gels were set using Mini-Protean III (Bio-Rad) gel casting apparatus with 1.5 mm spacers. Casting plates were cleaned with 70% ethanol (Fisher Scientific) before being assembled in a gel casting apparatus and filled to
approximately two thirds with the resolving gel. Once set, the stacking gel was poured over the resolving gel and a 10 or 15-well comb was inserted immediately. After the stacking gel had set, the combs were removed and the wells were rinsed with ddH₂O water.

<table>
<thead>
<tr>
<th>5% Stacking polyacrylamide gel (2 gels)</th>
<th>10% Resolving polyacrylamide gel (2 gels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.9 ml distilled water</td>
<td>6.8 ml distilled water</td>
</tr>
<tr>
<td>1.7 ml 30% Acrylamide (Sigma)</td>
<td>8.4 ml 30% Acrylamide (Sigma)</td>
</tr>
<tr>
<td>100 μl 10% (w/v) Sodium dodecyl sulphate (SDS, Sigma)</td>
<td>250 μl 10% (w/v) Sodium dodecyl sulphate (SDS, Sigma)</td>
</tr>
<tr>
<td>1.3 ml 1M Tris-HCl pH 6.8 (Sigma)</td>
<td>9.4 ml 1M Tris-HCl pH 8.8 (Sigma)</td>
</tr>
<tr>
<td>66 μl 25% (w/v) Ammonium persulphate (APS, Sigma)</td>
<td>72 μl 25% (w/v) Ammonium persulphate (APS, Sigma)</td>
</tr>
<tr>
<td>13.2 μl N,N,N,N-tetramethylethylenediamine (TEMED, Sigma)</td>
<td>13.2 μl N,N,N,N-tetramethylethylenediamine (TEMED, Sigma)</td>
</tr>
</tbody>
</table>

Table 2.6 Composition of gels for SDS-PAGE.

<table>
<thead>
<tr>
<th>Laemmli Buffer</th>
<th>5x running buffer (1L)</th>
<th>1x Tris-Glycine transfer buffer (1L)</th>
<th>Stripping buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125 M Tris-HCl pH 6.8 (Sigma)</td>
<td>950 ml deionized H2O</td>
<td>800 ml deionized H2O</td>
<td>62.5 mM Tris-HCl pH 6.8 (Sigma)</td>
</tr>
<tr>
<td>4% (w/v) SDS (Sigma)</td>
<td>15.1 g Tris base (Sigma)</td>
<td>200 ml methanol (Fisher Scientific)</td>
<td>2% w/v SDS (Sigma)</td>
</tr>
<tr>
<td>40% v/v glycerol (Sigma)</td>
<td>94 g Glycine (Sigma)</td>
<td>2.9 g Tris base (Sigma)</td>
<td>0.7% v/v beta-mercaptopethanol (Sigma)</td>
</tr>
<tr>
<td>0.1% w/v Bromophenol Blue (Sigma)</td>
<td>50 ml 10% (w/v) SDS (Sigma)</td>
<td>14.5 g Glycine (Sigma)</td>
<td></td>
</tr>
<tr>
<td>6% (v/v) beta-mercaptopethanol (Sigma)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.7 Composition of buffers used in Western analysis.
2.7.3.3. Protein separation by SDS-PAGE

Protein separation was performed using the Mini-Protean III (Bio-Rad) electrophoresis tank containing sufficient amount of SDS-PAGE running buffer (see Table 2.7 for composition). Full-range Rainbow molecular weight marker (Amersham) was loaded into the first lane of each gel and prepared protein samples were loaded into the appropriate remaining wells and gels were run at 120-200V until the dye front reached the end of the gel.

2.7.3.4. Transfer of proteins to nitrocellulose membranes

Before transfer, Amersham Hybond-ECL nitrocellulose membrane (GE Healthcare) was cut to a required size and soaked in transfer buffer prior to use. The membrane sandwich was assembled according to manufacturer’s instructions in a wet electroblotting system (Flowgen Biosciences) using sponges, 3MM blotting paper (Whatman), membrane and the prepared gel as shown in Figure 2.3. Air bubbles were carefully rolled out after the addition of each layer. The blotting tank was fitted with blots so that current runs through gel towards the filter, filled with transfer buffer and placed in ice and run at 100V for 1 hour.

Figure 2.3. Blotting apparatus set-up for Western Analysis

2.7.3.5. Generic protocol for probing of membranes

Upon completion of the transfer, membranes were washed briefly in PBS/T. To prevent non-specific binding, blots were incubated in 5% skimmed milk powder in TBS/T under agitation for at least 1 hour at room temperature. Membranes were incubated with
agitation in blocking solution containing the appropriate dilution of primary antibodies under conditions specified in Table 2.8. After cultivation, membranes were then washed in PBS/T 3 x 5 min followed by cultivation with appropriate secondary antibody under conditions detailed in Table 2.8. Subsequently, membranes were washed again in PBS/T 3 X 5 min prior to protein detection. Appropriate amount of ECL reagent kit (Amersham) was used to develop membranes according to manufacturer’s instructions. After incubation, blots were then wrapped in cling film prior to signal detection.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>HP1γ</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Millipore 05-690</td>
<td>Sigma A5316</td>
</tr>
<tr>
<td>Primary Antibody conditions</td>
<td>1:1000 in 1% BSA in PBS, O/N at 4°C</td>
<td>1:5000 in 5% milk in TBS/T, 1 h at RT</td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td>HRP-conjugated anti-mouse (GE Healthcare) 1:3000 in 1% BSA in PBS, 1 h at RT</td>
<td>HRP-conjugated anti-mouse (GE Healthcare) 1:2000 in 5% milk in TBS/T, 1 h at RT</td>
</tr>
<tr>
<td>Detection Reagents</td>
<td>ECL Advance (GE Healthcare)</td>
<td>ECL</td>
</tr>
<tr>
<td>Target Protein Size</td>
<td>25 kDa</td>
<td>48 kDa</td>
</tr>
</tbody>
</table>

*Table 2.8: Antibody-specific conditions for Western blotting analysis.*

### 2.7.3.6. Visualisation of protein bands

Immobilised immuno-labelled proteins were detected using ECL/ECL+ reagent (GE Healthcare, UK) according to manufacturer’s instruction. Prepared ECL/ECL+ solutions were mixed well and cultivated for 1 minute, before addition on the membrane (1 ml per membrane). After removal of excess reagent, the membrane was placed in a molecular imager system (Gel Doc XR, Biorad) and exposed for varying lengths of time. Pictures of protein bands and molecular weight marker were captured and realigned and the target protein was identified by comparison to the molecular weight marker.

### 2.7.3.7. Stripping and reprobing of membranes

Membranes were stripped before being incubated with subsequent antibody of interest. Membranes were washed in PBS/T and incubated in stripping buffer (see Table 2.7 for composition) for 30 min at 55°C under gentle agitation. Membranes were washed in
PBS/T, blocked in milk blocking solution and probed with required antibodies overnight. The visualisation was performed as described in section 2.7.3.6.
2.8. Gene expression analysis

2.8.1. Isolation of RNA

To prevent degradation of RNA, care was taken during RNA isolation to ensure that all plastic and glassware was RNase-free. All solutions were made using RNase-free water (Sigma) or Diethyl pyrocarbonate (DEPC, Sigma)-treated water. Working surfaces and pipettes were treated with RNaseZAP (Sigma). Sterile filtered pipette tips were used throughout.

2.8.1.1. Homogenisation of whole tissues and epithelial cell extracts

Small intestine or epithelial cell extracts were placed in homogenising lysis matrix D tubes (MP Biomedicals) containing 1 ml Trizol reagent (Invitrogen) and 1.4 mm ceramic beads, and were homogenised using Precellys 24 homogeniser (Bertin Technologies) at 6500 RPM for 2 x 45 second cycles. The samples were then centrifuged at 13,000 rpm for 10 minutes at 4°C to pellet the cell debris and the ceramic beads.

2.8.1.2. RNA extraction

After homogenisation and centrifugation of samples, the supernatants were placed in a fresh set of RNase/DNase free 1.5 ml eppendorf tubes and 200 µl of chloroform (Fisher scientific) was added to each tube in a fume hood. The samples were then placed on ice with occasional shaking. The tubes were then centrifuged at 13,000 rpm for 15 min at 4°C to separate the solution phases. The top, aqueous phase containing RNA was removed and transferred into clean 1.5 ml eppendorf tubes containing 400 µl of the pre-chilled isopropanol (Fisher scientific). Tubes were then shaken well and incubated at 4°C for 1 hour or overnight to precipitate RNA. The samples were then centrifuged as aforementioned to collect RNA pellets, which were washed with 500 µl of ice-cold 75% ethanol (Fisher scientific), then the ethanol was discarded and the pellets were left to air-dry at room temperature for 10 minutes. To dissolve the pellet, 100 µl of RNase-free water (Sigma) was added and tubes were heated to 65°C for 10 minutes.
2.8.1.3. RNA Purification

Purification of RNA was achieved using the RNeasy Mini kit (Qiagen). All centrifugation steps were performed at 13,000 rpm in a refrigerated bench top centrifuge at 4°C. The 100 µl RNA from the previous step was used for purification and the kit was used according to the manufacturer’s instructions. The concentration and purity of RNA was assessed in a 1 µl sample using NanoDrop 1000 (Thermo Scientific), then the samples were stored at -80°C until usage.

2.8.1.4. Determination of RNA Quality

The quality of RNA was checked using denaturing agarose gel electrophoresis. RNA samples containing 2 µg of RNA were mixed with the appropriate amount of RNA 5 X loading buffer (See Table 2.9), incubated at 65°C for 10 min and then quenched on ice before being loaded onto the gel. Samples were loaded onto a denaturing gel (1% agarose (Eurogentech), in 1 X FA gel buffer. The gel was then run in FA gel running buffer at 120 V for 30 min. RNA was visualised under UV light on a GelDoc (BioRad). Assessment of RNA quality was determined by the sharpness of 18S and 28S ribosomal RNA bands.

<table>
<thead>
<tr>
<th>5 X RNA Loading Buffer</th>
<th>1 X FA Gel Buffer</th>
<th>1 X FA Gel Running Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25% Bromophenol blue (Sigma)</td>
<td>20 mM 3-(N-Morpholino) propanesulfonic acid (MOPS, Sigma)</td>
<td>20 mM 3-(N-Morpholino) propanesulfonic acid (MOPS, Sigma)</td>
</tr>
<tr>
<td>4 mM EDTA (Sigma)</td>
<td>5 mM sodium acetate (Sigma)</td>
<td>5 mM sodium acetate (Sigma),</td>
</tr>
<tr>
<td>0.9 M formaldehyde (Sigma)</td>
<td>1 mM EDTA (Sigma)</td>
<td>1 mM EDTA (Sigma),</td>
</tr>
<tr>
<td>20% glycerol (Sigma)</td>
<td>0.25 M formaldehyde (Sigma)</td>
<td>0.25 M formaldehyde (Sigma)</td>
</tr>
<tr>
<td>30% formamide (Sigma)</td>
<td>0.1 µg/ml Ethidium bromide (Sigma)</td>
<td></td>
</tr>
<tr>
<td>1 X FA gel buffer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.9: Composition of FA gel buffers
2.8.2. Preparation of cDNA for quantitative analysis

2.8.2.1. DNase treatment of RNA samples

The purified RNA samples were DNase treated to eliminate any remnants of trace DNA. A total of 10 µg of RNA was treated 10 µl of RQ1 enzyme (Invitrogen) and 5 µl RQ1 buffer (Invitrogen) and the total volume were made up to 50 µl with RNase free-water. The mix was incubated at 30°C for half an hour and the reaction was stopped by adding 5 µl of stop solution and heating to 65°C for 10 minutes.

2.8.2.2. Reverse transcription

For the cDNA synthesis, the DNase treated RNA yielded at a concentration of 1 µg/5.5 µl was used. The reverse transcription enzyme mix was prepared by adding 2 µl of random primers (100 ng/µl (Invitrogen)), 2 µl of dNTP (10mM (Invitrogen)), 12 µl of DNase treated RNA and 11 µl of RNase free-water. The mix was heated at 65°C for 5 min before returning to ice, 8 µl of First strand buffer (5x (Invitrogen)) and 4 µl of 0.1 M Dithiothreitol (DTT, Invitrogen) was added to tubes and incubated at room temperature for 2 min prior adding 1 µl of Superscript II or III reverse transcriptase (Invitrogen). Reverse transcription was carried out under following conditions: 25°C for 10 min, 42°C for 50 min and the enzyme was inactivated at 70°C for 15 min. After cDNA preparation was complete, 160 µl of RNase-free water was added to each sample to make up to 200 µl total cDNA solution. Negative control cDNA samples were prepared in the same way but excluding Superscript enzyme to the enzyme mix.
2.8.3. Quantitative real-time (qRT) PCR analysis

2.8.3.1. Primer design for qRT-PCR

Primer sets for qRT-PCR analysis were designed to ensure the PCR product yield was between 100-200 bp in size and have an annealing temperature close to 60°C, and where possible, primers were designed to reside across exon boundaries to avoid amplification of genomic DNA. Primers (Rac1, Rac1b, ATM, CDC25C, CCNE1, DDX11, PIM1 and CAV2) were designed using Primer3 web-based program (http://bioinfo.ut.ee/primer3/) and primer sequences were checked for mis-priming using BLAST engine against Ensembl database (http://www.ensembl.org/Multi/blastview) and synthesised by Sigma-Genosys. Madeleine Young kindly provided the Notch-targeted primer sequences (Delta1, nRarp, Hes1, mTert, Lrig1, Jagged1 and Jagged2). Primer sequences (Axin2, CD44, c-Myc, Cyclin D1, E-cadherin and Beta-Actin) were obtained from the literature (Reed et al., 2008b). The primer sequences used are provided in Table 2.10.
<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer Sequence (5' to 3')</th>
<th>Reverse Primer Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axin-2</td>
<td>GCT CCA GAA GAT CAC AAA GAG C</td>
<td>AGC TTT GAG CCT TCA GCA TC</td>
</tr>
<tr>
<td>CD44</td>
<td>ATC GCG GTC AAT AGT AGG AGA A</td>
<td>AAA TGC ACC ATT TCC TGA GAC T</td>
</tr>
<tr>
<td>c-Myc</td>
<td>CTA GTG CTG CAT GAG GAC ACA C</td>
<td>GTA GTT GTG CTG GTG AGT GGA G</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>ACG ATT TCA TCG AAC ACT TCC T</td>
<td>GGT CAC ACT TGA TGA CTC TGG A</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>CAG ATG ATG ATA CCC GGG ACA A</td>
<td>GGA GCC ACA TCA TTT CGA GTC A</td>
</tr>
<tr>
<td>Beta-Actin</td>
<td>TGT TAC CAA CTG GGA CGA CA</td>
<td>GGG GTG TTG AAG GTC TCA AA</td>
</tr>
<tr>
<td>EphB3</td>
<td>TAA CGC TGT GGA GGT CTC TGT A</td>
<td>CCT TGC TTT GCT TTG TAA CTC C</td>
</tr>
<tr>
<td>Msi-1</td>
<td>CCT GGT TAC ACC TAC CAG TCC C</td>
<td>AGA GCC TGT CCC TCG AAC TAC</td>
</tr>
<tr>
<td>Rac1</td>
<td>TGT CCG AGC TTT CCC GAA AG</td>
<td>CGG CCA TTT CCT GTC TCT TCT</td>
</tr>
<tr>
<td>Rac1b</td>
<td>GAC GGA GCT GTA GGT AAA ACT TG</td>
<td>ATC GCC AAT CGG CTT GTC TT</td>
</tr>
<tr>
<td>ATM</td>
<td>AGA ACC AGC TGC TAG ATC CG</td>
<td>GGG ACT TCT GTG AGG GGG AA</td>
</tr>
<tr>
<td>CDC25C</td>
<td>ACG TTT GGG AGA GAG TGG AAG G</td>
<td>TCA TTC GTG GGA GTC TCC GT</td>
</tr>
<tr>
<td>CCNE1</td>
<td>TCT AGC CCC ACC CCT TTA GT</td>
<td>TTT AAA TGT CCC GCT CGA AGC</td>
</tr>
<tr>
<td>DDX11</td>
<td>GTG AGT GTG AGG GAT GAG GG</td>
<td>CTA AAC GCA GCA CGG TGG</td>
</tr>
<tr>
<td>PIM1</td>
<td>GCG ACA TCA AGG AGC AGA AC</td>
<td>TCA GAG CTG GCA GGG GTG</td>
</tr>
<tr>
<td>CAV2</td>
<td>GTT CCG ACT GCA AGC CAA G</td>
<td>GAG GCT AGG CAG GGC GAA</td>
</tr>
<tr>
<td>Delta1</td>
<td>CAA CTG TCC ATA GTG CAA TGG</td>
<td>CTG AGG TGT AAG ATG GAA GCG</td>
</tr>
<tr>
<td>nRarp</td>
<td>GCG TGG TTA TGG GAG AAA GAT</td>
<td>GGG AGA GGA AAA GAG GAA TGA</td>
</tr>
<tr>
<td>Hes1</td>
<td>GTG GTG CGT AAC GCA GTG TC</td>
<td>TMA GAA GAG AGA GTG GGG CTA</td>
</tr>
<tr>
<td>mTert</td>
<td>TGG CTT GCT GCT GGA CAC TC</td>
<td>TGA GCC TCG TCT TAA TTG AGG TC</td>
</tr>
<tr>
<td>Lrig1</td>
<td>GGG TCC ACg GCT ATG ATG AGC T</td>
<td>CCG CCT GTG AGG ACT GAA AAC GC</td>
</tr>
<tr>
<td>Jagged1</td>
<td>TGC AGC TGT CAA TCA CTT CG</td>
<td>CAG AAT GAC GCT CTC GTC G</td>
</tr>
<tr>
<td>Jagged2</td>
<td>GTC TTT CCC GAT GGG AGT T</td>
<td>GTT TCC ACC TCG TCC GC</td>
</tr>
</tbody>
</table>

Table 2.10: QRT-PCR primer sequences
2.8.3.2. qRT-PCR reaction

All qRT-PCR reactions were run on StepOnePlus real-time PCR system in conjunction with StepOne software v2.1 (Applied Biosystems). Each reaction was performed in a minimum of three technical and three biological replicates. In addition to this, control samples devoid of cDNA served as negative controls were run to ensure no contaminants were present in cDNA samples. A housekeeping gene (typically β-actin) was used on each plate as a reference gene.

The traditional qRT-PCR reaction reagent SYBR Green qPCR mix (Fast Mix, Applied Biosystems) that fluoresces when bound to double stranded DNA was used. qRT-PCR reactions were carried out in triplicate in MicroAmp 96-well PCR plates 0.1 ml wells (Applied Biosystems), and sealed with MicroAmp heat activated optical adhesive film lids (Applied Biosystems). Each reaction contained 10 ng of cDNA, 10 µl Fast Mix SyBR green mix (Applied Biosystems), 0.5 µl of 10 nM Primers (forward and reverse) and made up to 20 µl with DNase/RNase-free water. qRT-PCR reactions were performed using the StepOne Plus Real-Time PCR System (Applied Biosystems), all reaction were carried out under the same cycling conditions: 95°C for 15 min for initial denaturing cDNA, followed by 30 cycles of a denaturation step at 95°C for 30 seconds, and an annealing step at 60°C for 30 seconds followed by an elongation step at 72°C for 30 seconds. qRT-PCR reactions for the intestinal stem cell markers (Lgr5, Ascl2, Olfm4, Bmi1 and β-actin) were performed using TaqMan assay (Applied Biosystems) following the manufacturer’s instructions and using custom design TaqMan probes (Applied Biosystems). Forward and reverse primers (100 mM) were mixed and diluted to 10 mM concentration. TaqMan reactions were run using the following conditions 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. The experimental data was collected automatically using StepOne software (Applied Biosystems).
2.8.3.3. Analysis of qRT-PCR data

Data was initially visually examined using Step One Plus software. Melting curves were briefly analysed to confirm that each reaction yielded only one product at the expected melting temperature and formation of primer dimers was minimal.

Data was analysed using the $2^{\Delta \Delta C_T}$ method (Livak and Schmittgen, 2001). Cycle time ($C_T$) values for each reaction was recorded and duplicates averaged. $\Delta C_T$ values were calculated using $C_T$ value of the reference gene from the same sample for target gene of interest. The average $\Delta C_T$ across all biological replicates was then calculated, and $\Delta \Delta C_T$ was calculated as the difference in $\Delta C_T$ average between genotypes. The average $\Delta \Delta C_T$ from each biological group was then transformed into fold change according to the formula $2^{\Delta \Delta C_T}$. 
2.9. Cell culture maintenance and storage

2.9.1. Maintenance of cell line

The human embryonic kidney cells (HEK-293) were kindly provided by Prof. Trevor Dale's lab (School of Bioscience, Cardiff University). HEK-293 cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), supplemented with L-glutamine (2 mM, Invitrogen), 10% v/v foetal bovine serum (FBS, Sigma), penicillin (50 μg/ml, Invitrogen) and streptomycin (50 μg/ml, Invitrogen). The cell line was incubated at 37°C and 5% CO$_2$ in T25 or T80 tissue culture flasks (Nunc, Leics, U.K.) and were routinely passage every 2-3 days at a split ratio of 1:2 - 1:5 once they became 80 - 90% confluent. Growth media was aspirated and the cells were washed with 1 X PBS (Invitrogen) to remove any remaining media. The wash was removed and 2 ml for T25 or 4 ml for T80 tissue culture flasks of 0.25% Trypsin/EDTA (Invitrogen) was added to the flask followed by 5-10 minutes incubation at 37°C and the detachment of cells was observed under microscope. An appropriate volume of growth medium was added to the cells and these were then split at an appropriate ratio by the removal of excess cell suspension into waste. The remaining volume of cell suspension was made up to 5 ml for T25 or 15 ml for T80 tissue culture flasks with complete growth medium.

2.9.2. Long term cell storage

To maintain sufficient aliquots of same passage number cell lines, cells were frozen and cryo-stored using Mr. Frosty (Fischer Scientific). Respective cells were detached using 0.25% Trypsin/EDTA (Invitrogen) and re-suspended in 10 ml of growth medium in a 15 ml falcon tube, followed by centrifugation at 1100 rpm for 5 minutes at room temperature. The supernatant was aspirated and the pellet of cell was re-suspended in 1 or 2 ml of freezing medium (complete medium containing 10% v/v dimethyl sulfoxide (DMSO, Sigma)) and aliquoted into two 1 ml or one 2 ml Cryo-tube vials (Nunc, Leics, U.K.). The tube(s) were then placed into Mr. Frosty with Iso-propanol (Fischer Scientific) to facilitate slow freezing at -80°C for 24 hours before being transferred into either -80°C freezer for up to 2 weeks or liquid nitrogen storage for long-term storage. When cells were retrieved from Cryo-storage,
they were quickly defrosted in a 37°C water bath to avoid DMSO toxicity before being re-suspended in 10 ml of complete growth media and centrifuged at 1100 rpm at room temperature for 5 minutes. The cell pellet was re-suspended in 5 ml of complete growth medium and transferred to a T25 or T80 flask.

2.9.3. Cell counting

In order to allow the seeding of cells at appropriate density, cells were counted using an Improved Neubauer Counting Chamber (Hawksley). Cells were detached using 0.25% Trypsin/EDTA (Invitrogen) and re-suspended in an appropriate volume of complete growth medium and 10 µl of cell suspension was loaded into the counting chamber. Cells were counted under a microscope in four 1 mm² squares and the number of cells per ml of suspension was determined by calculating the average count per square and multiplying it by $1 \times 10^4$.

2.9.4. Transfection and Luciferase reporter assays

2.9.4.1. Transient siRNA transfection

In order to achieve CBX3 knock down in HEK-293 cells, siCBX3 (FlexiTube, QIAGEN) were used. The negative control siRNA (AllStar Negative Control siRNA, QIAGEN) was designed to have minimal targeting of known genes in human. The siRNA (total amount of 5 nmol) was re-suspended according to manufacturer protocol in 250 µl of RNase-free water to obtain required concentration of 20 µM.

Cells were transfected with siRNA using lipofectamine RNAiMAX (Invitrogen). Volumes and concentrations of the reagents used in different tissue culture plates are outlined in Table 2.11. The appropriate volume of siRNA was diluted in serum-free OPTIMEM (Invitrogen) in the well of a tissue culture plate and mixed gently to cover the entire well surface. Appropriate volume of Lipofactamine RNAiMAX was subsequently added to each well containing the siRNA in OPTIMEM. The complex was mixed and incubated for 20 minutes at room temperature to allow the siRNA-Lipofectamine complexes
to form. Meanwhile, cells were diluted in antibiotic-free growth medium (for seeding concentrations see Table 2.12.). Cells were then carefully added to each well containing siRNA-Lipofectamine complexes and were incubated at 37°C in a 5% CO2 incubator for 48 hours before being harvested and analysed.

<table>
<thead>
<tr>
<th>Tissue culture vessel</th>
<th>Number of cells</th>
<th>Volume of Complete medium (ml)</th>
<th>Opti-MEM dilution medium</th>
<th>Final siRNA conc. (nM)</th>
<th>Volume of 20 µM siRNA</th>
<th>Lipofectamin RNAiMAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well</td>
<td>$2 \times 10^4$</td>
<td>0.1</td>
<td>20 µl</td>
<td>20</td>
<td>0.06 µl</td>
<td>0.3 µl</td>
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<tr>
<td>24-well</td>
<td>$1 \times 10^5$</td>
<td>0.5</td>
<td>100 µl</td>
<td>20</td>
<td>0.8 µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>6-well</td>
<td>$5 \times 10^5$</td>
<td>2.0</td>
<td>500 µl</td>
<td>20</td>
<td>4.5 µl</td>
<td>7.0 µl</td>
</tr>
</tbody>
</table>

Table 2.11. Volumes and concentration of reagents for transient siRNA transfection in different culture plates.

<table>
<thead>
<tr>
<th>Tissue culture vessel</th>
<th>Number of cells</th>
<th>Volume of Complete medium (ml)</th>
<th>Opti-MEM dilution medium</th>
<th>Plasmid DNA</th>
<th>Volume of Transfectin Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well</td>
<td>$2 \times 10^4$</td>
<td>0.1</td>
<td>25 µl</td>
<td>0.1 µg</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>24-well</td>
<td>$1 \times 10^5$</td>
<td>0.5</td>
<td>50 µl</td>
<td>0.6 µg</td>
<td>1.8 µl</td>
</tr>
<tr>
<td>6-well</td>
<td>$5 \times 10^5$</td>
<td>2.0</td>
<td>250 µl</td>
<td>2.9 µg</td>
<td>8.7 µl</td>
</tr>
</tbody>
</table>

Table 2.12. Volumes and concentration of reagents for transient transfection for cells with plasmid DNA.

2.9.4.2. Transfection of cells with reporter plasmids

The TCF/LEF reporter plasmid, TOP-flash, and its mutant control, FOP-flash, were provided by Trevor Dale’s lab (School of Bioscience, Cardiff University). For measuring transfection efficiency, cells were co-transfected with a non–Tcf/Lef-dependent β-gal coding vector, pcDNA-LacZ. In brief, HEK-293 cells were transfected with appropriate amount of siRNA (see Table 2.11) cultured in a 96-well plate to reach 60-80% confluence at the time of DNA plasmid transfection. The next day, cells were transfected in triplicates with 0.1 µg (100 ng) total DNA per well. Each of the plasmids were transfected at 0.033 µg/well, for pTOP-flash (which contains consensus sequence of Tcf/Lef enhancer upstream of the luciferase enzyme-coding region), pFOP-flash (control), pcDNA-lacZ (transfection control), ΔNLRP6, TCF4 or pcDNA3.1. Vector alone was included in the experiments. Transfection was carried out using Transfectin (BioRad) as described in Table 2.12. After four hours incubation, media...
and DNA complexes were aspirated and replaced with fresh antibiotic-free medium and the cells were incubated for 48 hours at 37°C in 5% CO₂ before being lysed.

2.9.4.3. TCF/LEF luciferase reporter assays

After 48 hours post-transfection with luciferase reporter plasmids, cells were lysed in 50 µl GLO-lysis buffer (Promega) per well for 30 minutes on a rocker at room temperature. The lysates were equally aliquoted into two replicate white-walled and clear-bottomed plates (Corning). BrightGLO substrate and BetaGLO substrate (Promega) were used on each plate for the quantification of luciferase and β-gal activity respectively. Both activities were measured using a BMG Fluostar multi-platform plate reader using the luminescence mode. Tcf-mediated gene transcription was determined by the ratio of pTOP-flash/pFOP-flash luciferase activity, each normalized to β-gal enzymatic activity levels. The mean values of the normalized ratios were compared.
2.10. Statistical analysis

2.10.1. Kaplan-Meier survival analysis

Survival analysis and generation of survival curves was performed using the Kaplan-Meier method. All analyses were performed with the aid of SPSS software (Version 20). Statistical significance between survival times of different experimental cohorts was examined using the Log-Rank method. A significant difference between survival probabilities was accepted if p value was less or equal 0.05.

2.10.2. Kolmogorov-Smirnov analysis

Kolmogorov-Smirnov test was used to determine if two data sets differ significantly. All analyses were performed using the SPSS software (Version 20). The two-tailed test was used throughout and significant difference between distributions was accepted when p value was less or equal 0.05.

2.10.3. Mann-Whitney U-test

The Mann-Whitney U-test was used to determine statistical differences between non-parametric data sets. This was performed using the SPSS software (Version 20). A significant difference between means was accepted when p value was less or equal 0.05.

2.10.4. Student’s t-test

The Student’s T-test was used to test statistical differences between normally distributed data sets and between data sets with sample sizes of n = 3. This test was performed in Excel 2010 software.
Chapter Three

Investigating the effects of *Cbx3* loss in the small intestine
3. Investigating the effects of Cbx3 loss in the small intestine

3.1. Introduction

HP1γ plays a critical role in chromatin organisation and gene expression and a growing body of evidence has implicated HP1γ as a novel prognostic and therapeutic target in various types of cancer (Slezak et al., 2013; Takanashi et al., 2009). Furthermore, a number of studies have implicated HP1γ in the regulation of cell differentiation and cancer development. Decreased expression of HP1γ is observed during cell differentiation, which is unique to this isoform compared to HP1α and HP1β. In differentiated cells of various normal human tissues, HP1γ is absent or not detectable, suggesting that the loss of HP1γ is required for cell differentiation to occur (Takanashi et al., 2009). HP1γ has been documented to play a role in regulating cell proliferation, differentiation (Takanashi et al., 2009), male germ cell survival and spermatogenesis (Brown et al., 2010). Furthermore, upregulation of HP1γ is frequently observed in many cancer cell lines (including colon cell lines) and repression of HP1γ expression in selected cancer cell lines, which results in growth inhibition (Takanashi et al., 2009). Consistent with this, the deletion mutant of HP1γ lacks tumorigenesis activity in a mouse xenograft model (Sharma et al., 2003), suggesting that the expression of HP1γ may be important in the process of tumorigenesis. Given that HP1γ is closely linked to cell proliferation, differentiation and tumorigenesis, and little is known about the functional involvement of HP1γ in colorectal cancer development, it is of great interest to investigate the role of HP1γ in colorectal cancer initiation, progression and tumour development. The murine small intestinal epithelium is commonly used as a model tissue to study human CRC, particularly the early stages of this disease, because this tissue has been proven to be an excellent in vivo model system to study stem cell renewal (Fevr et al., 2007), cell differentiation and proliferation, (i.e. intestinal homeostasis) that is maintained by Wnt signalling (Korinek et al., 1997). It is of great interest to study the role of HP1γ in Wnt signalling therefore, it appears to be appropriate to investigate the consequences of Cbx3 loss in normal intestinal epithelium in order to further explore its role in cancer development.

Conditional deletion of Cbx3 was achieved using conditional transgenesis, involving LoxP-targeted Cbx3 alleles combined with the inducible VillinCreER transgene. In this
chapter the effects of epithelial-specific *Cbx3* deletion both on epithelial homeostasis and on the intestinal stem cell were characterised and discussed.

### 3.2. Results

#### 3.2.1. *VillinCreER* drives recombination in the small intestine with high efficiency

In order to examine the effects of *Cbx3* loss in the small intestinal epithelium, animals bearing the *VillinCreER* transgene were crossed with animals bearing LoxP-targeted *Cbx3* alleles (*Cbx3^{fl/fl}*). The VillinCreER is expressed along the entire length of the small and large intestine and its recombination is induced by treatment regime of 4 consecutive daily intraperitoneal administration of tamoxifen at a dose of 80 mg/kg (el Marjou et al., 2004).

Expression of *VillinCreER* recombinase in the small intestinal epithelium was confirmed using the ROSA26R LacZ reporter allele (abbreviated here as LacZ). The reporter construct consists of the LacZ β-galatosidase transgene with a LoxP flanked Stop-cassette (Soriano, 1999). In unrecurred cells, the Stop-cassette attenuates expression of the LacZ transgene. Following exposure to Cre recombinase, the LoxP flanked Stop-cassette is removed and the expression of β-galactosidase from the LacZ transgene is mediated. X-Gal substrate can be used as an indicator for cells expressing LacZ which results in a colourimetric change from colourless to blue, therefore allowing identification of cells expressing Cre recombinase (See Figure 3.1).
Figure 3.1: Assessment of VillinCreER-mediated recombination in the small intestinal epithelium. (A) The ROSA26R transgene was used to report recombination within the small intestine. LoxP sites flank a PGK-Neo-STOP cassette inserted in the LacZ transgene. Induction of Cre recombinase activity results in PGK-Neo-Stop cassette excision and expression of the LacZ transgene. Cells expressing β-galactosidase can be visualised by exposure to X-Gal substrate, which results in a colourimetric change from colourless to blue. (B) Treatment of mice with 4 consecutive daily doses of Tamoxifen at 80 mg/kg results in a high levels of X-Gal staining, indicating a high level of recombination of the small intestinal epithelium. (i) The LacZ positive staining is demonstrated in intestine of VillinCreER’LacZ’ mice 4 days post-induction. (ii) Absence of the LacZ transgene results in no staining. The pictures show 2x magnification in the upper panels and 4x magnification in the lower panels.
3.2.2. *Cbx3* loss under the control of *VillinCreER* recombination

Following the confirmation that the induction of *VillinCreER* activity results in efficient recombination within the intestinal epithelium, the loss of *Cbx3*, driven by *VillinCreER* mediated recombination, was examined and the immediate effects of *Cbx3* loss on small intestinal homeostasis assessed. Cohorts of experimental *VillinCreER*<sup>+</sup>*Cbx3<sup>fl/fl</sup>* and control *VillinCreER*<sup>+</sup>*Cbx3<sup>+/+</sup>* (where <sup>+/+</sup> denotes an absence of loxP sites) animals were induced with daily intraperitoneal injections of tamoxifen at 80 mg/kg for 4 days and animals were sacrificed 24 hours after the last dose and small intestines harvested. Loss of HP1γ protein from the epithelium following recombination was assessed qualitatively using immunohistochemistry and semi-quantitatively using western analysis. Immunohistochemical studies of HP1γ expression revealed strong staining in a mosaic pattern for HP1γ in the epithelium and stroma of tissue sections from control animals 4 days post-induction. In contrast, staining for HP1γ was absent from the epithelium of intestines *VillinCreER*<sup>+</sup>*Cbx3<sup>fl/fl</sup>* animals whilst the mosaic-staining pattern in the stroma was retained (Figure 3.2).

![Figure 3.2: Induction of *VillinCreER* recombinase drives complete intestinal epithelial loss of *Cbx3*. *VillinCreER*<sup>+</sup>*Cbx3<sup>fl/fl</sup>* mice were induced with 4 daily intraperitoneal injection of 80 mg/kg Tamoxifen along with *VillinCreER*<sup>+</sup>*Cbx3<sup>+/+</sup>* controls. Immunohistochemical analysis of HP1γ expression revealed close to complete loss of *Cbx3* expression in the small intestinal epithelium of *VillinCreER*<sup>+</sup>*Cbx3<sup>fl/fl</sup>* mice at day 4 post-induction. Scale bars represent 200 μm.](image-url)
Given that *VillinCreER* recombination mainly occurs within the epithelium of the intestine, Western analysis on protein extracted from epithelium was used to examine the loss of HP1γ protein in the *VillinCreER*Cbx3<sup>fl/fl</sup> animals. A substantial reduction of HP1γ protein is observed in the *VillinCreER*Cbx3<sup>0/0</sup> animals at 4, 7, 14, 22 and 366 days post-induction compared to the *VillinCreER*Cbx3<sup>+/+</sup> control animals (Figure 3.3).

Overall, these data indicate that induction of *VillinCreER* results in recombination within the intestinal epithelium. In addition, Cbx3 is excised specifically and efficiently from the small intestinal epithelium, resulting in dramatic loss of HP1γ protein 4 days post induction.

![Figure 3.3: Western analysis showed substantial reduction of HP1γ protein from 4 days onwards post induction. Western analysis of epithelial cell extract samples from *VillinCreER*Cbx3<sup>0/0</sup> animals confirms reduction of HP1γ protein at 4, 7, 14, 22 and 366 days post-induction. HP1γ protein (25kDa) is present in all control *VillinCreER*Cbx3<sup>+/+</sup> animals. β-actin (42kDa) was used as a loading control.](image)

3.2.3. Intestinal epithelial Cbx3 deficiency does not impair survival

Before studying the effect of Cbx3 loss in Wnt-activated intestines, it is critical to examine the effect of Cbx3 deletion on the lifespan of the animals. A cohort of *VillinCreER*Cbx3<sup>0/0</sup> (n = 5) animals was aged along with *VillinCreER*Cbx3<sup>+/+</sup> control cohorts (n = 3). Mice from both groups were induced with 4 daily intraperitoneal injections of 80 mg/kg Tamoxifen and they were regularly inspected for health status. All mice were aged to the expected end point 366 days post induction and the health status from the experimental cohort *VillinCreER*Cbx3<sup>0/0</sup> animals looked indistinguishable from the control animals. Survival analysis of *VillinCreER*Cbx3<sup>0/0</sup> (n = 5) and control (n = 3) animals demonstrated no significant difference between the cohorts (p > 0.1 Log-Rank test, n ≥ 3).
3.2.4. *Cbx3* deficiency has little immediate effects on small intestinal homeostasis

Although the loss of *Cbx3* from the intestinal epithelium did not affect survival, a more detailed characterisation of intestinal homeostasis was conducted at various time points (Day 4, 7, 14, 22 and 366). Upon dissection the phenotypic presentation of the intestinal epithelia from all the *Cbx3* deficient cohorts was indistinguishable from the controls. Consequently, a quantitative analysis of histological and functional parameters of the intestinal epitheliums was performed to assess parameters of intestinal homeostasis. The data revealed subtle differences between *Cbx3* deficient and control animals (results are collated in Table 3.1).
Chapter 3: Investigating the effects of Cbx3 loss in the small intestine

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype</th>
<th>Mean</th>
<th>± Standard Deviation</th>
<th>p Value (to WT)</th>
</tr>
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<td>Control WT</td>
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<td>0.001</td>
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<td>Apoptosis per half-crypt</td>
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<td>0.21</td>
<td>0.01</td>
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<tr>
<td>Caspase3 positive cells per half-crypt</td>
<td>Control WT</td>
<td>1.37</td>
<td>0.37</td>
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<td>1.18</td>
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<td>0.09</td>
<td>0.159</td>
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### Chapter 3: Investigating the effects of Cbx3 loss in the small intestine

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<td>1.01</td>
</tr>
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</table>

Table 3.1. Quantitative analysis of histological parameters between small intestinal tissues from control and VillinCreER<sup>+</sup>Cbx3<sup>fl/fl</sup> animals.

Quantitative analysis of histological parameters such as crypt and villus length, apoptosis and proliferation were quantified and compared between small intestinal tissues from VillinCreER<sup>+</sup>Cbx3<sup>fl/fl</sup> (marked here as Control WT) and VillinCreER<sup>+</sup>Cbx3<sup>fl/fl</sup> animals at various time points post induction.
Chapter 3: Investigating the effects of Cbx3 loss in the small intestine

Figure 3.4: Quantitative analysis of the morphological and functional parameters of small intestinal epithelium following Cbx3 deletion. Experimental VillinCreER’Cbx3<sup>fl/fl</sup> animals along with controls were induced with 4 daily i.p. 80 mg/kg Tamoxifen and samples of the small intestine were harvested at day 4, 7, 14, 22 and 366 post induction. Histological parameters such as crypt length (A), villus length (B), apoptosis (C), percentage of apoptotic cells in crypt (D), mitosis (G) and percentage of mitotic bodies in crypt (H), were scored on H&E stained sections from control and VillinCreER’Cbx3<sup>fl/fl</sup> small intestines. The average number of cleaved Caspase 3 (E), Percentage of Caspase 3-positive cells in crypt (F), Ki67 (I) and percentage of Ki67 positive cells in crypt or villus were scored on respective immunostained sections. The positioning of Ki67 positive cells (K) in control and VillinCreER’Cbx3<sup>fl/fl</sup> small intestines at different time points were analysed by cumulative frequency analysis (Kolmogorov-Smirnov Z test, p = 0.297, n ≥ 3). Error bars represent standard deviation.
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A  
Ki67-positive cells (Ki67 IHC)

Figure 3.5: Immunohistochemical staining of Ki67 and cleaved Caspase-3. Immunohistochemical staining of (A) Ki67 and (B) cleaved Caspase-3 positive cells were performed in control and VillinCreER$^{+}$Cbx3$^{fl/fl}$ small intestines at different time points and selected representative sections are shown. Scale bars indicate 100 μm.

The crypt and villus sizes were scored as the average number of epithelial cells from the base of the crypt to the crypt-villus junction and from the crypt-villus junction to the top of villus respectively. Although no differences were observed in the crypt length 4 days post induction, a significant increase in the number of cells within the crypt was observed at both 7 and 22 days post induction (Figure 3.4A). However, the lack of significant differences at days 14 and 366 renders it difficult to draw an easy conclusion about the role of Cbx3 in regulating crypt length. Conversely, the length of villus was not significantly changed at any of the time points (Figure 3.4B).

To gain a clearly understanding, markers of cell proliferation and cell death were also examined in detail. Levels of apoptosis and mitosis within the crypt were scored from H&E stained sections and quantified as average number of apoptotic bodies and mitotic...
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figures per half-crypt respectively. No significant alteration in the numbers of apoptotic bodies was observed in any of the time points analysed Figure 3.4C and Figure 3.5B). Furthermore, the numbers of apoptotic bodies were quoted as percentage of total crypt cells to take account of the differences in crypt length. No significant changes in the percentage of apoptotic index in crypt regions were detected in any of the time points examined (Figure 3.4D). In order to validate apoptotic levels obtained from quantification of apoptotic bodies, sections of the Cbx3-deficient and control intestine were stained with the cell death marker cleaved Caspase-3. Scoring of cleaved Caspase-3 positive cells detected no significant differences following Cbx3 loss at any of the time points (Figure 3.4E). Further examination of the percentage of cleaved Caspase-3 positive cells in crypt revealed no significant differences following Cbx3 loss at all time points examined.

Quantification of mitotic figures revealed no difference in mitosis levels in Cbx3-deficient animals on 4, 7 and 22 days post-induction versus control animals. Whereas analysis of mitotic figures between Cbx3-deficient epitheliums on 14 days and 366 days post induction and control intestines demonstrated a significant increase in the average number of mitotic bodies (Figure 3.4G). Further examination of the percentage of mitotic cells in crypt showed no significant difference across all time points examined. In order to validate mitosis levels obtained from quantification of mitotic figures, sections of the Cbx3-deficient and control intestine were stained with Ki67, a cell proliferation marker. Quantification of the Ki67 positive cells detected no change in the average number of proliferating cells per half-crypt Cbx3-deficient samples on day 4, 14 and 366 post-induction verses control. However, a significant increase in the average number of Ki67 positive cells was detected in Cbx3-deficient epithelium on day 7 and 22 post induction (Figure 3.4I and Figure 3.5A), which correlates with the increase in crypt length shown in (Figure 3.4A). Furthermore, the relative distribution of Ki67 positive cells in crypt or villus as a percentage of the total Ki67 positive cells in the small intestines of wildtype control and VillinCreER* Cbx3<sup>0/f0</sup> small intestines were examined at different time points. The data revealed no significant difference between the distribution patterns between the control and Cbx3-deficient samples, all of which were with the majority of proliferative cells confined to the crypt (Figure 3.4J). This observation, therefore, further confirmed that the increase in Ki67 expressing cells on Day 7 and Day 22 samples compared to control samples was attributed
to the increase in crypt sizes solely but not an increase in proliferative activity in the epithelium. Additionally, distribution of the cells stained positive for Ki67 was analysed in order to examine the shape of the proliferative compartment. Analysis of the cumulative frequency of Ki67 positive cells in the crypt demonstrated no difference in distribution of proliferating cells across all Cbx3-deficient and control samples (Kolmogorov-Smirnov Z test, p = 0.297, n ≥ 3, Figure 3.4K).

Overall, quantitative analysis of the histological and functional parameters of the small intestinal epithelium showed that Cbx3 loss is tolerated within the small intestinal epithelium and does not perturb the normal architecture of the intestinal epithelium.
3.2.5. Cbx3 deficiency has no effect on cell migration of the intestinal epithelium

In order to examine the effects of Cbx3 loss on epithelial cell migration a BrdU pulse-chase experiment was performed. Cohorts of VillinCreER\textsuperscript{+}Cbx3\textsuperscript{fl/fl} and control mice (n = 4) were administrated with BrdU reagent on day 4 post-induction and one group of mice from both cohorts was sacrificed 2 hours and another group of mice was sacrificed 24 hours post-BrdU treatment to assess cell migration. The positions of the BrdU-labelled cells was then scored and their cumulative frequency was analysed as an indicator of migratory ability of the epithelial cells in Cbx3-deficient and control intestine.

Positional quantification of BrdU-positive cells 2 hours post-labelling revealed no significant difference in the distribution of BrdU positive cells between control and experimental animals, with positive cells being confined to the crypt (Kolmogorov-Smirnov Z test, p = 0.771, n = 4, Figure 3.6). Similarly, cumulative frequency analysis of BrdU-labelled cells 24 hours post-labelling displayed no significant changes in the migratory ability of the Cbx3-deficient epithelium (Kolmogorov-Smirnov Z test, p = 0.997, n = 4, Figure 3.6).
Figure 3.6: Cbx3 deficiency in small intestinal epithelium had no effects on cell migration. Cumulative frequency analysis of BrdU immunostaining 2 hours and 24 hours post-labelling revealed no significant changes in cell migration in the small intestinal epithelium of VillinCreER<sup>−/−</sup>Cbx3<sup>+/−</sup> compared to control mice 4 days post-induction (Kolmogorov-Smirnov Z test, 2 hours p = 0.771, n = 4; 24 hours p = 0.997, n = 4).

3.2.6. Intestinal epithelial cells differentiate normally in the absence of Cbx3

Intestinal homeostasis is highly regulated and maintained between cell proliferation and differentiation. In order to further characterise the effects of Cbx3 loss on small intestinal homeostasis the major differentiated cell types present in the small intestinal epithelium in Cbx3-deficient and control mice at day 4, 7, 14, 22 and 366 post induction were examined. The results of quantitative analysis of differentiated cells in control and Cbx3-deficient animals are collated in Table 3.2.
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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>p Value (to WT)</th>
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</thead>
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<tr>
<td><strong>Goblet cells per half crypt and villus</strong></td>
<td>Control WT</td>
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</tr>
<tr>
<td></td>
<td>$Cbx3^{+/+}$D4</td>
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<tr>
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<td>0.21</td>
<td>0.169</td>
</tr>
<tr>
<td></td>
<td>$Cbx3^{+/+}$D366</td>
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<td>0.08</td>
<td>0.120</td>
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<td><strong>Enteroendocrine cells per half crypt and villus</strong></td>
<td>Control WT</td>
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<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>0.22</td>
<td>0.978</td>
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<tr>
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<td>0.16</td>
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<tr>
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<td>0.424</td>
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</tbody>
</table>

Table 3.2: Quantitative analysis of Cbx3-deficient epithelia reveals no significant difference on cell differentiation compared to controls.

Small intestinal epithelia from $VillinCreER^{+/-}Cbx3^{-/+}$ (marked here as Control WT) and $VillinCreER^{+/-}Cbx3^{-/-}$ animals at various time points post-induction were examined and demonstrated no significant alternations on cell differentiation compared to controls ($n \geq 3$).
In the small intestine, enterocytes responsible for absorption constitute the majority of the cells in the villus. Using alkaline phosphatase, which specifically stains the brush border of the enterocytes, insight into the state of the enterocytes in the Cbx3-deficient epithelium compared to the controls could be gained. Alkaline phosphatase staining patterns revealed no alteration in the location of the brush border or differentiation of enterocytes in the context of Cbx3 deficiency (Figure 3.8A).

Goblet cells represent the second most abundant cell type in the small intestinal epithelium. Alcian blue staining was carried out to access changes in Goblet cell differentiation upon Cbx3 loss (Figure 3.8B). Quantification of the positively stained cells revealed no significant changes in number of Goblet cells between experimental and control animals (Figure 3.7A).

Enteroendocrine cells represent another secretory cell lineage of the small intestinal epithelium. In order to assess the frequency of the positively stained enteroendocrine cells, Grimelius staining was carried out on small intestinal sections from Cbx3-deficient intestine and controls (Figure 3.8C). Scoring of the cells stained for Grimelius showed no difference between experimental and control animals (Figure 3.7B).

The last member of the secretory cell lineage studied is Paneth cell. Tissue from control and Cbx3-deficient animals at various time points were stained immunohistochemically against lysozyme, a marker of Paneth cells (Figure 3.8D). Positively stained cells were scored and showed no difference in the Paneth cell numbers between experimental and control animals (Figure 3.7C). The staining of Paneth cells were found to be located at the base of the crypt and their position of the lysozyme positive cells showed no changes in the loss of Cbx3.

Overall, these observations demonstrate that small intestinal epithelial cells are capable to differentiate normally in the absence of Cbx3 from day 4 to day 366 post-induction, as all mature cell types revealed no differences between Cbx3-deficient and control intestines.
Figure 3.7: Quantification analysis of cell differentiation upon loss of Cbx3 within the small intestine.

The average number Goblet (A), Enteroendocrine (B) and Paneth (C) cells per half-crypt and villus was scored in both the wildtype control and VillinCreER\textsuperscript{+} Cbx3\textsuperscript{fl/fl} animals at day 4, 7, 14, 22 and 366 post induction. No significant difference was observed in the number of all the parameters tested above between control and experimental mice. Error bars represent standard deviation. Exact values, standard deviations, respective p values are provided in Table 3.2.
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Figure 3.8: Identification of differentiated epithelial cell types following Cbx3 loss. Small intestinal sections of Cbx3-deficient and control animals were subjected to cell type-specific stains to visualise Enterocytes (A), Goblet cells (B), Enteroendocrine cells (C) and Paneth cells (D). The presence of Enterocytes, Goblet, enteroendocrine and Paneth cells were not affected by Cbx3 loss. Scale bars indicate 100 μm.
3.2.7. Cbx3 loss alters intestinal stem cell gene expression

Previous evidence suggested that loss of Cbx3 is required for cell differentiation to occur; therefore it would be of great interest to investigate the consequences of Cbx3 loss on the normal intestinal stem cell population.

To assess the effects of Cbx3 loss on the small intestinal stem cell population, the expression levels of the intestinal stem cell markers Ascl2, Lgr5, Bmi1, Msi1 and Olfm4 were analysed (Potten et al., 2003; Barker et al., 2007b; Sangiorgi and Capecchi, 2008; van der Flier et al., 2009a; van der Flier et al., 2009b). Epithelium cell extracts from the small intestine of VillinCreER’T’Cbx3fl/fl and control mice at day 4 post-induction were used for qRT-PCR analysis to minimise interference from the stromal compartments of the intestine. The qRT-PCR expression analysis revealed a significant increased expression of Ascl2 in Cbx3 deficient epithelium compared to wildtype epithelium (p = 0.04, n = 3, Figure 3.9). However, this observation is not detected in the expression of Lgr5 (p = 0.287), Bmi1 (p = 0.344), Msi1 (p = 0.803) and Olfm4 (p = 0.696) following loss of Cbx3 from the intestinal epithelium (Figure 3.9). Thus the differences in the profiles of these surrogate markers of stem cell function do not paint an entirely clear picture of Cbx3 deficiency on intestinal stem cell compartment. One limitation is that three biological replicates may not be sufficient to yield accurate data, therefore increasing the sample size in future qRT-PCR analysis could be performed in order to improve the statistical power of the candidate intestinal stem cell genes.

In situ hybridisation using RNA probes for Olfm4 was performed to investigate further the effect of Cbx3 loss on the intestinal stem cell compartment. The in situ hybridisation revealed no alteration of Olfm4 staining of the VillinCreER’T’Cbx3fl/fl and control epithelium (Figure 3.10), consistent with the qRT-PCR data and indicate that loss of Cbx3 has no effect on Olfm4 expression.
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Figure 3.9: Cbx3 loss in the small intestinal epithelium increases Ascl2 expression. Quantitative RT-PCR analysis of the small intestinal epithelium 4 days post-induction revealed significantly increased expression level of Ascl2 in the small intestinal epithelium of VillinCreER<sup>Cbx3<sup>fl/fl</sup></sup> mice compared to control animals (p = 0.04, n = 3). No significant changes in the level of Lgr5, Bmi1, Msi1 and Olfm4 markers was observed in Cbx3-deficient intestinal epithelium compared to controls (Two-tailed T-test, p > 0.05, n = 3).

Figure 3.10: In situ hybridisation for Olfm4 expression in control and VillinCreER<sup>Cbx3<sup>fl/fl</sup></sup> intestines. In situ hybridisation analysis of the stem cell marker Olfm4 in the small intestine of VillinCreER<sup>Cbx3<sup>fl/fl</sup></sup> mice compared to control mice at day 4 post-induction demonstrated no alteration of Olfm4 expression following Cbx3 loss. Scale bars represent 100 μm.
3.2.8. *Cbx3* deficiency leads to reduced heterochromatic marks

It is known that *Cbx3* is a regulator of the epigenome, with critical roles in establishing, spreading and maintaining heterochromatin (Maison and Almouzni, 2004b). HP1γ, coded by the gene *Cbx3*, has been reported to be the reader of repressive methylation at histone H3K9 trimethylation, and mediate heterochromatin spread (Jacobs and Khorasanizadeh, 2002b; Nielsen et al., 2002b). It is therefore hypothesized that depletion of *Cbx3* would result in a complete loss of H3K9me3 marks in intestines.

Immunofluorescence analysis was performed in small intestinal tissue sections of control wildtype and *VillinCreER*^*Cbx3^0/0^ animals. Fluorescence microscopy images of intestines stained with antibodies against HP1γ (red) and H3K9me3 (green) revealed that both HP1γ and H3K9me3 signals overlapped substantially with that of DNA (DAPI, blue) (Figure 3.11A). The level of H3K9me3 was substantially lowered in the absence of *Cbx3* in the *VillinCreER*^*Cbx3^0/0^ intestinal epithelium compared to the control intestinal epithelium (Figure 3.11B), suggesting HP1γ plays some role in establishing and/or maintaining H3K9me3 in the intestinal homeostasis.

![Immunofluorescence analysis of HP1γ and H3K9me3 in small intestine of wildtype and *VillinCreER*^*Cbx3^0/0^ day 4 post induction.](image)

Confocal analysis of HP1γ and H3K9me3 in wildtype (A) and *VillinCreER*^*Cbx3^0/0^ (B) are illustrated in Red (Alexi 594) and Green (Alexi 488), respectively. These images are representative of small intestines analysed in each cohort (n = 3). Nuclei were counterstained with DAPI (4', 6-diamidino-2-phenylindole; blue).
After identifying that Cbx3 loss causes reduction in H3K9me3 level in the VillinCreER\(^r\)Cbx3\(^{fl/fl}\) intestines, it would be interesting to investigate whether another heterochromatic marker H4K20me3 would also be affected in the absence of Cbx3. H4K20me3, together with H3K9me3 and HP1\(\gamma\), were found to be essential for proper establishment of a compact heterochromatin structure (Dambacher, Hahn and Schotta, 2013). Therefore immunofluorescence analysis was performed on small intestinal tissue sections from wildtype and VillinCre\(^r\)Cbx3\(^{fl/fl}\) mice. Fluorescence microscopy images of intestines stained with antibodies against H4K20me3 correlated with the staining patterns observed in H3K9me3, with intense signals on the large matter of DAPI-dense facultative heterochromatin in the wildtype intestine (Figure 3.12A) and marked reduction of signals in Cbx3-deficient intestinal epithelia (Figure 3.12B). These findings suggest that HP1\(\gamma\) is involved in the establishment of both H3K9me3 and H4K20me3 signatures at heterochromatin in intestinal homeostasis.
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Figure 3.12: Immunofluorescence analysis of HP1γ and H4K20me3 in small intestine of wildtype and VillinCreER\(^{+}\)Cbx3\(^{fl/fl}\) mice day 4 post-induction. Confocal images of control (A) and VillinCreER\(^{+}\)Cbx3\(^{fl/fl}\) (B) small intestines stained with anti-HP1γ antibody (red) and anti-H4K20me3 antibody (green). Presented images are representative of small intestinal tissues analysed in each cohort (n = 3). Nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole; blue).

Despite the fact that the most common of HP1γ functions is the formation of heterochromatin, HP1γ is present in both heterochromatin and euchromatin (Minc, Courvalin and Buendia, 2000), it would be of great interest to investigate the importance of HP1γ to a euchromatic marker, H3K4me3. H3K4me3 is an active marker associated with actively transcribed genes (Hon, Hawkins and Ren, 2009). Therefore, immunofluorescence analysis of HP1γ and H3K4me3 was performed in the small intestine of the wildtype and
VillinCreER\textsuperscript{+}Cbx3\textsuperscript{fl/fl} animals. In contrast to the heterochromatic markers, the staining pattern of H3K4me3 was found to be mostly unchanged following the loss of Cbx3 (Figure 3.13), suggesting the active euchromatic marker H3K4me3 is present in small intestinal homeostasis, regardless of the presence or absence of Cbx3.

![Immunofluorescence analysis of HP1γ and H3K4me3](image)

Figure 3.13: Immunofluorescence analysis of HP1γ and H3K4me3 in small intestine of (A) wildtype and (B) VillinCreER\textsuperscript{+}Cbx3\textsuperscript{fl/fl} mice day 4 post-induction. Confocal images of small intestines stained with anti-HP1γ antibody (green) and anti-H3K4me3 antibody (red). These images are representative of small intestinal tissues analysed in each cohort (n = 3). Nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole; blue).
3.3. Discussion

Previous studies have suggested a role for Cbx3 (encoding the HP1γ protein) in cell lineage differentiation. Cbx3 negatively regulates adipocyte differentiation from preadipocyte cells and is required for male germ cell survival and spermatogenesis. Another study also suggested Cbx3 plays a critical role in the epigenetic regulation of both cell differentiation and cancer development. However, little is known about the functional involvement of Cbx3 in intestinal epithelial cell differentiation and development (Cammas et al., 2002; Cammas et al., 2004; Takanashi et al., 2009). This chapter describes for the first time, to our knowledge, the phenotypes of Cbx3 deficiency in small intestinal homeostasis, using conditional transgenics, involving LoxP-targeted Cbx3 alleles together with the inducible VillinCreER transgene.

Following generation of VillinCreER<sup>+/−</sup>Cbx3<sup>fl/fl</sup> and control animals, the loss of Cbx3 has been confirmed from the intestinal epithelium following induction of Cre recombinase activity by treatment with 4 consecutive daily doses of Tamoxifen at 80 mg/kg. Recombination within the small intestinal epithelium was assessed using the ROSA26R transgene reporter and visualised by X-Gal staining for β-galatosidase activity. This confirmed that the induction of VillinCreER activity resulted in efficient recombination within the intestinal epithelium. The loss of HP1γ protein from epithelium of VillinCreER<sup>+/−</sup>Cbx3<sup>fl/fl</sup> animals following recombination was also confirmed, both by Immunohistochemical studies and western analysis. Immunohistochemical studies of HP1γ revealed near-100% loss of HP1γ from the epithelium, whilst mosaic-staining pattern of HP1γ still remained in the stroma. Western analysis on protein extracted from epithelium further supported the fact that induction results in a substantial reduction of HP1γ protein 4 days post induction. This therefore indicates that induction of VillinCreER results in efficient recombination within the intestinal epithelium, resulting in dramatic loss of HP1γ protein 4 days post-induction.

Furthermore, the effect of Cbx3 deletion on the lifespan of the mice was also examined. A cohort of VillinCreER<sup>+/−</sup>Cbx3<sup>0/0</sup> was aged along with control VillinCreER<sup>+/−</sup>Cbx3<sup>+/+</sup> mice and both groups were induced with 4 daily intraperitoneal injections of 80 mg/kg
Tamoxifen and were regulated checked for health status. The epithelial Cbx3 deficiency did not affect health status of the experimental mice 366 days after induction.

3.3.1. Epithelial Cbx3 is not required for normal intestinal homeostasis

Having established that Cbx3 is lost from the intestinal epithelium in VillinCreER<sup>+</sup>Cbx3<sup>+/−</sup> animals, and that the loss of Cbx3 from the intestinal epithelium did not affect survival in our mouse models, the effect of Cbx3 deletion at various time points (Day 4, 7, 14, 22 and 366) on intestinal homeostasis were then examined. As Cbx3 has been demonstrated to play an important role in cell proliferation and differentiation, one could predict that Cbx3 loss would result in dramatic changes in the intestinal homeostasis.

In contrast to this hypothesis, deletion of Cbx3 from the epithelium has no dramatic effect upon homeostasis up to 366 days post-induction. No perturbations in gross histology were identified, and no change in proliferative and apoptotic status were observed, either from immunohistochemical studies or from scoring of mitotic or apoptotic index from H&E-stained sections. Furthermore, analysis of the migratory ability of Cbx3-deficinet epithelial cells revealed no alteration in migration. Although majority of the histological and quantitative analysis revealed no abnormalities in the general crypt-villus architecture despite the loss of Cbx3 from the epithelial cells. One notable difference is on the crypt sizes, which were quantified as significantly larger at both 7 and 22 days post-induction although these increases were not prominent under histological inspection. Despite this finding, Cbx3-deficient intestines displayed no significant changes in the villus sizes at any of the time points. The increase in crypt size observed at day 7 and 22 is therefore likely to be attributed to high variation and lower sample size. In addition, the lack of age-matched controls (data from all the experimental cohorts were compared with data from the day 4 control cohort) and the lack of significant differences at days 14 and 366 render it difficult to draw an easy conclusion about the role of Cbx3 in regulating crypt length. Using age-matched controls and increasing the sample number in each cohort in future studies would reduce variation in data and increase the statistical power of the experiment.

Takanashi and colleagues have demonstrated that the loss of Cbx3 is required for cell differentiation to occur (Takanashi et al., 2009). Therefore it might be predicted that
Cbx3 loss would result in increased differentiation of stem cell to mature epithelial cell types. However, qualitative and quantitative analysis of the normal differentiation of epithelial cells revealed that the presence, location and frequency of mature cell types was unaffected by Cbx3 loss across all time points. These data therefore indicate that Cbx3 is not necessary for normal development of the epithelium and deletion of Cbx3 from the epithelium exhibits no effect on the normal intestinal homeostasis.

3.3.2. Cbx3 loss in the epithelium alters Ascl2 stem cell gene expression

Previous studies from the literature indicate that Cbx3 plays a role in stem cell maintenance in adipocyte, male germ cell and cardiovascular system (Xiao et al., 2011; Brown et al., 2010). However, little is known about the functional involvement of Cbx3 in intestinal stem cell maintenance.

To examine the effects of Cbx3 loss on the small intestinal stem cell population, the expression levels of a number of the intestinal stem cell markers Ascl2, Lgr5, Bmi1, Msi1 and Olfm4 were assessed using qRT-PCR. The qRT-PCR analysis expression analysis showed no change of expression level in the crypt base columnar stem cell markers (Lgr5, Msi1 and Olfm4) and the +4 stem cell marker (Bmi1) following Cbx3 loss. Furthermore, In situ hybridisation using RNA probes for Olfm4 was performed and this confirmed that there was no alteration in the stem cell compartment of the epithelium.

However, Cbx3 loss up-regulated one of the stem cell markers, Ascl2, in the small intestinal epithelium. Ascl2, one of the CBC stem cell markers, was identified as an important regulator of intestinal stem cell fate, as its conditional deletion in the intestinal epithelium resulted in a rapid and selective loss of the Lgr5+ stem cell compartment, suggesting Ascl2 is essential for the maintenance of Lgr5 stem cells in the adult intestinal epithelium (van der Flier et al., 2009). Ascl2 expression level was significantly increased following the loss of Cbx3, indicating Cbx3 plays an active role on regulating Ascl2 expression and keeping a balance between stem cell maintenance and differentiation to maintain epithelial homeostasis. Previous data from this chapter demonstrated that the deletion of Cbx3 has no effect on cell differentiation and intestinal homeostasis, despite the fact that Cbx3 loss results in a significant increase in Ascl2 expression, this does not translate into any
cellular or phenotypic effects. This may be due to the highly plastic system operates between the intestinal stem cell populations, the effect of Ascl2 up-regulation following Cbx3 loss is likely to be diluted by the niche environment at the crypt base. Similarly, Ascl2 upregulation in normal intestinal homeostasis and in a Wnt-activated intestines has been reported to result in no alternation in the stem cell compartment of the intestine and no changes in survival in the Apc\textsuperscript{min} mouse suggesting that elevated levels of Ascl2 does not have an impact on the overall intestinal homeostasis and overall survival (Reed et al., 2012). Furthermore, Ascl2 is a Wnt target gene and it promotes cell growth and migration in colon cancer (Kwon et al., 2013). This observation raises a number of interesting questions with regard to the role of Cbx3 on colon cancer development and therefore provides directions for further investigation in the next chapter.

3.3.3. Cbx3-deficient intestinal epithelium displays diminished heterochromatic marks

Extensive studies have revealed that Cbx3 is a regulator of the epigenome, with roles in establishing, spreading and maintaining heterochromatin, through its ability to bind to H3K9me3, which is an epigenetic marker for gene silencing in the context of a histone code (Eissenberg, 2001; Bannister et al., 2001; Lachner et al., 2001; Maison and Almouzni, 2004). In this study, immunofluorescence analysis was performed in small intestinal tissue sections of control wildtype and VillinCreER*\textsuperscript{Cbx3\textsuperscript{fl/fl}} animals and the level of H3K9me3 was reduced in the absence of Cbx3 in the VillinCreER*\textsuperscript{Cbx3\textsuperscript{fl/fl}} intestinal epitheliums compared to the control intestinal epithelia, suggesting HP1\textgamma is involved in establishing and / or maintaining H3K9me3 in the intestinal homeostasis.

H4K20me3, another silencing heterochromatic marker, was found to be essential for proper establishment of a compact heterochromatin structure (Dambacher, Hahn and Schotta, 2013). Therefore, immunofluorescence analysis was also carried out in small intestinal tissue sections of control wildtype and VillinCreER*\textsuperscript{Cbx3\textsuperscript{fl/fl}} animals and the level of H4K20me3 was found to be substantially lowered following the loss of Cbx3. A sequential pathway could subsequently be proposed to connect these two histone methylation systems: H3K9me3 is established by Suv39h1 and Suv39h2 enzymes (Rea et al., 2000). HP1\textgamma
binds to histone methylation marks such as H4K20me3 and in turn recruit more Suv39h enzymes to maintain and propagate H3K9me3 as well as Suv4-20h methyltransferases, which then establish H4K20me3 (Schotta et al., 2004). Consistent with this sequential pathway, H3K9me3 and H4K20me3 are lost in the absence of HP1γ; these findings suggest that HP1γ is involved in the establishment and maintenance of both H3K9me3 and H4K20me3 signatures at heterochromatin in intestinal homeostasis.

3.3.4. Summary

In summary, three major conclusions can be drawn from the data presented in this chapter. Firstly, Cbx3 deficiency had no immediate effects on intestinal homeostasis and intestinal cell lineage differentiation, and epithelial Cbx3 deficiency does not affect life span of mice up to 366 days post-induction, indicating that Cbx3 is redundant with respect to epithelial homeostasis and survival. Secondly, Cbx3 deficiency has no the overt effect on the majority of the intestinal stem cell markers, apart from Ascl2. Despite the fact that Cbx3 deletion results in significant increase in Ascl2 expression level, intestinal homeostasis and cell differentiation were unaltered, indicating Cbx3-mediated up-regulation of Ascl2 alone might not be sufficient to alter intestinal stem cell maintenance and differentiation and therefore it has no effect on overall intestinal homeostasis. Thirdly, HP1γ plays a role in the establishment and maintenance of both H3K9me3 and H4K20me3 markers at heterochromatin in intestinal homeostasis.
Chapter Four

Investigating the effects of $Cbx3$ loss in Wnt-activated small intestine
Chapter 4: Investigating the effects of Cbx3 loss in Wnt-activated small intestine

4. Investigating the effects of Cbx3 loss in Wnt-activated small intestine

4.1. Introduction

Wnt signaling is well documented as a critical regulator of intestinal homeostasis and it is aberrantly activated in more than 90% of CRC cases (Clevers, 2006). The vast majority of mutations leading to aberrant Wnt signaling activation in CRC involve the Adenomatous polyposis poly gene (Apc) (Kinzler and Vogelstein, 1996). The role of activated Wnt signaling in homeostasis and tumorigenesis of the intestine has been extensively studied using murine models of Apc mutation (Taketo, 2006). Studies of constitutive homozygous disruption of Apc results in early embryonic lethality (Moser et al., 1995) whereas studies of heterozygous inactivation of Apc using different approaches lead to tumour predisposition syndrome, reminiscent of human FAP (Moser, Pitot and Dove, 1990; Shibata et al., 1997). Conditional deletion of both alleles of Apc in the adult intestine using the Cre-loxP system results in rapid disruption of intestinal epithelial homeostasis, observations includes hyperproliferation and attenuation of epithelial migration. Use of such model has indicated that mice following conditional deletion of Apc could live up to 5 days post induction (Sansom et al., 2004).

Cbx3 has been reported to be frequently upregulated in various cancer cell lines (including colon cell lines) and the transient knock down of Cbx3 inhibited cancer cell line growth (Takanashi et al., 2009). Furthermore, depletion of HP1γ attenuated tumorigenesis activity in a mouse xenograft model (Sharma et al., 2003), indicating that expression of HP1γ causes acceleration and progression of tumorigenesis.

To date, other than the acute up-regulation of Cbx3 expression following Wnt signalling activation, nothing is known about the biological involvement of Cbx3 in Wnt-activated small intestines. In this chapter, the role of Cbx3 in Wnt-activated small intestines was investigated by studying the impact of Cbx3 deficiency on Wnt driven carcinogenesis. Activation of Wnt signalling was achieved by conditional deletion of both copies of Apc using the Cre-loxP approach. Therefore, conditional homozygous deletion of Cbx3 and Apc was used to examine the role of Cbx3 loss in the context of acute Wnt driven carcinogenesis in the murine small intestinal epithelium.
4.2. Results

4.2.1. Prolonged lifespan of Cbx3 deficient mice in the context of acute Wnt driven carcinogenesis

Before studying the immediate, acute effects of combined loss of both Apc and Cbx3 on the intestinal epithelium, it is critical to examine whether Cbx3 loss will affect the lifespan of the animals with homozygous loss of Apc. In order to do this, the Cre-loxP conditional transgenic system was used. Experimental animals VillinCreERT2Apcfl/flCbx3fl/fl (n = 5) and controls VillinCreERT2Apcfl/fl (n = 5) were induced with 4 daily intraperitoneal injections of 80 mg/kg Tamoxifen and were sacrificed once ill health was observed.

Homozygous inactivation of Apc in the small intestinal epithelium, as previously described, induced acute aberrant Wnt activation, which results in rapid and catastrophic perturbation of epithelial homeostasis within 5 days of Apc loss (Sansom et al., 2004b). Consistently, control cohorts (VillinCreERT2Apclox/lox) developed signs of ill health and had to be sacrificed by day 4 post induction (Figure 4.1, median survival 4 days). Conversely, experimental animals (VillinCreERT2Apclox/loxCbx3lox/lox) with additional homozygous loss of Cbx3 displayed no signs of ill health on day 4 but survived significantly longer with some mice surviving past 7 days (Figure 4.1, median survival 7 days, Log-Rank test p < 0.01).
Chapter 4: Investigating the effects of Cbx3 loss in Wnt-activated small intestine

Figure 4.1. The additional loss of Cbx3 prolonged survival of mice with homozygous Apc loss. Cohort of VillinCreER\textsuperscript{+}Apc\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} animals were induced with four daily intraperitoneal injections of 80 mg/kg Tamoxifen along with VillinCreER\textsuperscript{+}Apc\textsuperscript{fl/fl} control animals (n = 5). Mice were closely monitored daily until signs of ill health are observed. Survival data was presented as a Kaplan-Meier plot. Analysis of survival probability revealed a significantly increased survival of VillinCreER\textsuperscript{+}Apc\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} mice (median survival 7 days) compared to the VillinCreER\textsuperscript{+}Apc\textsuperscript{fl/fl} control animals (median survival 4 days, Log-Rank test p = 0.003, n = 5).

4.2.2. No change in gross intestinal structure associated with Cbx3 loss in acute Wnt activated intestinal epithelium

Considering the additional loss of Cbx3 from the acute Wnt activated intestinal epithelium prolonged median survival by 3 days, the consequence of Cbx3 loss in the associated phenotypes that accompany aberrant Wnt pathway activation were investigated. Experimental cohorts VillinCreER\textsuperscript{+}Apc\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} and VillinCreER\textsuperscript{+}Apc\textsuperscript{fl/fl}, and control wildtype were induced and culled at day 4 post induction. Intestines were removed, fixed and sectioned before being stained for histological assessment. Quantitative analysis of histological parameters (crypt and villus size, apoptosis and mitosis levels) revealed that the experimental mice shared histological features of Wnt activated intestinal epithelium and there are no significant alternations between VillinCreER\textsuperscript{+}Apc\textsuperscript{fl/fl} and double knock-out VillinCreER\textsuperscript{+}Apc\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} animals. The results are collated in Table 4.1.
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Quantitative analysis of the effect of Cbx3 loss on the histological parameters in Wnt-activated intestinal epithelium.

Quantitative analysis of histological parameters such as crypt and villus size, apoptosis and mitosis levels, in the context of Wnt activated small intestinal epithelium, showed no significant alternations between VillinCreER\(^{+}\)Apc\(^{f/f}\) and VillinCreER\(^{+}\)Apc\(^{f/f}\)\(\text{Cbx3}^{f/f}\) animals.

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Mean ± Standard Deviation</th>
<th>p Value</th>
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<tbody>
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<td>Crypt Length (cells)</td>
<td>Control WT</td>
<td>26.62 ± 1.21</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Apc(^{f/f})</td>
<td>51.87 ± 2.59</td>
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<td>Apc(^{f/f})(\text{Cbx3}^{f/f})</td>
<td>48.96 ± 1.64</td>
<td></td>
</tr>
<tr>
<td>Villus Length (cells)</td>
<td>Control WT</td>
<td>64.37 ± 3.59</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Apc(^{f/f})</td>
<td>52.70 ± 3.09</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>Apc(^{f/f})(\text{Cbx3}^{f/f})</td>
<td>54.30 ± 2.79</td>
<td></td>
</tr>
<tr>
<td>Apoptosis per half-crypt</td>
<td>Control WT</td>
<td>1.09 ± 0.04</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>Apc(^{f/f})</td>
<td>2.27 ± 0.19</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>Apc(^{f/f})(\text{Cbx3}^{f/f})</td>
<td>2.52 ± 0.23</td>
<td></td>
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<tr>
<td>Caspase3 positive cells per half-crypt</td>
<td>Control WT</td>
<td>0.79 ± 0.19</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Apc(^{f/f})</td>
<td>3.10 ± 0.39</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Apc(^{f/f})(\text{Cbx3}^{f/f})</td>
<td>2.65 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>Mitosis per half-crypt</td>
<td>Control WT</td>
<td>0.70 ± 0.20</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>Apc(^{f/f})</td>
<td>1.60 ± 0.42</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td>Apc(^{f/f})(\text{Cbx3}^{f/f})</td>
<td>1.20 ± 0.36</td>
<td></td>
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<tr>
<td>Ki67 positive cells per half-crypt</td>
<td>Control WT</td>
<td>24.75 ± 1.03</td>
<td>0.001</td>
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The size of crypt and villus were quantified as the average number of cells between the crypt base and the crypt-villus junction and between the crypt-villus junction and the tip of the villus respectively. Average number of cells (± standard deviation) per crypt was found to be significantly longer in the intestinal epithelium of \textit{VillinCreER’Apc}\textsuperscript{fl/fl} and \textit{VillinCreER’Apc}\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} mice compared to control wildtype animals (p = 0.001). On the other hand, animals from both experimental groups displayed significantly shorter villi compared to control mice (p < 0.05). Quantification of the crypt and villus length revealed no difference between the \textit{Apc} deficient epithelium and animals with combined loss of \textit{Apc} and \textit{Cbx3} (p = 0.548 and p = 0.206 respectively, n ≥ 3, Figure 4.2A-B).

Apoptosis and mitosis levels were scored on H&E stained sections and quantified as average number of apoptotic bodies and mitotic figures per half-crypt respectively. Quantification of apoptotic bodies within crypts was significantly increased in the experimental animals compared to controls (p < 0.05). Scoring of cleaved Caspase-3 positive cells revealed a similar change in apoptosis levels (p = 0.001). \textit{VillinCreER’Apc}\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} intestine displayed no significant changes of apoptotic and Caspase-3 level compared to \textit{VillinCreER’Apc}\textsuperscript{fl/fl} intestine (p = 0.875 and p = 0.392 respectively, n ≥ 3, Figure 4.2C, E and H).

Quantification of mitotic figures revealed significant elevation of mitotic bodies in both experimental cohorts compared to control animals (p < 0.05). Similarly, quantification of Ki67 positive cells detected significantly higher number of proliferating cells in experimental mice compared to control mice (p = 0.001). However, no significant difference was observed between the \textit{VillinCreER’Apc}\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} intestine compared to \textit{VillinCreER’Apc}\textsuperscript{fl/fl} intestine (p = 0.158 and p = 0.685 respectively, n ≥ 3, Figure 4.2D, F and I). Additionally, distribution of proliferating cells was assessed by scoring positions of Ki67 positive cells. Cumulative frequency analysis of Ki67 positive cell revealed significant expansion of the proliferation compartment in \textit{VillinCreER’Apc}\textsuperscript{fl/fl} and \textit{VillinCreER’Apc}\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} epithelium compared to control mice (Kolmogorov-Smirnov Z test p < 0.01, n ≥ 3, Figure 4.2G). However, there is no significant variation in the distribution of Ki67 positive cells in the epitheliums between the two experimental groups.

Overall, these data demonstrate that loss of \textit{Cbx3} has no effect on the associated phenotypes observed in Wnt activated small intestinal epithelium.
Figure 4.2: Quantification analysis of the effect of short-term Cbx3 deficiency in Wnt-activated small intestinal epithelium. Experimental VillinCreER'Apc<sup>fl/fl</sup> and VillinCreER'Apc<sup>fl/fl</sup>Cbx3<sup>fl/fl</sup> mice together with control wildtype mice were induced with 4 daily i.p. injections of 80 mg/kg Tamoxifen and samples were harvested at day 4 post induction. (A-F) Histological parameters such as crypt length (A), villus length (B), apoptosis (C) and mitosis (D) were scored on H&E stained sections of small intestinal epithelium.
from control, *VillinCreER*^Ap$c^p_l$ and *VillinCreER*^Ap$c^p_l$*Cbx3*^p_l$. The average number of cleaved Caspase-3 (E) and Ki67 (F) positive cells per half-crypt were scored on respective immunostained sections and selected representative pictures are shown (H & I). (G) Cumulative frequency analysis of Ki67 positive cells revealed an expansion of the proliferative compartment in *VillinCreER*^Ap$c^p_l$ and *VillinCreER*^Ap$c^p_l$*Cbx3*^p_l* mice compared to control animals (for both comparison Kolmogorov-Smirnov Z test $p < 0.001$, $n = 4$). Comparison of cumulative frequency of Ki67 positive cells between the experimental groups revealed no alteration between the two experimental groups (Kolmogorov-Smirnov Z test $p = 0.707$, $n = 4$). Scale bars represent 100 μm. Error bars represent standard deviation. A single asterisk mark statistically significant $p$ value $< 0.05$ and double asterisks mark statistically significant $p$ value $< 0.01$ between groups. Exact values, standard deviation, $p$ values are provided in Table 4.1.

### 4.2.3. *Cbx3* loss in the context of *Apc* deficiency has no effect on cell migration

In order to investigate the effects of *Cbx3* loss on cell migration in *Apc* deficient small intestinal epithelium, BrdU was administrated at 2 hours and 24 hours prior to dissection of the mice. The positions of BrdU labelled cells 2 hours and 24 hours post labelling were quantified and compared, and cumulative frequency distributions of cell position were generated. Cumulative distribution analysis of BrdU positive cells detected an expansion of BrdU-positive cells in both experimental groups *VillinCreER*^Ap$c^p_l$ and *VillinCreER*^Ap$c^p_l$*Cbx3*^p_l* compared to control mice (Kolmogorov-Smirnov Z test $p < 0.001$, $n = 4$, Figure 4.3). However, the analysis of BrdU staining 24 hours post labelling displayed no significant changes in cell migration between two experimental groups *VillinCreER*^Ap$c^p_l$ and *VillinCreER*^Ap$c^p_l$*Cbx3*^p_l* intestines (Kolmogorov-Smirnov Z test $p > 0.05$, $n = 4$, Figure 4.3). Overall, *Cbx3* deficiency does not have any impacts on migratory ability in the intestines in the context of *Apc* deletion.
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Figure 4.3: Cbx3 loss does not affect cell migration in Apc-deficient small intestinal epithelium. Cumulative frequency analysis of BrdU staining 2 hours and 24 hours post-labelling detected a significant expansion of BrdU positive cells in the small intestine of the 2 experimental groups VillinCreER*Apc<sup>fl/fl</sup> and VillinCreER*Apc<sup>fl/fl</sup> Cbx3<sup>fl/fl</sup> mice compared to control animals (for both comparisons Kolmogorov-Smirnov Z test \( p < 0.001, n = 4 \)), however the analysis revealed no alteration in cell migration in VillinCreER*Apc<sup>fl/fl</sup> Cbx3<sup>fl/fl</sup> intestine compared to VillinCreER*Apc<sup>fl/fl</sup> intestine.

4.2.4. Cbx3 deficiency impacts on certain cell type differentiation in the context of Wnt-activated small intestinal epithelium

One of the most important signalling pathways that control cell fate decisions and differentiation in the small intestine is Wnt pathway (Fre et al., 2009). Therefore, to further characterise the effects of Cbx3 loss on cell differentiation in the context of aberrant Wnt activation, quantitative analysis of the major mature cell types present in the small intestinal epithelium of control wildtype, VillinCreER*Apc<sup>fl/fl</sup> and VillinCreER*Apc<sup>fl/fl</sup> Cbx3<sup>fl/fl</sup> mice was undertaken (Figure 4.4 and Figure 4.5) and the results are summarised in Table 4.2.
Chapter 4: Investigating the effects of Cbx3 loss in Wnt-activated small intestine

Table 4.2: Quantification analysis of Cbx3 loss modulates cell type-specific responses to aberrant Wnt signalling activation. Experimental VillinCre\(^{+/R}\) and VillinCre\(^{+/R}\)Ap\(^c^{+/R}\)Cbx3\(^{+/R}\) mice together with control wildtype mice were induced with 4 daily i.p. 80 mg/kg Tamoxifen and samples were harvested at day 4 post-induction. Cell type-specific stains were performed to study differentiation of various mature cell types in the small intestinal epithelium. Frequency of Goblet, Enteroendocrine and Paneth cells was quantified and compared between the three groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype</th>
<th>Mean ± Standard Deviation</th>
<th>p Value</th>
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<tr>
<td></td>
<td></td>
<td>WT</td>
<td>WT</td>
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<tr>
<td></td>
<td></td>
<td>vs</td>
<td>vs</td>
</tr>
<tr>
<td>Goblet cells per half crypt and villus</td>
<td>Control WT</td>
<td>6.98 ± 0.57</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>Ap(^c^{+/R})</td>
<td>5.09 ± 0.22</td>
<td>0.192</td>
</tr>
<tr>
<td></td>
<td>Ap(^c^{+/R})Cbx3(^{+/R})</td>
<td>5.20 ± 0.73</td>
<td>0.351</td>
</tr>
<tr>
<td>Enteroendocrine cells per half crypt and villus</td>
<td>Control WT</td>
<td>0.76 ± 0.05</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td>Ap(^c^{+/R})</td>
<td>0.75 ± 0.04</td>
<td>0.542</td>
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<td></td>
<td>Ap(^c^{+/R})Cbx3(^{+/R})</td>
<td>0.89 ± 0.08</td>
<td>0.489</td>
</tr>
<tr>
<td>Paneth cells per half crypt and villus</td>
<td>Control WT</td>
<td>4.54 ± 1.61</td>
<td>0.249</td>
</tr>
<tr>
<td></td>
<td>Ap(^c^{+/R})</td>
<td>5.56 ± 0.53</td>
<td>0.815</td>
</tr>
<tr>
<td></td>
<td>Ap(^c^{+/R})Cbx3(^{+/R})</td>
<td>4.56 ± 0.38</td>
<td>0.055</td>
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Alkaline phosphatase was used to visualise the brush border of the enterocytes in small intestinal epithelium. A consistent decrease in staining was observed between VillinCre\(^{+/R}\)Ap\(^c^{+/R}\)Cbx3\(^{+/R}\) compared to VillinCre\(^{+/R}\)Ap\(^c^{+/R}\) epitheliums (Figure 4.4A).

Alcian blue staining was undertaken to identify Goblet cells in the small intestinal epithelium (Figure 4.4B). Scoring of the cells stained with Alcian blue showed a significant reduction in the numbers of Goblet cells per half-crypt-villus structure in the epithelium of VillinCre\(^{+/R}\)Ap\(^c^{+/R}\) mice compared to the control animals (p < 0.01, n ≥ 3, Figure 4.5A). No significant changes were detected in Goblet cell numbers from VillinCre\(^{+/R}\)Ap\(^c^{+/R}\)Cbx3\(^{+/R}\) and VillinCre\(^{+/R}\)Ap\(^c^{+/R}\) or control epitheliums (p > 0.05, n ≥ 3).

Grimelius staining was used to detect Enteroendocrine cells in the small intestinal epithelium (Figure 4.4C). Quantification of the positively stained cells revealed no differences in the number of Enteroendocrine cells per half-crypt-villus structure in control, VillinCre\(^{+/R}\)Ap\(^c^{+/R}\) and VillinCre\(^{+/R}\)Ap\(^c^{+/R}\)Cbx3\(^{+/R}\) animals (p > 0.05, n ≥ 3, Figure 4.5B).
Lastly, immunohistochemical staining of lysozyme expression was carried out to detect Paneth cells in the small intestinal epithelium (Figure 4.4D). Scoring of lysozyme positive cells in the intestinal epithelium of double knock out VillinCreER\(^{+/lo}\)Apc\(^{fl/fl}\)Cbx3\(^{fl/fl}\) mice detected a reduced number of Paneth cells compared to VillinCreER\(^{+/lo}\)Apc\(^{fl/fl}\) mice, however the change was not significantly different (\(p = 0.055, n \geq 3\), Figure 4.5C). In addition, the position of the Lysozyme positive cells was scored in control, VillinCreER\(^{+/lo}\)Apc\(^{fl/fl}\) and VillinCreER\(^{+/lo}\)Apc\(^{fl/fl}\)Cbx3\(^{lo/lo}\) epithelium. Cumulative distribution analysis on Paneth cell position revealed a significant expansion of the Paneth cell population in both VillinCreER\(^{+/lo}\)Apc\(^{fl/fl}\) and VillinCreER\(^{+/lo}\)Apc\(^{fl/fl}\)Cbx3\(^{lo/lo}\) epithelia compared to the control epithelium (Kolmogorov-Smirnov Z test, both \(p < 0.001, n \geq 3\), Figure 4.5D). Analysis of the Paneth cell position did not differ significantly between the two experimental groups VillinCreER\(^{+/lo}\)Apc\(^{fl/fl}\) and VillinCreER\(^{+/lo}\)Apc\(^{fl/fl}\)Cbx3\(^{lo/lo}\) (Kolmogorov-Smirnov Z test, \(p = 0.10, n \geq 3\), Figure 4.5D).
Figure 4.4: Identification of differentiated epithelial cell types upon Cbx3 loss in Wnt-activated small intestinal epithelium.

Small intestinal sections of VillinCreER\(^{+}\)Apc\(^{fl/fl}\) and VillinCreER\(^{+}\)Apc\(^{fl/fl}\) Cbx3\(^{fl/fl}\) mice at day 4 post induction were subjected to cell-type specific stains to visualise Enterocytes (A), Goblet cells (B), Enteroendocrine cells (C) and Paneth cells (D). Alkaline phosphatase staining revealed a slightly diminished brush border in the small intestinal epithelium of VillinCreER\(^{+}\)Apc\(^{fl/fl}\) Cbx3\(^{fl/fl}\) mice (A). No apparent differences were detected in differentiation of Goblet and enteroendocrine cell lineages (B and C), whereas a reduction in Paneth cell number is shown between the experimental and control cohorts. However, these changes were not significantly different (D). Scale bars indicate 100μm.
Figure 4.5: Quantification analysis of cell differentiation upon Cbx3 loss in Wnt-activated small intestine.

The average number of Goblet (A), Enteroendocrine (B) and Paneth (C) cells per half-crypt and villus was scored in the small intestine of control, VillinCreER\textsuperscript{+}Apc\textsuperscript{+/+} and VillinCreER\textsuperscript{+}Apc\textsuperscript{+/+}/Cbx3\textsuperscript{fl/fl} mice at day 4 post induction. The number of Goblet cells was significantly reduced in VillinCreER\textsuperscript{+}Apc\textsuperscript{+/+} mice compared to control animals (p = 0.016, n ≥ 3), no changes between the groups were observed in differentiation of Enteroendocrine cells. The number of Paneth cells was found to be reduced in double knock out mice compared to Apc deficient animals, however the change was not significantly different (p = 0.055, n ≥ 3). Error bars represent standard deviation. Exact values, standard deviations and p values are provided in Table 4.2. (D) Cumulative frequency analysis of Paneth cell distribution detected a significant expansion of Paneth cell location throughout the crypt in VillinCreER\textsuperscript{+}Apc\textsuperscript{+/+} and VillinCreER\textsuperscript{+}Apc\textsuperscript{+/+}/Cbx3\textsuperscript{fl/fl} small intestine compared to control animals (Kolmogorov-Smirnov Z test, both p < 0.001, n ≥ 3).
4.2.5. *Cbx3* loss shows a tendency to attenuate the expression of Wnt target genes in the Wnt activated small intestine

Down-regulation of *Cbx3* expression was previously reported to lead to growth inhibition of cancer cell lines (including colorectal cancer cell lines) (Takanashi et al., 2009b). The dysregulation of the Wnt signalling pathway has been considered to play an important role in colorectal carcinogenesis and since little is known about the functional involvement of *Cbx3* in Wnt signalling regulation and colorectal cancer development, it was of great interest to investigate the consequence of *Cbx3* loss on Wnt target gene activation in the small intestinal epithelium.

Quantitative analysis of Wnt target gene expression was performed on intestinal samples from control, VillinCreER′Apc^fl/fl^ and VillinCreER′Apc^fl/fl^Cbx3^fl/fl^ animals (n = 6). Total mRNA was extracted from the small intestinal epithelium and subjected to qRT-PCR analysis in order to assess the expression levels of a subset of Wnt target genes. A significantly elevated level of expression of *Axin2*, *c-Myc*, *CD44* and *CyclinD1* was demonstrated in the VillinCreER′Apc^fl/fl^ and VillinCreER′Apc^fl/fl^Cbx3^fl/fl^ intestines compared to wildtype (p < 0.05, n = 6). Notably, the additional loss of *Cbx3* displayed a tendency to attenuate *Axin2*, *c-Myc*, *CD44*, *CyclinD1* and *EphB3* up-regulation although these reductions were not statistically significant (Figure 4.6).

Given that the loss of *Cbx3* has been shown to prolong survival of mice with homozygous Apc loss (see Section 4.2.1), and *Cbx3* loss attenuates the activation of the Wnt target genes in an Apc deficient intestine, an established *in vitro* Wnt-responsive Topflash analysis was performed to further study the effect of *Cbx3* loss on Wnt activity.
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Figure 4.6: Cbx3 loss shows a tendency to attenuate expression of the Wnt target genes.
qRT-PCR analysis of Wnt targets: Axin2, cMyc, CD44, CyclinD1, EphB3 and E-cadherin revealed slight reduction of a range of Wnt target gene expression in the small intestinal epithelium of VillinCreER^Apc^{fl/fl}Cbx3^{fl/fl} compared to VillinCreER^Apc^{fl/fl} animals at day 4 post induction. Asterisks mark significant changes with p value < 0.05. For all comparisons n = 6.

4.2.6. Transient knock down of CBX3 exhibits inhibitory effects on Wnt signalling

To investigate if Cbx3 is a potential Wnt regulator, luciferase assays were employed in HEK-293 cells through the use of the Wnt-responsive Topflash system.

Transient knock down of CBX3 using siRNA were performed 24 hours prior to transfection of TCF/β-catenin-responsive luciferase reporter, pTOPFLASH or its mutated variant, pFOPFLASH into HEK-293 cells in conjunction with a Wnt pathway activator, ΔNLRP6 (constitutively active LRP6 receptor) or TCF4-VP16 (constitutively active form of TCF4 transcription factor). The luciferase activities were determined from cell lysates 48 hours after transfection.

In this experiment, siRNA was used to down-regulate the expression of CBX3 and the resulting TCF/β-catenin-dependent transcription was analysed. First, the efficiency of CBX3 siRNA towards CBX3 mRNA using qRT-PCR was tested (as shown in Figure 4.7A). The CBX3 siRNA substantially reduced the levels of CBX3 mRNA (8.7 fold reduction) compared to the control scrambled siRNA. Subsequently, the Topflash reporter plasmids were introduced using transfectin; the plasmids used include the pTOPFLASH reporter, pFOPFLASH (control) and pLacZ (transfection efficiency). To stimulate Wnt signalling the cells were co-transfected
with the ΔNLRP6 construct or the TCF4-VP16 construct. Forty-eight hours post-transfection the cells were harvested, cell lysates prepared and luciferase activity measured. CBX3 down-regulation resulted in a reduction of ΔNLRP6 (41%) and TCF4 (27%) stimulation of TCF/β-catenin-dependent transcription or Topflash activity compared to the control cells.

Thus, the loss of CBX3 demonstrated a reduction of Wnt activity from the receptor level to transactivation of Wnt target gene level, thereby demonstrating that CBX3 positively contributes to Wnt signalling activation.

**Figure 4.7**: The effect of transient knock down of CBX3 on Topflash activity. (A) qRT-PCR was performed using human CBX3-specific primers on HEK-293 cells and HEK-293 cells stably transfected with siRNA toward CBX3. Scramble siRNA was included as control. CBX3 transcripts were significantly reduced in cells transfected with CBX3 siRNA compared to the Scramble siRNA control, data was collected 72 hours after siRNA transfection (8.7 fold reduction, p < 0.01, n = 3). (B) Topflash was activated by transfection of ΔNLRP6 or TCF4 in HEK-293 cells. Transient knock down of CBX3 using CBX3 siRNA 24 hours prior transfection of Topflash activator NLRP6 or TCF4 (plotted as percentages of the Scramble siRNA value) reduced total Topflash activity by 41% (p = 0.04, n = 3) and 27% (p = 0.10, n = 3), data collected 48 hours post-transfection. A single asterisk denotes statistically significant p value < 0.05 and double asterisks marks statistically significant p value < 0.01.
4.2.7. Cbx3 loss does not affect Wnt-activated stem cell gene expression

Studies have shown that Cbx3 is upregulated in various cancer cell lines including colon cell lines. Considering Cbx3 has a potential to attenuate Wnt pathway activation in small intestinal epithelium and inactivation of CBX3 in vitro suppressed Wnt activation. The consequences of Cbx3 loss on the expression levels of the intestinal stem cell genes was examined to see if Cbx3 loss would suppress the expansion of the stem cell population in Wnt activated intestinal epithelium. In order to do this, the intestinal stem cell markers Ascl2, Lgr5, Msi1, Bmi1 and Olfm4 were analysed by qRT-PCR using RNA samples extracted from small intestinal epithelium of control, VillinCreER^Apc^{fl/fl} and VillinCreER^Apc^{fl/fl}Cbx3^{fl/fl} mice.

Expression levels of Ascl2, Lgr5 and Msi1 were significantly increased in VillinCreER^Apc^{fl/fl} and VillinCreER^Apc^{fl/fl}Cbx3^{fl/fl} compared to control animals (Figure 4.5, for Ascl2 \( p < 0.01 \) and for Lgr5 and Msi1 \( p < 0.05, n = 6 \), indicating the stem cell population were hugely expanded following the loss of Apc and acute Wnt activation. However, no significant difference was detected in the stem cell markers in VillinCreER^Apc^{fl/fl}Cbx3^{fl/fl} epithelium compared to VillinCreER^Apc^{fl/fl} epithelium (\( p > 0.05, n = 6 \)), suggesting that the status of Cbx3 does not contribute to the expansion of the stem cell population.

In addition, in situ hybridisation analysis of Olfm4 was performed to further assess the effect of Cbx3 loss on the state of the stem cell population in Wnt-activated intestinal epithelium. Small intestinal samples from VillinCreER^Apc^{fl/fl} and VillinCreER^Apc^{fl/fl}Cbx3^{fl/fl} animals harvested 4 days post induction were stained using an anti-Olfm4 riboprobe (Figure 4.9). The small intestinal epithelium of VillinCreER^Apc^{fl/fl} and VillinCreER^Apc^{fl/fl}Cbx3^{fl/fl} animals displayed Olfm4 staining throughout the crypt length. The additional loss of Cbx3 in context of Apc deficiency revealed no alteration in the stem cell compartment in the small intestinal epithelium of VillinCreER^Apc^{fl/fl}Cbx3^{fl/fl} compared to VillinCreER^Apc^{fl/fl} animals.

Previous studies demonstrated that the loss of Cbx3 is required for cell differentiation to occur and therefore leads to a reduction in stem cell numbers (Takanashi et al., 2009e). These observations are therefore at odds with published data, in that deletion of Cbx3 from the epithelium did not result in a reduction in stem cell markers or compartment within the Wnt-activated intestines.
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Figure 4.8: Cbx3 loss does not attenuate Wnt-driven upregulation of small intestinal stem cell gene expression. Quantitative RT-PCR expression analysis revealed a significant up-regulation of the stem cell markers Ascl2, Lgr5, Msi1, Bmi1 and Olfm4 in the small intestinal epithelium of VillinCreER'Apc\textsuperscript{fl/fl} and VillinCreER'Apc\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} mice compared to wildtype control at day 4 post-induction (for single asterisk p value < 0.05 and double asterisks p value < 0.01, n = 6). Expression levels of all stem cell markers revealed no significant differences in the small intestine of VillinCreER'Apc\textsuperscript{fl/fl} mice compared to VillinCreER'Apc\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} mice.

Figure 4.9: The additional Cbx3 loss does not alter stem cell compartment. \textit{In situ} hybridisation analysis of Olfm4 expression revealed no expansion or reduction of the stem cell compartment in the small intestinal epithelium of VillinCreER'Apc\textsuperscript{fl/fl}Cbx3\textsuperscript{fl/fl} compared to VillinCreER'Apc\textsuperscript{fl/fl} animals. Scale bars represent 100 μm.
**4.2.8. Cbx3 loss does not affect Wnt-activated Notch target gene expression**

Notch signalling pathway has been shown to be critical in driving enterocyte differentiation (Fre et al., 2005). The loss of Cbx3 was found to suppress enterocyte differentiation in VillinCreER<sup>+</sup>Apc<sup>fl/fl</sup>Cbx3<sup>fl/fl</sup> compared to VillinCreER<sup>+</sup>Apc<sup>fl/fl</sup> epitheliums (Figure 4.4A) and therefore it can be hypothesised that Cbx3 loss could potentially attenuate Notch signalling to a level sufficient to impair enterocyte differentiation.

In order to investigate the effects of Cbx3 deficiency on Notch signalling in the Wnt-activated intestinal epithelium, the expression levels of a subset of Notch signalling target genes were examined. Total mRNA was extracted from the intestinal epithelium of control, VillinCreER<sup>+</sup>Apc<sup>fl/fl</sup> and VillinCreER<sup>+</sup>Apc<sup>fl/fl</sup>Cbx3<sup>fl/fl</sup> mice at day 4 post-induction and used for qRT-PCR. Expression analysis of the Notch target genes: Delta1, nRarp, Hes1, mTert, Lrig1, Jagged1 and Jagged2 revealed significantly increased expression levels in both VillinCreER<sup>+</sup>Apc<sup>fl/fl</sup> and VillinCreER<sup>+</sup>Apc<sup>fl/fl</sup>Cbx3<sup>fl/fl</sup> epithelium compared to wildtype control epithelium (p < 0.05, n = 6, Figure 4.10). The expression analysis of the Cbx3 deficient epithelium demonstrated a slight decrease in Delta1, nRarp, Hes1, Lrig1 and Jagged2 expression compared to the VillinCreER<sup>+</sup>Apc<sup>fl/fl</sup> samples, and however the changes were not significantly different.

![Graph showing expression levels of Notch target genes](image)

**Figure 4.10:** Cbx3 loss does not affect expression of Notch target genes. qRT-PCR analysis revealed a significant up-regulation of a range of Notch target genes (Delta1, nRarp, Hes1, mTert, Lrig1, Jagged1 and Jagged2) in the small intestinal epithelium of VillinCreER<sup>+</sup>Apc<sup>fl/fl</sup> and VillinCreER<sup>+</sup>Apc<sup>fl/fl</sup>Cbx3<sup>fl/fl</sup> animals compared to control samples at day 4 post-induction. However, there is no significant change in expression of the Notch target genes between the two experimental groups. Asterisks represent the pair-wise comparisons that were found to be significantly different (p < 0.05, n = 6).
4.2.9. *Cbx3* loss alters Wnt target Splice variants but has mild effect on cell cycle gene expression in Wnt-activated intestine

The Wnt target gene, *Rac1*, was shown to be essential for tumour formation *in vivo* (Matos and Jordan, 2008) and a novel splice variant of *Rac1*, designated *Rac1b*, is expressed in a range of cancer cells including colon carcinoma cells (Singh *et al.*, 2004). *Cbx3* has been demonstrated to play a role in RNA splicing due to its interaction with splicesome (Smallwood *et al.*, 2012).

The expression levels of the Wnt target gene *Rac1* and its splice variant *Rac1b* was determined using qRT-PCR analysis. cDNA was made from RNA extracted from control, *VillinCreER^*Apc^{fl/fl^} and *VillinCreER^*Apc^{fl/fl^}Cbx3^{fl/fl^} epithelium at day 4 post induction and used for qRT-PCR analysis.

Expression levels of the Wnt target gene *Rac1* and its splice variant *Rac1b* increased significantly in *VillinCreER^*Apc^{fl/fl^} epithelium compared to control epithelium (p < 0.05, n = 6). Although both *Rac1* and *Rac1b* expression was reduced in the *VillinCreER^*Apc^{fl/fl^}Cbx3^{fl/fl^} epithelium compared to the *VillinCreER^*Apc^{fl/fl^} epithelium, only expression of *Rac1b* was found to be significantly repressed following the loss of *Cbx3* in the Wnt activated intestine (Figure 4.11). This observation is consistent with the hypothesis that loss of *Cbx3* impedes RNA splicing and therefore leads to a reduced level of *Rac1b* in the small intestinal epithelium.

Previous work has demonstrated that the role of *Cbx3* on cell cycle regulation in *Caenorhabditis elegans* and human cells (Smallwood *et al.*, 2012). It is therefore of great interest to investigate the effect of *Cbx3* loss on the expression level of a range of cell cycle genes. qRT-PCR analysis of the cell cycle genes detected a significantly reduced expression of *ATM* and a significantly elevated expression of *CDC25C* in both *VillinCreER^*Apc^{fl/fl^} and *VillinCreER^*Apc^{fl/fl^}Cbx3^{fl/fl^} epithelium compared to the control epithelium (p < 0.05, n = 6). *Cbx3* loss shows a tendency to reduce expression of *CCNE1, PIM2* and *CAV2* in the Wnt-activated *VillinCreER^*Apc^{fl/fl^}Cbx3^{fl/fl^} intestines, although the changes are not statistically significant.
Together these observations suggest that Cbx3 deficiency is likely to suppress RNA splicing and therefore attenuate the level of Rac1b, and Cbx3 loss has mildly attenuated cell cycling gene expression in the Wnt activated intestines.

![Figure 4.11: Cbx3 loss significantly depletes the expression of Wnt target splice variant Rac1b in Wnt-activated intestine. Quantitative RT-PCR analysis of the small intestinal epithelium 4 days post-induction revealed significantly increased expression levels of the Wnt-target splice gene Rac1b, as well as the cell cycle genes CDC25C and PIM1 (apart from ATM which showed a reduction) in the small intestinal epithelium of VillinCreER’Apcfl/fl mice compared to control wildtype mice (p < 0.05, n = 6). The additional loss of Cbx3 in the small intestinal epithelium significantly reduced expression of a Wnt target splice variant Rac1b in the VillinCreER’Apcfl/flCbx3fl/fl animals compared to VillinCreER’Apcfl/fl animals (p < 0.05, n = 6).](image)

4.2.10. HP1γ and H3K9me3 are mutually exclusive in normal intestinal homeostasis and acute Wnt activated intestine

HP1γ, coded by the gene Cbx3, differs from other members of heterochromatin protein 1 and it is present in both heterochromatin and euchromatin (Minc, Courvalin and Buendia, 2000). Previous evidence has proposed that HP1γ, bound to methylated H3K9, mediates heterochromatin formation, rendering the chromatin inaccessible for transcription (Jacobs and Khorasanizadeh, 2002b; Nielsen et al., 2002b). Therefore, the association between HP1γ and methylated H3K9 marks in normal intestinal homeostasis and in acute Wnt activated intestine was investigated. Immunofluorescence co-localisation analysis was carried out in small intestinal tissue sections of control wildtype, VillinCreER’Cbx3fl/fl, VillinCreER’Apcfl/fl and VillinCreER’Apcfl/flCbx3fl/fl animals.
Analysis of H3K9me3 expression showed nuclear localisation of H3K9me3 on the large matter of DAPI-dense facultative heterochromatin in all intestines. Staining of H3K9me3 was found to be more intense following the loss of Apc compared to wildtype animals. However, in contrast to the published literature no co-localisation between HP1γ and H3K9me3 was observed in all intestines (Figure 4.12, Figure 4.13). Furthermore, HP1γ and H3K9me3 also established a mutually exclusive relationship in the Wnt-activated small intestines (Figure 4.12, Figure 4.13). Interestingly, the level of H3K9me3 was substantially lowered in the absence of HP1γ in the VillinCreER⁺Cbx3⁻/⁻ intestinal epithelium (Chapter 3, Figure 3.12), suggesting that despite being mutually exclusive, HP1γ plays some role in establishing and / or maintaining H3K9me3 via docking on other heterochromatin marks (such as H4K20me3) to indirectly regulate H3K9me3. The lack of HP1γ-H3K9me3 co-localisation could be caused by H3S10 phosphorylation, which was reported to antagonise binding of HP1 to H3K9me3 (Fischle et al., 2005). Detailed expression analysis by immunohistochemistry of H3S10 phosphorylation status is essential in future studies to determine whether the dissociation of HP1γ and H3K9me3 is caused by phosphorylation of H3S10 in the normal and Wnt-activated intestinal epithelium.

Figure 4.12: Immunofluorescence analysis of HP1γ and H3K9me3 in small intestine of wildtype and VillinCreER⁺APC⁻/⁻ day 4 post-induction. Confocal analysis of small intestines double-labelled with HP1γ antibody (Alexa 594, red) and H3K9me3 antibody (Alexa 488, green) in control wildtype (A) and VillinCreER⁺APC⁻/⁻ (B) intestines. These images are representative of small intestines analysed in each cohorts (n = 3). Nuclei were counterstained with DAPI (4’, 6-diamidino-2-phenylindole; blue).
In order to confirm the mutually exclusive patterns observed between HP1γ and H3K9me3 in small intestinal tissues, co-localisation analysis using histograms displaying measured fluorescence intensity along a chosen distance across different cells in the small intestine was undertaken. Figure 4.13 shows representative regions that were scanned sequentially using appropriate excitation and emission settings for recordings of Alexa 594 (HP1γ in Red) and Alexa 488 (H3K9me3 in Green). The merged images of the dual-labelled HP1γ and H3K9me3 in small intestines of wildtype (Left panel) and VillinCreER\(^{+}\)/APC\(^{fl/fl}\) (Right Panel) showed distinct red and green labelling. The histograms (Figure 4.13A-H) representing the lines arbitrarily drawn upon red / green composite images across four different cells in the crypt regions of wildtype and VillinCreER\(^{+}\)/APC\(^{fl/fl}\) small intestine are consistent to our observations, the red and green fluorescence intensity peaked independently and there is no correlation between the staining patterns in both models, confirming that the immunostained HP1γ (Red) and H3K9me3 (Green) are mutually exclusive.

Figure 4.13: Confocal microscopic analysis of immunostained HP1γ and H3K9me3 in small intestine of wildtype and VillinCreER\(^{+}\)/APC\(^{fl/fl}\) mice day 4 post-induction. (Top) Co-localisation of HP1γ (red) and H3K9me3 (green) in small intestines of WT and APC\(^{fl/fl}\) mice revealed distinct patterns of staining in HP1γ and H3K9me3. (A-H) Histograms display measured fluorescence intensity along the lines marked on the top merged image. Line traces were generated with a line drawn arbitrarily upon red/green composite epithelial cells of small intestinal images.
4.2.11. Difference in HP1γ and H4K20me3 association patterns in small intestinal homeostasis and acute Wnt activated intestine

Given that the relationship between HP1γ and H3K9me3 is mutually exclusive in small intestinal homeostasis and acute Wnt activated intestines, the localisation of another heterochromatin repressive marker, H4K20me3, was also explored. H4K20me3 has been shown to have a significant correlation with HP1γ expression in normal tissue samples and a dissociation of the correlation between HP1γ expression and H4K20me3 may reflect the malfunction of epigenetic control in cancer tissues (Takanashi et al., 2009). Therefore this correlation was examined in the small intestine of the wildtype animals and following Apc deletion in VillinCreER\(^{+/+}\)Apc\(^{fl/fl}\) animals. Immunofluorescence analysis was performed on small intestinal tissue sections from wildtype and VillinCreER\(^{+/+}\)Apc\(^{fl/fl}\) mice.

Immunofluorescence analysis of H4K20me3 expression showed intense staining in stromal compartments, crypts and along villi of small intestine across both the cohorts. A prominent increase in HP1γ expression was detected in the absence of Apc at day 4 post induction, compared to wildtype (Figure 4.14A and F), which is consistent with our preliminary data. H4K20me3 levels remained similar between VillinCreER\(^{+/+}\)Apc\(^{fl/fl}\) small intestine compared to wildtype intestine, although the pattern of staining changed from more foci in wildtype to more diffuse in VillinCreER\(^{+/+}\)Apc\(^{fl/fl}\) model (Figure 4.14B and G). Interestingly, the double staining (HP1γ and H4K20me3) clearly demonstrated co-localisation of HP1γ with H4K20me3 at the crypt and along the villus of the small intestine in wildtype mice in accordance to expectations (Figure 4.14D). The association of HP1γ with H4K20me3 in the small intestine of the wildtype mouse is lost in the absence of Apc (Figure 4.14D and I), expression of HP1γ and H4K20me3 appeared to be mutually exclusive in VillinCreER\(^{+/+}\)Apc\(^{fl/fl}\) animals.
In order to examine the co-localisation of HP1γ from H4K20me3 we examined the fluorescence intensity of HP1γ (Alexa 594, red) and H4K20me3 (Alexa 488, green) within the merged images, along the chosen distance across four different cells in the crypt and villus of small intestines, respectively. This analysis confirmed the co-localisation of HP1γ and H3K9me3 in small intestines of wildtype (Top panel, Figure 4.15) and the dissociation of the two markers following the loss of Apc (Bottom panel, Figure 4.15). The histograms illustrating the lines arbitrarily drawn upon red / green composite images across four different cells in the crypts (Figure 4.15A-D, I-L) and villus regions (Figure 4.15E-H, M-P) in
small intestine of wildtype and VillinCreER'Apc^{fl/fl} mice demonstrated consistent results with our previous observations. In the wildtype cohort, the red and green fluorescence intensity peaked at exact locations and the staining patterns appeared to be dependent on each other, whereas in the small intestinal tissues of VillinCreER'Apc^{fl/fl} the histograms showed that the red and green fluorescence intensity peaked independently and there was no correlation between the staining patterns. Taken together, these data suggest that HP1γ (Red) and H4K20me3 (Green) are associated in both crypt and villus of small intestine in wild-type mice. However, this association pattern is lost in the absence of Apc in the small intestine. This could be a potentially important mechanism by which dissociation of HP1γ and H4K20me3 are required for epigenetic malfunction in Wnt activated intestine.
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Figure 4.15: Confocal microscopy analysis of immunostained HP1γ and H4K20me3 in small intestine of wildtype and VillinCreER<sup>APC<sup>fl/fl</sup></sup> mice. Co-localisation of HP1γ (red) and H4K20me4 (green) in small intestine of wild-type mouse is shown in the Top panels and <sup>APC<sup>fl/fl</sup></sup> mouse model is shown in the Bottom panels. Histograms display measured fluorescence intensity along the lines marked on the merged images of the wildtype mouse (A-H) and <sup>APC<sup>fl/fl</sup></sup> mouse model (I-P).
4.2.12. HP1α displaces HP1γ to the H4K20me3 marks in acute Wnt activated intestine

Considering the proposed dissociation of HP1γ and H4K20me3 as a sign for epigenetic malfunction in cancer development (Takanashi et al., 2009), further investigation was undertaken to see whether this dissociation pattern was caused by displacement of other heterochromatin protein members in the Wnt activated small intestine. To this end, immunofluorescence analysis of other HP1 family members was performed on small intestinal tissue sections from wildtype and VillinCreER\(^{+}\)Apc\(^{fl/fl}\) mice.

Immunofluorescence analysis of H4K20me3 expression showed intense staining in stromal compartments, crypts and along villi of small intestine across both the cohorts, as observed in the previous section. Expression of HP1α was detected in high level in more differentiated cells higher up along the villus of both wildtype and VillinCreER\(^{+}\)Apc\(^{fl/fl}\) intestines (Figure 4.16). The double staining of HP1α and H4K20me3 expression demonstrated little association patterns of HP1α with H4K20me3 at the crypt and along the villus of the small intestine in wildtype mice (Figure 4.16D). Conversely, expression of HP1α with H4K20me3 in the small intestine of VillinCreER\(^{+}\)Apc\(^{fl/fl}\) was found to be fully co-localised (Figure 4.16I).
Based on these observations, the co-localisation of HP1α from H4K20me3 was examined by analysing fluorescence intensity of HP1α (Alexa 488, green) and H4K20me3 (Alexa 594, red) within the merged images, along the chosen distance across a number of cells in the crypt (Figure 4.17 Top and bottom panels, D-F) and villus (Figure 4.17 Top and bottom panels, A-C) of small intestines, respectively. This analysis confirmed the partial association pattern of HP1α and H4K20me3 in small intestines of wildtype (Top panel, Figure 4.17) and the full co-localisation of the two markers following the loss of Apc (Bottom panel Figure 4.17). The histograms illustrating the lines arbitrarily drawn upon red/green
composite images across four different cells in the crypts (D-F) and villus regions (A-C) in small intestine of wildtype and VillinCreER\(^{+}\)Apc\(^{fl/fl}\) mice demonstrated consistent results with our previous observations. In the wildtype intestine, the histograms showed that the green and red fluorescence intensity partially peaked together and partially peaked independently and there was partial correlation between the staining patterns of HP1\(\alpha\) and H4K20me3. Conversely, in the small intestinal tissues of VillinCreER\(^{+}\)Apc\(^{fl/fl}\) the red and green fluorescence intensity peaked at exact locations and the staining patterns appeared to be dependent on each other. Collectively, these data indicate that HP1\(\alpha\) (Green) and H4K20me4 (Red) are partially associated in both crypt and villus of small intestine in wildtype mice, whereas this association pattern become fully established in the absence of Apc in the small intestine. This observation, along with the dissociation of HP1\(\gamma\) from H4K20me3 in acute Wnt activated intestines, strongly suggests that the displacement of HP1\(\gamma\) by HP1\(\alpha\) from H4K20me3 in Wnt activated small intestines.
Figure 4.17: Confocal microscopy analysis of immunostained HP1α and H4K20me3 in small intestine of wildtype and VillinCreER^APC^fl/fl small intestines. In the top panel, co-localisation of HP1α (green) and H4K20me3 (red) in small intestines of wildtype revealed partially distinct patterns of staining in HP1α and H4K20me3. In contrast, HP1α and H4K20me3 expressions showed co-localisation in VillinCreER^APC^fl/fl intestine. Histograms display measured fluorescence intensity along the lines marked on the top merged image. Line traces were generated with a line drawn arbitrarily upon red/green composite epithelial cells of small intestinal images (Top and bottom panels A-F).
4.2.13. Difference in HP1γ and H3K4me3 association patterns in small intestinal homeostasis and acute Wnt-activated intestine

It has been previously shown that HP1γ is fully colocalised with H4K20me3 in small intestinal homeostasis and it is dissociated from H4K20me3 in acute Wnt activated intestines. The biological significance of this association patterns was unclear and therefore the localisation of a euchromatin active marker, H3K4me3 was investigated. Histone H3K4 trimethylation (H3K4me3) is an active marker in the epigenetic code, it is enriched downstream of the active transcription start site (Hon, Hawkins and Ren, 2009). Since HP1α and HP1β are localised in heterochromatin, whereas HP1γ is present in both heterochromatin and euchromatin (Minc, Courvalin and Buendia, 2000), for it would be of great interest to determine whether HP1γ interacts with the active euchromatic marks. Therefore, immunofluorescence analysis of HP1γ and H3K4me3 was performed in the small intestine of the wildtype animals and following Apc deletion in VillinCreER\(^{+}\)Apc\(^{B/−}\) animals.

The overall staining pattern of H3K4me3 is mostly diffuse in both wildtype and Wnt-activated intestines. Double immunofluorescence analysis of HP1γ and H3K4me3 demonstrated a partial co-localisation pattern was observed between the two markers at the crypt and along the villus of the small intestinal epithelium of the wildtype animals (Figure 4.18). On the other hand, the staining of HP1γ and H3K4me3 clearly became more fully colocalised especially at the crypt and along the villus of the small intestinal epithelium of VillinCreER\(^{+}\)Apc\(^{B/−}\) animals (Figure 4.18).
Figure 4.18: Co-localisation of HP1γ and H3K4me3 in small intestine of wildtype and VillinCreER’Apc^{fl/fl} mice day 4 post induction. Confocal images of small intestines stained with anti-HP1γ antibody (green) and anti-H3K4me3 antibody (red). These images are representative of small intestinal tissues analysed in each cohort (n = 3). Nuclei were stained with DAPI (4’, 6-diamidino-2-phenylindole; blue).

Having analysing the immunofluorescence images visually, the co-localisation of HP1γ from H3K4me3 was examined by analysing fluorescence intensity of HP1γ (Alexa 488, green) and H3K4me3 (Alexa 594, red) within the merged images, across a number of cells in the crypt (Figure 4.19) Top and bottom panels, A-C) and villus (Figure 4.19 Top and bottom panels, D-F) of small intestines, respectively. The histogram analysis confirmed the partial association pattern of HP1γ and H3K4me3 in small intestines of wildtype (Top panel, Figure 4.19) as the green and red fluorescence intensity partially peaked together and partially peaked independently. While in the small intestinal tissues of VillinCreER’Apc^{fl/fl} mice, the green and red fluorescence intensity peaked uniformly, confirming that the co-localisation pattern seen in the Wnt-activated intestine (Bottom panel Figure 4.19). Collectively, these data indicate that HP1γ (Green) and H3K4me4 (Red) are partially associated in both crypt and villus of small intestine in wildtype mice, whereas this association pattern became fully colocalized in the absence of Apc in the small intestine, indicating a role of HP1γ in transcriptional activation in euchromatin.
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Figure 4.19 Confocal microscopy analysis of immunostained HP1γ and H3K4me3 in small intestine of wildtype and VillinCreER<sup>APC<sup>fl/fl</sup> animals.
In the top panel, co-localisation of HP1γ (green) and H3K4me3 (red) in wildtype small intestines revealed partially distinct patterns of staining in HP1γ and H3K4me3. In contrast, HP1γ and H3K4me3 expressions showed more co-localisation in VillinCreER<sup>APC<sup>fl/fl</sup> intestine. Histograms display measured fluorescence intensity along the lines marked on the top merged image. Line traces were generated with a line drawn arbitrarily upon red/green composite epithelial cells of small intestinal images (Top and bottom panels A-F).
4.2.14. HP1α and H3K4me3 are mutually exclusive in normal intestinal homeostasis and acute Wnt activated intestine

Having observed that the joint presence of HP1γ and H3K4me3 in the Wnt-activated intestine as a sign of gene activation in euchromatin, it would be interesting to investigate whether there is any interaction between HP1α and H3K4me3 and immunofluorescence analysis was performed on small intestinal tissue sections from wildtype and *VillinCreER/\*Apc^fl/fl* mice. Literature has indicated that HP1α is enriched at sites of heterochromatin, while H3K4me3 is mark of active euchromatin, therefore one would predict that there would be no association between HP1α and H3K4me3.

Consistent with our hypothesis, the double staining of HP1α and H3K4me3 demonstrated little to no association patterns between the two marks at the crypt and along the villus of the small intestine in wildtype mice (Figure 4.16D). Similarly, expression of HP1α with H3K4me3 in the small intestine of *VillinCreER/\*Apc^fl/fl* was also found to be mutually exclusive (Figure 4.20).

![Figure 4.20: Co-localisation of HP1α and H3K4me3 in small intestine of wildtype and *VillinCreER/\*Apc^fl/fl* Day 4 post induction. Confocal images of small intestines stained with anti-HP1α antibody (green) and anti-H3K4me3 antibody (red). These images are representative of small intestinal tissues analysed in each cohorts (n = 3). Nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole; blue).](image-url)
In order to confirm the mutually exclusive patterns observed between HP1α and H3K4me3 in small intestinal tissues, co-localisation analysis using histograms displaying measured fluorescence intensity along a chosen distance across different cells in the small intestine was performed. The merged images of the dual-labelled HP1α and H3K4me3 in small intestines of wildtype (top panel) and VillinCreER\(^{APC^{fl/fl}}\) (bottom Panel) showed distinct red and green labelling. In the wildtype intestine, the histograms showed that the green and red fluorescence intensity peaked independently and similar pattern is observed in the small intestinal tissues of VillinCreER\(^{APC^{fl/fl}}\). Taken together, these data indicate that HP1α and H3K4me3 are mutually exclusive in both crypt and villus of wildtype and Wnt-activated intestines, indicating that the two markers are localised at different nuclear locations, HP1α is enriched at sites of heterochromatin, whereas H3K4me3 is an active mark located in the euchromatin.
Figure 4.21: Confocal microscopy analysis of immunostained HP1α and H3K4me3 in small intestine of wildtype and VillinCreER\(^{ERT2}\) APC\(^{flox/flox}\) animals.

In the top panel, co-localisation of HP1α (green) and H3K4me3 (red) in small intestines of wildtype revealed distinct patterns of staining in HP1α and H3K4me3. Similarly, HP1α and H3K4me3 expressions also are mutually exclusive in VillinCreER\(^{ERT2}\) APC\(^{flox/flox}\) intestine. Histograms display measured fluorescence intensity along the lines marked on the top merged image. Line traces were generated with a line drawn arbitrarily upon red/green composite epithelial cells of small intestinal images (Top and bottom panels A-F).
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4.3. Discussion

Previous work has demonstrated that epithelial deletion of Cbx3 results in no immediate perturbation of intestinal homeostasis. Studies from the literature have shown that Cbx3 is over-expressed in various cancer cell lines (including colon cell lines) and the deletion of Cbx3 inhibited cancer cell line growth (Takanashi et al., 2009). Furthermore, work described in our preliminary work has indicated that over-expression of Cbx3 was observed in tumour samples of colorectal cancer patients compared to normal tissues. Consistent with this, conditional deletion of both alleles of Apc in the murine intestine using the Cre-loxP system results in an increased expression level of Cbx3 and up-regulation of HP1γ protein. Taken together, these observations suggested that Cbx3 has a role in intestinal carcinogenesis. Investigation of the effects of Cbx3 deficiency on aberrant Wnt signaling is of importance for the understanding of the potential biological functions of Cbx3 in cancer development. In this chapter, the effects of epithelial-specific Cbx3 loss in the context of activated Wnt signaling was examined using conditional transgenesis with Lox-P targeted Cbx3 and Apc alleles together with the inducible VillinCreER transgene.

4.3.1. Epithelial Cbx3 deficiency prolongs survival but has no effect on gross intestinal structure in the context of Apc deficiency

The effect of Cbx3 deletion following acute activation of Wnt signaling by deletion of both alleles of Apc was examined. Firstly, VillinCreER\(^{+/-}\)Apc\(^{0/0}\)Cbx3\(^{0/0}\) and VillinCreER\(^{+/-}\)Apc\(^{0/0}\)Cbx3\(^{0/0}\) animals were generated, induced and closely monitored for signs of ill health. Intestinal deletion of both alleles of Apc is catastrophic for intestinal homeostasis, resulting in morbidity within 5 days (Sansom et al., 2004). Consistently, VillinCreER\(^{+/-}\)Apc\(^{0/0}\) mice were sacrificed 4 days post induction due to ill health whereas VillinCreER\(^{+/-}\)Apc\(^{0/0}\)Cbx3\(^{0/0}\) mice with the additional homozygous loss of Cbx3 outlived VillinCreER\(^{+/-}\)Apc\(^{0/0}\) mice by 3 days (with median survival 7 days). This observation is unprecedented and multiple mechanisms that might have contributed to the improved animal survival in VillinCreER\(^{+/-}\)Apc\(^{0/0}\)Cbx3\(^{0/0}\) mice include the suppression of Wnt activation at the receptor level observed in the Topflash assay, the diminished enterocyte brush border and the reduction of Rac1b splice variant expression level. These notions would be individually discussed in the subsequent sections.
In this section, the further histological parameters were investigated in order to gain an insight to this observation.

Having established that deletion of Cbx3 prolonged lifespan of mice with homozygous loss of Apc, the consequence of Cbx3 loss on cell differentiation in the aberrant Wnt-activated intestine were investigated. Quantitative measurements of histological parameters, such as crypt length, apoptotic index, cleaved Caspase3, mitotic figures and Ki67 were all significantly increased in Wnt activated epithelium in both Cbx3 deficient and proficient tissue compared to control mice, consistent with previous observations of conditional Apc deletion in the small intestine (Sansom et al., 2004). No effect was observed between the two experimental cohorts VillinCreER\(^+\)Apc\(^{fl/fl}\)Cbx3\(^{fl/fl}\) and VillinCreER\(^+\)Apc\(^{fl/fl}\) across all these parameters. Additionally, Cbx3 loss in the context of Apc deficiency exhibited no effect on cell migration.

Having observed no dramatic histological changes between VillinCreER\(^+\)Apc\(^{fl/+}\)Cbx3\(^{fl/+}\) and VillinCreER\(^+\)Apc\(^{fl/+}\) intestines, qualitative and quantitative analysis of the normal intestinal cell differentiation was examined. Visualization of the enterocytes from the absorptive lineage in the Cbx3 deficient Wnt activated small intestinal epithelium revealed a consistent decrease in brush border staining compared to VillinCreER\(^+\)Apc\(^{fl/+}\) intestines, indicating Cbx3 might play a role in regulating enterocyte maturation. However, quantitative analysis of the Goblet cells, Enteroendocrine cells and Paneth cells from the secretory lineage revealed that the presence, location and frequency of these mature cell types was unaffected by Cbx3 loss in the Apc deficient intestines. Although Cbx3 loss did not diminish the entire absorptive lineage, the thin brush border of enterocytes were observed in Cbx3-deficient epithelium compared to control epithelium. Notch signaling has been reported to play a crucial role in differentiation of enterocyte, as constitutive activation of the Notch pathway substantially reduced differentiation of secretory cells and promoted differentiation of post-mitotic cells into adsorptive enterocytes (Fre et al., 2005). In contrast, blockade of Notch signaling in rodent intestine with the Notch inhibitors has been found to result in expansion of secretory cells at the expense of enterocytes (Milnao et al, 2004). In the current study short-term Cbx3 loss was found to perturb the development of the brush border without affecting the differentiation of other secretory cell lineages. It could be hypothesized that Cbx3 loss does not impair
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enterocyte differentiation, but rather suppresses expression of the brush border components. Expression analysis of other enterocyte-specific markers, such as Villin may be required to determine whether Cbx3 loss suppresses enterocyte differentiation.

Taken together, these data suggest that the immediate effect of Cbx3 loss in the context of aberrant Wnt signaling activation is prolonged lifespan of Apc-deficient mice without altering the intestinal histology and cell differentiation of the secretory cell types.

4.3.2. Cbx3 loss exhibits inhibitory effects on Wnt signaling

Evidence from the literature indicates that down regulation of Cbx3 inhibited growth of cancer cell lines (Takanashi et al., 2009). Previous data has demonstrated that Cbx3 loss prolonged life span of Wnt-activated mice. Therefore the functional interaction between Cbx3 and Wnt signalling in vivo in murine small intestinal epithelium and in vitro in a Wnt-responsive Topflash system was investigated.

In order to examine the consequence of Cbx3 deficiency on Wnt target gene activation in the small intestinal epithelium, the expression levels of a number of Wnt target genes (Axin2, c-Myc, CD44, CyclinD1, EphB3 and E-cadherin) was assessed. Quantitative analysis of Wnt target gene expression revealed that a subset of Wnt target genes (Axin2, c-Myc, CD44 and CyclinD1) was found to be substantially upregulated following the loss of Apc in both Cbx3 deficient and proficient intestinal epithelium. The Wnt target genes show a slight reduction in the small intestinal epithelium of VillinCreER\(^{+}\)Apc\(^{fl/fl}\)Cbx3\(^{fl/fl}\) mice compared to VillinCreER\(^{+}\)Apc\(^{fl/fl}\) mice, although these reductions were not statistically significant.

To further investigate whether Cbx3 has a role in Wnt activity, luciferase assays were utilised in HEK-293 cells by use of the Wnt-responsive Topflash system. In order to determine at which level Cbx3 is modulating the Wnt pathway, pathway activators (\(\Delta NLRP6\) [constitutively active LRP6 receptor] and TCF4-VP16 [constitutively active form of TCF4 transcription factor]) were used to induce Wnt signalling at distinct levels (Ewan et al., 2010). Transient knock down of CBX3 using CBX3 siRNA 24 hours prior transfection of Topflash activator \(\Delta NLRP6\) or TCF4-VP16 reduced total Topflash activity 48 hours post-
transfection by 41% and 27%, respectively. This data suggest that CBX3 mainly acted at LRP6 receptor level based on the observation that the loss of CBX3 blocked TCF-dependent transcription induced by a constitutively active LRP6 receptor, but less extent of blockage observed by a constitutively active TCF-VP16 fusion protein, indicating that CBX3 functions at receptor level within the Wnt signalling cascade.

In summary, depletion of Cbx3 has shown a noticeable inhibitory activity against Wnt target gene expression in the epithelium of Wnt-activated intestine. Concordantly, the Topflash assay confirmed that the loss of Cbx3 significantly suppressed Wnt activation by 41%, at the receptor level. Furthermore, the repressed Wnt activation may account for the prolonged survival observed previously in epithelial Cbx3 deficient mice with activated Wnt signalling.

4.3.3. Cbx3 loss in the Wnt-activated epithelium does not alter stem cell compartment

Previous evidence suggested Cbx3 is required for cell differentiation to occur in adipocyte, male germ cell and cardiovascular system (Xiao et al., 2011; Brown et al., 2010b). However, Cbx3 deficiency had no impact on cell differentiation in the Wnt-activated intestines as previously analysed. Next, the expression analysis of a range of intestinal stem cell markers (Ascl2, Lgr5, Msi1, Bmi1 and Olfm4) revealed no significant difference between VillinCreER$^{+/+}$Cbx3$^{+/+}$ epithelium compared to VillinCreER$^{+/+}$ epithelium. In situ hybridisation analysis of Olfm4 was also performed to further assess the effect of Cbx3 loss on the state of the stem cell population in Wnt-activated intestinal epithelium. The additional loss of Cbx3 in context of Apc deficiency indicated again no alteration in the stem cell compartment in the small intestinal epithelium of VillinCreER$^{+/+}$Cbx3$^{+/+}$ compared to VillinCreER$^{+/+}$ animals.

These observations are compelling evidence for the fact that epithelial Cbx3 deficiency does not modulate the stem cell genes and compartment in the intestine.
4.3.4. Cbx3 loss has mild effect on Notch and cell cycle gene expression in Wnt-activated intestine

Notch signalling pathway also plays a critical role in intestinal homeostasis and intestinal stem cell maintenance and furthermore, Notch has been shown to be critical in driving enterocyte differentiation (Fre et al., 2005). Previous data has shown that Cbx3 was found to suppress enterocyte differentiation in VillinCreER\(^+\)Apc\(^{fl/fl}\)Cbx3\(^{fl/fl}\) compared to VillinCreER\(^+\)Apc\(^{fl/fl}\) epitheliums and therefore one could hypothesise that Cbx3 loss could potentially attenuate Notch signalling to a level sufficient to impair enterocyte differentiation. Therefore the effects of Cbx3 loss on Notch target gene expression was investigated in Wnt activated intestinal epithelium.

In order to investigate the effects of Cbx3 deficiency on Notch signalling in the Wnt-activated intestinal epithelium, the expression levels of a subset of Notch signalling target genes were examined. Expression analysis of all examined Notch target gene: Delta1, nRarp, Hes1, mTert, Lrig1, Jagged1 and Jagged2 revealed significantly increased expression levels in both Cbx3 deficient and proficient epithelium with Apc loss. The expression analysis of the Cbx3 deficient epithelium demonstrated a trend of decrease in Delta1 nRarp, Hes1, Lrig1 and Jagged2 expression compared to the VillinCreER\(^+\)Apc\(^{fl/fl}\) samples, however the changes did not reach statistical significance. Even though the downregulation of Notch target genes was not statistically significant, the effect of this was sufficient to be translated into cellular effect on the differentiation of secretory enterocyte.

Previous work has demonstrated that the role of Cbx3 on cell cycle regulation in Caenorhabditis elegans and human cells (Black et al., 2010). Additionally, the involvement of Cbx3 in cell cycle regulation is critical in controlling levels of primordial germ cells in mouse embryos (Abe et al., 2011). It is therefore of great interest to investigate the effect of Cbx3 loss on the level of a range of cell cycle genes in the Wnt-activated intestine. Expression analysis of the cell cycle genes detected a significantly reduced expression of ATM and a significantly elevated expression of CDC25C in both VillinCreER\(^+\)Apc\(^{fl/fl}\) and VillinCreER\(^+\)Apc\(^{fl/fl}\)Cbx3\(^{fl/fl}\) epithelium. However, there are no significant differences in the cell cycle gene expression levels between the two experimental groups VillinCreER\(^+\)Apc\(^{fl/fl}\) and VillinCreER\(^+\)Apc\(^{fl/fl}\)Cbx3\(^{fl/fl}\). A trend of reduction is observed in expression of CCNE1,
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*PIM1* and *CAV2*, which are thought to be involved in cancer progression, following the loss of *Cbx3* in the Wnt-activated intestines.

Collectively, these data indicate that *Cbx3* loss has mild modulation on Notch signalling and cell cycle regulation in the Wnt-activated intestines.

4.3.5. *Cbx3* loss alters Wnt target splice variant *Rac1b* expression in Wnt-activated intestine

Previously published data has indicated a role for *Cbx3* in aiding in efficient cotranscriptional RNA processing through recruitment of the splicing machinery. Depletion of *Cbx3* diminished the recruitment of splicing factors such as SNRNP70 and SRSF1 to gene bodies, resulting in RNA processing defect and subsequently leading to an increase in unspliced transcripts of its target genes (Saint-Andre *et al.*, 2011; Smallwood *et al.*, 2012). Alternative splicing of the tumour-related *Rac1b* is regulated by SRSF1 in colorectal cells (Goncalves *et al.*, 2014). *Rac1b* was identified in malignant colorectal tumours as an alternative splice variant of *Rac1* (Schneizer *et al.*, 2000; Fiegen *et al.*, 2004). A number of studies have reported that *Rac1b* plays an important role in tumorigenesis via augmenting Wnt signalling, promoting tumour cell survival (Matos *et al.*, 2008) and increasing the invasive and metastatic properties of colorectal cancer cells (Esufali *et al.*, 2007). However, the mechanisms of how changes in alternative splicing lead to *Rac1b* overexpression remain unclear. Since *Rac1* is a Wnt target gene which would be upregulated following *Apc* loss in the intestinal epithelium, therefore, one would predict that deletion of *Cbx3* would lead to *Rac1b* splicing defect and therefore a lower expression of *Rac1b* would be observed.

The expression levels of the Wnt target gene *Rac1* and its splice variant *Rac1b* was determined using qRT-PCR analysis. As expected, expression levels of the Wnt target gene *Rac1* and its splice variant *Rac1b* increased significantly in *Apc* deficient epithelium. The splice variant *Rac1b* was significantly repressed following the loss of *Cbx3* in the Wnt activated intestine, consistent with the hypothesis that loss of *Cbx3* impairs RNA splicing and therefore leads to a reduced level of *Rac1b* in the small intestinal epithelium. Since *Rac1b* is a product of alternative processing that can activate Wnt signalling (Esufali *et al.*, 2007), a decrease in *Rac1b* level in *Cbx3* deficient epithelium would suggest an attenuation of the
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Wnt target gene expression in the context of Apc deficiency. However, gene expression analysis of Wnt signalling in Cbx3 deficient epithelium was found to be unaltered compared to Apc deficient epithelium alone. It has been reported that the expression of Rac1b is controlled not only by levels of Wnt but also PI3K signalling (Goncalves, Matos and Jordan, 2009; Esufali, Charames and Bapat, 2007). Therefore, expression analysis of PI3K target genes should be explored to determine whether Cbx3 loss plays a role in PI3K and hence the regulation of Rac1 splicing.

4.3.6. HP1α displaces HP1γ to the Heterochromatin mark H4K20me3 in Wnt activated intestine

Previous evidence has suggested that H4K20 trimethylation (H4K20me3) is essential for the maintenance of perinatal, non-cancerous cell states and confers heterochromatin maintenance capability (Fraga et al., 2005; Pogribny et al., 2006; Schotta et al., 2008). Trimethylation specifically requires the recruitment of the Sur4-20h enzymes by HP1 (Schotta et al., 2004). H4K20me3 has been shown to have a significant correlation with HP1γ expression in normal tissue samples and a dissociation of the correlation between HP1γ expression and H4K20me3 may reflect the malfunction of epigenetic control in cancer tissues (Takanashi et al., 2009). Therefore, this correlation was examined in the wildtype intestine and Apc-deficient intestine. Immunofluorescence analysis was performed on small intestinal tissue sections and joint presence of HP1γ with H4K20me3 was detected at the crypt and along the villus of the wildtype intestine. In accordance to expectation, this co-localisation of HP1γ with H4K20me3 is a signature of the normal intestinal homeostasis. In the Wnt-activated epithelium, the association of HP1γ with H4K20me3 is lost in the absence of Apc, expression of HP1γ and H4K20me3 appeared to be mutually exclusive in VillinCreER"Apc<sup>fl/fl</sup>" animals. These observations suggest that the co-localisation of HP1γ and H4K20me3 is important for heterochromatin maintenance required for normal intestinal homeostasis, whereas the loss of HP1γ-H4K20me3 association could be an important mechanism required for epigenetic malfunction in Wnt-activated intestine.

Considering the dissociation of the correlation between HP1γ and H4K20me3 may reflect the malfunction of epigenetic control in cancer development, further investigation
was aimed to see whether this dissociation pattern was caused by displacement of other heterochromatin protein members in the Wnt activated small intestine. Immunofluorescence analysis of the other HP1 family member HP1α was performed on small intestinal tissue sections from wildtype and Wnt-activated mice. Little association patterns between HP1α and H4K20me3 was observed in the small intestine in wildtype mice whereas the expression of HP1α with H4K20me3 in the Wnt-activated intestine was found to be fully co-localised. This observation, along with the dissociation of HP1γ from H4K20me3 in acute Wnt activated intestines, strongly suggests that the displacement of HP1γ by HP1α from H4K20me3 in Wnt activated small intestines. Studies from the literature have demonstrated that the role of HP1α in breast cancer (Takanashi et al., 2009) and a correlation between HP1α and H4K20me3 levels is observed in ES cells (Wongtawan et al., 2011). These observation from the literature together with our data suggest that the role of HP1α in aberrant Wnt-activated intestinal cancer.

Together, we propose that the H4K20me3 signature in heterochromatin, while bound by HP1γ contributes to maintaining normal intestinal homeostasis by providing a gene-repressive compartment in the nucleus, however when bound by HP1α it retains the dynamics to adapt to chromatin structural changes during carcinogenesis.

4.3.7. HP1γ interacts with the active Euchromatin mark H3K4me3 in Wnt activated intestine

Histone H3K4 trimethylation (H3K4me3) is an active marker in the epigenetic code, it is enriched downstream of the active transcription start site in the euchromatin (Hon, Hawkins and Ren, 2009). Since HP1γ is present in both heterochromatin and euchromatin (Minc, Courvalin and Buendia, 2000). It would be of great interest to see whether HP1γ has any interaction with the active euchromatic marks, immunofluorescence analysis of HP1γ and H3K4me3 was performed in the small intestine of the wildtype animals and following Apc deletion in VillinCreER\textsuperscript{Apc\textsuperscript{fl/fl}} animals.
Double immunofluorescence analysis of HP1γ and H3K4me3 demonstrated partial co-localising pattern is observed between the two markers in wildtype intestine whereas the association pattern of HP1γ and H3K4me3 clearly become more fully colocalised especially in the Wnt-activated intestine, indicating a role of HP1γ in transcriptional activation in euchromatin.

Immunofluorescence analysis was also performed to investigate whether there is any interaction between HP1α and H3K4me3 in small intestinal tissue sections from wildtype and VillinCreER\(^{+/−}\)Apc\(^{fl/fl}\) mice. The co-localisation analysis demonstrated that HP1α and H3K4me3 are mutually exclusive in both crypt and villus of wildtype and Wnt-activated intestines, indicating that the two markers are localised at different nuclear locations, HP1α is enriched at sites of heterochromatin, whereas H3K4me3 is an active mark located in the euchromatin. This observation suggests that despite the significant degree of sequence conversation shared between the HP1 isotypes, different members of the HP1 family have distinct and non-redundant functions.
4.3.8. Summary

In summary, four major conclusions can be drawn from the data presented in this chapter. First, the loss of Cbx3 substantially suppressed Wnt activation at the receptor level in a Topflash assay. This observation is supported by the prolonged lifespan of Cbx3 deficient mice following the conditional loss of both alleles of Apc gene with no alteration in gross intestinal structure. Secondly, Cbx3 deficiency does not impact on the stem cell compartment although the enterocyte bush border is noticeably diminished following the loss of Cbx3 in the context of Wnt activated small intestinal epithelium. This observation could be caused by the down-regulation of Notch target genes following the epithelial Cbx3 loss in Wnt-activated intestines. Thirdly, Cbx3 plays a crucial role in RNA splicing and upon deletion of Cbx3 the tumour-related splice variant Rac1b was found to be significantly repressed in the Wnt activated intestine. Fourthly, the joint presence of HP1γ and H4K20me3 appears to be essential for heterochromatin maintenance required for normal intestinal homeostasis, whereas the loss of HP1γ-H4K20me3 association, caused by HP1α displacing HP1γ to the H4K20me3 marks, could be an important mechanism required for epigenetic malfunction in Wnt-activated intestine.
Chapter Five

Analysing the long-term consequences of *Cbx3* loss in the context of Wnt-driven tumorigenesis
Chapter 5: Analysing the long-term consequences of Cbx3 loss in the context of Wnt-driven tumorigenesis

5. Analysing the long-term consequences of Cbx3 loss in the context of Wnt-driven tumorigenesis

5.1. Introduction

Previous work by others and that described in Chapter 4 indicates that Cbx3 has the potential to regulate Wnt signalling pathway by modulating heterochromatin organisation via its association with H4K20me3 (Schotta et al., 2004). Loss of CBX3 expression has been shown to result in diminished levels of Wnt activation in vitro (Chapter 4). Studies have demonstrated that the deletion of mutant of HP1γ lacks tumorigenesis activity in a mouse xenograft model (Sharma et al., 2003), indicating that the expression of HP1γ contributes to cancer formation.

Therefore, it is interesting to investigate the effects of Cbx3 loss in Wnt-driven tumorigenesis. Conditional deletion of both copies of Apc in the adult intestine using the Cre-loxP approach resulted in rapid and catastrophic disruption of epithelial homeostasis and animals lived up to 4 days post induction (Chapter 4). Heterozygous inactivation of Apc is a well-established approach for induction of Wnt-driven tumorigenesis in the gastrointestinal tract (Shibata et al., 1997).

In this chapter, the consequence of epithelial-specific Cbx3 loss in the context of Wnt-activated tumorigenesis was examined utilising a mouse model possessing a heterozygous mutation of the Apc gene.
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5.2. Results

5.2.1. Cbx3 loss in the context of Apc heterozygosity has a tendency to reduce lifespan of male mice

In order to examine the effects of Cbx3 deficiency on the survival of mice with heterozygous inactivation of Apc, mice from VillinCreER^{+/-}Apc^{fl/+}Cbx3^{fl/fl} and VillinCreER^{+/-}Apc^{fl/+}Cbx3^{fl/+} along with the control VillinCreER^{+/-}Apc^{fl/+}Cbx3^{+/+} mice (for all cohorts n > 13) were induced with four daily intraperitoneal injections of 80 mg/kg Tamoxifen, were aged to 500 days and regularly inspected for signs of ill health, and sacrificed when needed. The majority of animals developed signs of ill health due to extensive tumour burden and had to be sacrificed prior to the 500 days time point. Analysis of the survival probability revealed a trend of reduced survival (but not significant) between the experimental VillinCreER^{+/-}Apc^{fl/+}Cbx3^{fl/fl} cohort (median survival of 197 days post induction) and control animals (median survival of 282 days post induction, Log Rank Test: p = 0.128, n > 13, Figure 5.1).

It is known that survival rates associated with intestinal tumorigenesis can vary between the genders (Colorectal Cancer Facts & Figures 2014-2016) and given that the experimental cohorts contained animals of both genders, the survival phenomenon was further explored. The effects of Cbx3 deficiency on survival were generated for both female and male mice with heterozygous deletion of Apc. Analysis of the survival probability demonstrated that the reduced survival between VillinCreER^{+/-}Apc^{fl/+}Cbx3^{fl/fl} and control cohorts was more prominent in male compared to female cohorts (Figure 5.2). The median survival of the male control cohorts (n = 8) was 225 days post induction, the VillinCreER^{+/-}Apc^{fl/+}Cbx3^{fl/+} cohort (n = 12) was 268 days and the VillinCreER^{+/-}Apc^{fl/+}Cbx3^{fl/fl} cohort (n = 12) was reduced to 179 days, although this was not proven to be statistically significant (Log Rank Test: p = 0.082, n > 8, Figure 5.2B). On the other hand, the median survival of the female control cohort (n = 5) was 295 days, the VillinCreER^{+/-}Apc^{fl/+}Cbx3^{fl/+} cohort (n = 5) was 317 days and the VillinCreER^{+/-}Apc^{fl/+}Cbx3^{fl/fl} cohort (n = 8) was reduced to 260 days (Log Rank Test: p = 0.724, n > 5, Figure 5.2A). However, the Kaplan-Meier survival analysis shows a moderate reduction in median survival in male cohorts following homozygous deletion of Cbx3 compared to control (46 days reduction), with the last survived male mouse sacrificed to 317 days post induction due to ill health.
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Figure 5.1: Cbx3 deficiency moderately impairs survival of mice with heterozygous Apc loss. Cohorts of VillinCreER\(^{ERT2}\)/Cbx3\(^{fl/fl}\) and VillinCreER\(^{ERT2}\)/Cbx3\(^{fl/+}\) mice were induced with 4 daily intraperitoneal injections of 80 mg/kg Tamoxifen along with VillinCreER\(^{ERT2}\)/Cbx3\(^{fl/+}\) control animals (n > 13). Experimental mice were aged to 500 days post induction or sacrificed when ill health was observed. Survival data was presented as a Kaplan-Meier plot. Analysis of cumulative survival revealed a reduced survival of VillinCreER\(^{ERT2}\)/Cbx3\(^{fl/fl}\) (Median survival: 197 days) compared to control animals (265 days) (Log Rank Test: p = 0.128, n > 13). No significant difference in survival was observed between experimental and control cohorts.

Figure 5.2: The loss of Cbx3 shows a trend to reduce survival of male mice following heterozygous loss of Apc. Experimental mice were aged to 500 days post induction or sacrificed when ill health was observed. Survival data for Female (A) and Male (B) mice were presented as Kaplan-Meier plots. Analysis of the cumulative survival of female mice demonstrated a slight reduction in the lifespan of VillinCreER\(^{ERT2}\)/Cbx3\(^{fl/+}\) (Median survival: 260 days) compared to control animals (295 days) (Log Rank Test: p = 0.724, n > 5). On the other hand, analysis of the cumulative survival of male mice demonstrated a greater tendency to reduce survival of VillinCreER\(^{ERT2}\)/Cbx3\(^{fl/+}\) (Median survival: 179 days) compared to control animals (225 days), although it did not reach the statistical significance (Log Rank Test: p = 0.082, n > 8).
5.2.2. Increased Wnt-driven tumorigenesis occurs in the small intestine following Cbx3 loss

Having identified that the loss of Cbx3 shortened the lifespan of mice with heterozygous loss of Apc, the incidence and rates of intestinal tumorigenesis in each cohort was investigated.

Intestines from VillinCreER\(^{+/+}\), VillinCreER\(^{+/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/+}\) and VillinCreER\(^{+/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) animals were dissected at the time of sacrifice and analysed for tumour burden and volume within the small and large intestines (Figure 5.3). Statistical analysis of tumour burden and size revealed significant changes in both experimental cohorts compared to control in the small intestine (for all comparisons \(p < 0.05\), \(n > 13\), Table 5.1). In small intestine, VillinCreER\(^{+/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) and VillinCreER\(^{+/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) animals were found to bear significantly more tumours and larger tumours than controls at the time of dissection.

Next, the tumour number and size was also examined in both female and male Intestines from VillinCreER\(^{+/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) and VillinCreER\(^{+/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) along with control animals (Figure 5.4). Significant increases in both tumour burden and size were observed in male mice between both experimental cohorts compared to control cohort in the small intestine, just as evident in the previous full cohort (for all comparisons \(p < 0.05\), \(n > 8\), Table 5.2B). Statistical analysis of tumour number in female mice also demonstrated a significant increase in tumour number in the VillinCreER\(^{+/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) cohort compared to controls (\(p < 0.05\), \(n > 5\), Table 5.2A). However, note that there was no significant difference in tumour size between the experimental cohorts and controls in the small intestine of female mice.

In the full cohort and in the subgroups according to gender, there is no clear relationship between genotype and tumour number and size in the large intestine. It is therefore obvious that in the context of Apc heterozygosity, Cbx3 loss increases tumour initiation and development in the small intestine, which may account for the trend in decreased survival observed in the VillinCreER\(^{+/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) cohort.
Chapter 5: Analysing the long-term consequences of Cbx3 loss in the context of Wnt-driven tumorigenesis

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Table 5.1: Analysis of small and large intestinal tumour burden observed in the experimental cohorts \( \text{VillinCreER Apco}^{+/-}\text{Cbx3}^{+/-} \) and \( \text{VillinCreER Apco}^{+/-}\text{Cbx3}^{+/-} \) along with control \( \text{VillinCreER Apco}^{+/-} \) animals.

Figure 5.3: The consequence on Cbx3 loss on intestinal tumorigenesis in mice with heterozygous loss of Apco.

Tumour burden was analysed in the small and large intestinal epithelium of \( \text{VillinCreER Apco}^{+/-} \), \( \text{VillinCreER Apco}^{+/-}\text{Cbx3}^{+/-} \) and \( \text{VillinCreER Apco}^{+/-}\text{Cbx3}^{+/-} \) animals at the time of dissection. Tumour burden and size in the small intestine significantly increased in both the experimental cohorts compared to control animals (for all comparisons *\( p < 0.05 \), **\( p < 0.01 \), n > 13). In contrast, statistical analysis of tumour burden and size revealed no significant differences between the cohorts in large intestine. N.S. indicates ‘not significant’. Exact values for tumour number, tumour size, standard deviation and \( p \) values are provided in Table 5.1.
Chapter 5: Analysing the long-term consequences of Cbx3 loss in the context of Wnt-driven tumorigenesis

A Female

<table>
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<th>Mean</th>
<th>Standard Deviation</th>
<th>$p$ Value</th>
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Table 5.2: Analysis of small and large intestinal tumour burden observed in the female (A) and male (B) experimental cohorts VillinCreER$^{ER}$/Apc$^{fl/+}$Cbx3$^{fl/+}$ and VillinCreER$^{ER}$/Apc$^{fl/+}$Cbx3$^{fl/fl}$ along with control Apc$^{fl/+}$ animals.

B Male

<table>
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Figure 5.4: The effect of Cbx3 loss on intestinal tumorigenesis in female and male mice with heterozygous loss of Apc.

(A) Tumour number in the female small intestine significantly increased in the VillinCreER$^{ER}$/Apc$^{fl/+}$Cbx3$^{fl/+}$ cohort compared to the control cohort ($p < 0.05$, n > 5). (B) Tumour burden and size in the male small intestine significantly increased in both the experimental cohorts compared to control animals (for all comparisons $^* p < 0.05$, $^{**} p < 0.01$, n > 8). On the contrary, no significant differences in the tumour number and size exist between the cohorts in the large intestine for both genders. N.S. indicates ‘not significant’. The exact values for tumour number, tumour size, standard deviation and $p$ values are provided in Table 5.2.
5.2.3. The tumours arising in Cbx3-deficient Apc heterozygous small intestine are more advanced compared to controls

As observed in the previous section, loss of Cbx3 substantially increased tumour burden and tumour size in the Apc heterozygous small intestines. Lesions can be classified histopathologically into different categories depending on their stage and invasive qualities. Thus, the tumour morphology was examined and the number of each tumour type observed was quantified in VillinCreER\(^\text{f/+}\), VillinCreER\(^{f/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/+}\) and VillinCreER\(^{f/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) intestines. For the purpose of this study, five categories were classified: single crypt lesion (lesion confined to a single crypt), microadenoma (lesions confined to no more than 5 crypts), small adenoma (lesions extended up to 15 crypts), large adenoma (lesions involving more than 15 crypts) and adenocarcinoma with submucosal invasion (lesions showing clear invasion into the submucosa). Although smooth muscle invasion can also be observed in more aggressive CRC mouse models, it has not been detected in the cohorts used; therefore this category has not been included (Outlined in Figure 5.5).

In the VillinCreER\(^{f/+}\) control cohort, 69% of lesions were found to be single crypt lesions and 19% of lesions were classed as large microadenoma. No lesions were identified as adenocarcinoma with submucosal invasion in control animals. In contrast to this, experimental animals VillinCreER\(^{f/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/+}\) and VillinCreER\(^{f/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) showed a reduction in the number of lesions classified as single crypt lesion (54% and 38% respectively). An increase in the incidence of adenoma (both small and large adenoma) was observed in both experimental cohorts: 12% of all lesions in the control cohorts were adenomas compared to 23% of all lessons in the VillinCreER\(^{f/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/+}\) cohort and 42% in the VillinCreER\(^{f/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) cohort (T-test \(p = 0.031\) in VillinCreER\(^{f/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/+}\) and \(p = 0.007\) in VillinCreER\(^{f/+}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) tumours, \(n > 13\), Figure 5.6A). Adenocarcinoma with submucosal invasion was also observed in both experimental cohorts following the deletion of Cbx3 but absent in the control cohort (Figure 5.6A).

Subgroup analysis according to gender showed that the majority of the advanced tumours in the experimental cohorts were found mainly in male animals; while the single crypt lesion constituted a larger fraction of the tumours in female mice. Tumours with homozygous loss of Cbx3 show a greater percentage of tumours that were graded small and large adenoma, and invasive adenocarcinomas in male mice (56%) than in the female mice.
(34%) (T-test $p = 0.026$, $n > 8$, Figure 5.6 B and C). All the invasive tumours (adenocarcinomas with submucosal invasion) were found in male experimental cohorts but not in female cohorts (Figure 5.6 B and C). Taken together, these findings suggest that, in the context of $Apc$ heterozygosity, additional loss of $Cbx3$ resulted in more advanced progression of tumours in male mice compared to tumours arising in female mice. This suggestion is consistent with the trend for reduced survival, increased tumour number and size in the male compared with the female mice.
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Figure 5.5: Histological categorisation of small intestinal tumour severity. Assessment of H&E stained sections of small intestinal tumours from VillinCreER'Apc<sup>fl/+</sup>, VillinCreER'Apc<sup>fl/+</sup>Cbx3<sup>fl/+</sup> and VillinCreER'Apc<sup>fl/+</sup>Cbx3<sup>fl/fl</sup> mice revealed varying grades of tumour severity. Lesions can be classified histopathologically into five categories: single crypt lesion (lesion confined to a single crypt), microadenoma (lesions confined to no more than 5 crypts), small adenoma (lesions extended up to 15 crypts), large adenoma (lesions involving more than 15 crypts) and adenocarcinoma with submucosal invasion (lesions showing clear invasion into the submucosa). Scale bars represent 100 μm.
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Figure 5.6: Increased severity of small intestinal tumours in mice with Cbx3-deficiency compared to control mice.

Intestinal tumours arising in experimental VillinCreER$^{+/-}$Ap$c^{fl/+}/Cbx3^{fl/+}$ and VillinCreER$^{+/-}$Ap$c^{fl/+}/Cbx3^{fl/fl}$ mice along with control VillinCreER$^{+/-}$Ap$c^{fl/+}$ mice were assessed histologically and categorised according to grade of severity of the tumour in full cohorts (A), female cohorts (B) and male cohorts (C). An increased proportion of lesions in experimental animals showed progression to small and large adenoma compared to controls (T-test $p = 0.031$ in VillinCreER$^{+/-}$Ap$c^{fl/+}/Cbx3^{fl/+}$ and $p = 0.007$ in VillinCreER$^{+/-}$Ap$c^{fl/+}/Cbx3^{fl/fl}$ tumours, n $> 13$) and a subset of lesions were also found to progress into adenocarcinoma with submucosal invasion in VillinCreER$^{+/-}$Ap$c^{fl/+}/Cbx3^{fl/+}$ and VillinCreER$^{+/-}$Ap$c^{fl/+}/Cbx3^{fl/fl}$ tumours in the full cohorts. Loss of Cbx3 resulted in more advanced progression of tumours mainly in male mice compared to tumours arising in female mice in the context of Apc heterozygosity (More advanced tumours include small, large adenomas and adenocarcinoma, T-test $p = 0.026$, n $> 8$).

5.2.4. Limited effects on Wnt, Notch, TGF-β and PI3K signaling in Cbx3-deficient Apc heterozygous tumours

Having found that tumours arising in VillinCreER$^{+/-}$Ap$c^{fl/+}/Cbx3^{fl/fl}$ mice show an overall increase in severity compared to that observed in the control Ap$c^{fl/+}$ mice, various signalling pathways known to govern stem cell maintenance and cell differentiation, including Wnt,
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Notch, TGF-β and PI3K were examined in both control and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) tumours. Tumours (including single crypt lesion, microadenoma, small adenoma, large adenoma and adenocarcinoma with submucosal invasion) and normal tissue from control VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) mice were subjected to immunohistochemistry against β-catenin, Notch1, PTEN, pAKT and Smad2/3.

First the normal intestinal tissues and tumours were examined using anti-β-catenin immunohistochemistry as a marker for Wnt-activation. Normal or non-tumour epithelial tissues in both control VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) intestines was observed to have a cytoplasmic and cell-surface staining pattern of β-catenin, whereas tumours arising in both control VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) intestines revealed both upregulation and nuclear localisation of β-catenin, consistent with activation of the Wnt pathway and indicative of loss of Apc (Figure 5.7). Furthermore, there was a site-dependent variability in the β-catenin staining patterns in small intestinal tumours. Tumours arising in VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) intestines revealed some well-differentiated tubular epithelial structures where weak cytoplasmic and nuclear localisation of β-catenin was observed. By contrast, strong nuclear localisation of β-catenin was detected in the dedifferentiated epithelial cells and invasive tumour cells in the intestinal tumours in both control VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) intestines, indicating the intratumour heterogeneity in Wnt-activated intestines. However, there is no variation in the β-catenin staining pattern between the control VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) intestines.

Secondly, an antibody which detects the activated intracellular Notch domain produced after γ-secretase cleavage was used to determine the level of Notch pathway activation. Normal intestines and tumours from both control VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) intestines were uniformly negative for Notch1 expression. The expression of Notch1 showed no differences across all types of tumours evaluated in both control VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) intestines (Figure 5.7), indicating that Notch signalling was not altered following the loss of Cbx3 in the small intestines.

Thirdly, anti-PTEN was used to confirm PTEN status in VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) intestines. PTEN immunohistochemistry can be observed in the stromal cells of both control VillinCreER\(^{Apc^0/+}\) and VillinCreER\(^{Apc^0/+Cbx3^{0/\beta}}\) intestines.
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Elevated level of diffuse positive cytoplasmic staining of PTEN was demonstrated in the crypt regions of intestines and single crypt lesions in VillinCreER^* Apc^{fl/+} Cbx3^{fl/fl} intestines. Tumours arising in both control VillinCreER^* Apc^{fl/+} and VillinCreER^* Apc^{fl/+} Cbx3^{fl/fl} intestines show intense positive cytoplasmic expression for PTEN across epithelial and stromal cells, with no obvious difference between the control VillinCreER^* Apc^{fl/+} and VillinCreER^* Apc^{fl/+} Cbx3^{fl/fl} intestines (Figure 5.8).

Next, in order to assess the activation status of the PI3K/AKT pathway following deletion of Apc, sections were stained immunohistochemically against activated Phospho-AKT^{Ser473} (pAKT). Weak staining of pAKT was observed at the bottom of the crypts in the normal intestines and the tumours of both control VillinCreER^* Apc^{fl/+} and VillinCreER^* Apc^{fl/+} Cbx3^{fl/fl} intestines. Cellular staining of pAKT was noted to be homogenously cytoplasmic, with no obvious difference between the control VillinCreER^* Apc^{fl/+} and VillinCreER^* Apc^{fl/+} Cbx3^{fl/fl} intestines (Figure 5.8).

Lastly, phosphorylated Smad2/3 levels were analysed in control VillinCreER^* Apc^{fl/+} and VillinCreER^* Apc^{fl/+} Cbx3^{fl/fl} intestines to evaluate the TGF-β growth inhibitory effects. Immunohistochemical staining of phospho-Smad2/3 was highly expressed and predominantly located in the nuclei of all normal intestinal epithelium and tumour cells in control VillinCreER^* Apc^{fl/+} and VillinCreER^* Apc^{fl/+} Cbx3^{fl/fl} intestines (Figure 5.8). No change in intensity of the phospho-Smad2/3 staining was observed between the two cohorts.
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Figure 5.7: Immunohistochemical analysis of β-catenin and Notch1 in the intestines and adenomas of VillinCreER’Apc\textsuperscript{fl/+} and VillinCreER’Apc\textsuperscript{fl/+}Cbx3\textsuperscript{fl/fl} animals. Anti-β-catenin immunohistochemistry revealed strong cytoplasmic staining of β-catenin in normal intestinal epithelium and nuclear localisation of β-catenin in tumours arising in both VillinCreER’Apc\textsuperscript{fl/+} and VillinCreER’Apc\textsuperscript{fl/+}Cbx3\textsuperscript{fl/fl} animals. Anti-Notch1 immunohistochemistry revealed little to no staining of Notch1 in both VillinCreER’Apc\textsuperscript{fl/+} and VillinCreER’Apc\textsuperscript{fl/+}Cbx3\textsuperscript{fl/fl} intestines and tumours. Scale bars indicate 100 μm.
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Figure 5.8: Immunohistochemical analysis of PTEN, pAKT and pSmad2/3 in intestines and adenomas of VillinCreER\(^{+}\)Apc\(^{−/−}\) and VillinCreER\(^{+}\)Apc\(^{−/−}\)Cbx3\(^{+/−}\) animals. Anti-PTEN immunohistochemistry detected cytoplasmic staining of PTEN in the crypt of normal intestines and single crypt lesions and intense cytoplasmic staining in tumours arising in VillinCreER\(^{+}\)Apc\(^{−/−}\) and VillinCreER\(^{+}\)Apc\(^{−/−}\)Cbx3\(^{+/−}\) animals. Anti-pAKT\(^{\text{ser473}}\) immunohistochemistry revealed little to no staining of pAKT in both VillinCreER\(^{+}\)Apc\(^{−/−}\) and VillinCreER\(^{+}\)Apc\(^{−/−}\)Cbx3\(^{+/−}\) intestines and tumours. Anti-pSmad2/3 immunohistochemistry was found ubiquitously positive in all cell nuclei of normal intestine and tumours of VillinCreER\(^{+}\)Apc\(^{−/−}\) and VillinCreER\(^{+}\)Apc\(^{−/−}\)Cbx3\(^{+/−}\) animals. Scale bars indicate 100 \(\mu m\).
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5.3. Discussion

Previous work has shown that epithelial deletion of Cbx3 results in no immediate perturbation of intestinal homeostasis and survival (Chapter 3), but prolonged survival and repressed splice variant Rac1b expression in epithelial Apc-deficient mice (Chapter 4). Evidence has demonstrated that the deletion of HP1γ lacks tumorigenesis activity in a mouse xenograft model (Sharma et al., 2003), suggesting that the expression of HP1γ contributes to cancer formation. It was therefore of great interest to examine the effects of epithelial-Cbx3 deficiency in the context of activated Wnt signalling, achieved by conditional deletion of one alleles of Apc.

5.3.1. Rapid progression of Wnt-initiated tumorigenesis in the context of Cbx3 deficiency

In order to test the long-term effects of Cbx3 loss in the context of activated Wnt signalling, either one or both alleles of Cbx3 were deleted in the context of Apc heterozygosity using the VillinCreER recombinase. Experimental cohorts of VillinCreER′Apcfl/fl/Cbx3fl/fl and VillinCreER′Apcfl/+/Cbx3fl/+ mice were aged along with VillinCreER′Apcfl/+ controls. Survival analysis of the full cohorts revealed a substantially reduced lifespan in VillinCreER′Apcfl/fl/Cbx3fl/fl animals with a medium survival of 197 days, compared to VillinCreER′Apcfl/+ control animals, which had a median survival time of 265 days post induction. Subgroup analysis according to gender demonstrated a more prominent reduction in median survival duration in male cohorts following homozygous deletion of Cbx3 (179 days) compared to control (225 days), with the last survived male VillinCreER′Apcfl/fl/Cbx3fl/fl mouse lived to 317 days post induction due to ill health. Upon dissection, VillinCreER′Apcfl/fl/Cbx3fl/fl mice were found to contain remarkably more and larger lesions within the small intestine. The number and size of the tumours in the small and large intestines of the experimental animals and control animals were quantified and measured at the time of dissection. The experimental animals (VillinCreER′Apcfl/+/Cbx3fl/fl and VillinCreER′Apcfl/fl/Cbx3fl/fl) were found to have a significant increase in tumour burden within the small intestine. Subgroup analysis according to gender showed that the observed
significant increase in tumour burden was mainly attributed to tumours in male mice. No difference in tumour burden was observed in the large intestine between all cohorts. Therefore, these observations indicate that additional deficiency of Cbx3 increases predisposition of tumour formation and development in the small intestine of male mice in an Apc heterozygous background.

The morphology of the small intestinal tumours was examined and categorised. Tumours arising in experimental animals VillinCreER\(^{Apc^{fl/+}-Cbx3^{fl/+}}\) and VillinCreER\(^{Apc^{fl/+}-Cbx3^{fl/fl}}\) were found to be more aggressive, with a higher number of lesions classified as small and large adenoma and also adenocarcinoma with submucosal invasion. In VillinCreER\(^{Apc^{fl/+}}\) control animals, the majority of small intestinal lesions were found to be single crypt lesion, with a small proportion of lesions found to be microadenoma and no invasive characteristics observed. Subgroup analysis according to gender showed that this pattern was not observed in female tumours but was highly related, and even accentuated, in male tumours. This observation is consistent with the previous observed trend for reduced survival, increased tumour burden in male compared to female mice.

It is therefore clear from these data that in the context of Apc heterozygosity, additional epithelial deletion of Cbx3 drives both an increase in multiplicity of small intestinal lesions and also drives progression of those lesions to a malignant phenotype in male cohorts but not female cohorts, indicating that lack of Cbx3 is associated with a more aggressive phenotype in male mice. In male control animals, severity of intestinal tumours was not observed to equal that of VillinCreER\(^{Apc^{fl/+}-Cbx3^{fl/+}}\) and VillinCreER\(^{Apc^{fl/+}-Cbx3^{fl/fl}}\) animals, even in very aged animals. This exemplifies the dramatic acceleration of disease progression resulting from epithelial Cbx3 loss in the context of Apc heterozygosity in male mice. Although the exact mechanism is currently unclear, previous evidence has shown that depletion of Cbx3 in adult male mice resulted in a severe hypogonadism (absence of hormone secretion) associated with a loss of germ cells and defeat in spermatogenesis, therefore the observed gender differences in survival duration and incidence of tumours may be due in part to hormonal differences between male and female mice (Brown et al., 2010; Cleveland et al., 2009; Patman, 2015). Additionally, estrogens (female hormones) were found to induce mismatch repair gene expression in colonic epithelial cells mediated
by estrogen receptor β (ERβ) in vitro and in vivo, suggesting estrogens in female mice play a protective role against CRC initiation and progression (He et al., 2012; Jin et al., 2010; Kennelly et al., 2008). Thus, there is an urgent need to examine the level of ERβ using immunohistochemistry in both male and female intestinal epitheliums and such information could shed light on the tumour susceptibility observed in male mice. Another possible reason for this marked shorter survival and increased in tumour severity in Cbx3-deficient male mice could be genomic instability as the loss of HP1γ results in loss of gene silencing, heterochromatin structure and transcriptional repression (Eissenberg and Elgin, 2000; Ebert et al., 2006; Smallwood et al., 2007), which predisposes to DNA strand breakage, DNA mismatches and recombination within derepressed repetitive sequences (Ehrlich, 2002). Links between hypomethylation and genomic instability have been demonstrated for many cancer types (Tsuda et al., 2002; Schulz et al., 2002). The lack of HP1γ may cause a global genomic demethylation in Cbx3 deficient epithelium and it therefore would be interesting to explore the global methylation signature of Cbx3 deficient epithelium, in male and female subgroups, compared to control epithelium to gain an insight into the role of HP1γ in heterochromatin marks and the DNA methylation in both male and female mice of colorectal cancer.

5.3.2. Limited effects on signalling pathways in Cbx3-deficient Wnt-activated small intestinal tumours

Previous data has demonstrated that the deletion of Cbx3 alone is no different from healthy wildtype, indicating that Cbx3 loss alone is unable to induce tumorigenesis (Chapter 3). However, additional loss of Cbx3 enhances tumour burden and progression in mice with heterozygous deletion of Apc, suggesting that Cbx3 loss accelerate Wnt-driven tumour formation. Therefore the activation of several key signalling pathways was tested, including Wnt, Notch, TGF-β and PI3K, in Apc heterozygous tumours with or without Cbx3 deletion. Immunohistochemistry was performed on tumour sections from all grades (single crypt lesion, microadenoma, small adenoma, large adenoma and adenocarcinoma with
submucosal invasion) and normal intestinal tissue from control VillinCreER\(^{+/−}\)Apc\(^{fl/+}\) and VillinCreER\(^{+/−}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) mice.

In the intestine, Wnt signalling plays an important role in the proliferation and maintenance of intestinal stem cells and progenitor cells (van de Wetering et al., 2002). Upregulation and strong nuclear localisation of β-catenin is a sign of Wnt pathway activation by Apc loss within the intestine (Sansom et al., 2004). Normal intestines were found to have a cytoplasmic staining pattern of β-catenin, whereas tumour tissues revealed both upregulation and nuclear localisation of β-catenin in control VillinCreER\(^{+/−}\)Apc\(^{fl/+}\) and VillinCreER\(^{+/−}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) intestines. This observation reflects Wnt activation as a result of Apc loss. Furthermore, β-catenin was detected strongly in the nuclei of the dedifferentiated epithelial cells and invasive tumour cells in the intestinal tumours in both control VillinCreER\(^{+/−}\)Apc\(^{fl/+}\) and VillinCreER\(^{+/−}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) intestines, indicating the intratumour heterogeneity in Wnt-activated intestines. However, there is no variation in the β-catenin staining pattern between the control VillinCreER\(^{+/−}\)Apc\(^{fl/+}\) and VillinCreER\(^{+/−}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) intestines.

In the intestine, Notch signaling is a crucial element for the maintenance of intestinal stem cells and for the regulation of cell differentiation. Notch signaling was thought to be oncogenic by positively regulating the proliferation of intestinal progenitors and imitating tumour formation in the intestine (Fre et al., 2011). Previously published data has demonstrated that the dual roles of Notch signaling in both enhancing tumour initiation and suppressing tumour progression. Furthermore, Notch signaling particularly has a suppressive role in Apc deficient tumours, suggesting that there is a negative correlation between Wnt and Notch signaling activation (Kim et al., 2012). As described above, I previously observed a dramatic increase in activation status of Wnt, as assessed by immunohistochemical staining for β-catenin, following heterozygous loss of Apc in both Cbx3-proficient and deficient intestines. Therefore, the activation status of Notch was examined in tumours arising in both VillinCreER\(^{+/−}\)Apc\(^{fl/+}\) control and VillinCreER\(^{+/−}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) intestines. The immunohistochemistry was performed using anti-activated Notch1 as a marker of Notch activation. Normal intestines and tumours from both control VillinCreER\(^{+/−}\)Apc\(^{fl/+}\) and VillinCreER\(^{+/−}\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) intestines were uniformly negative for
Chapter 5: Analysing the long-term consequences of Cbx3 loss in the context of Wnt-driven tumorigenesis

Notch1 expression, suggesting that a very low levels of Notch activation in the Wnt-activated intestines. However, Notch signalling was not altered following the loss of Cbx3 in the small intestines.

PTEN is an important tumour suppressor that negatively regulates the PI3K-AKT signalling pathway and it is frequency mutated in a wide range of human cancers and is associated with the apoptosis, proliferation and metastasis of tumour cells (Sansal and Sellers, 2004; Tamura et al., 1998). I then examined the normal intestinal tissues and tumours using anti-PTEN immunohistochemistry as a marker for PTEN status. Tumours arising in both control VillinCreER\(^+\)Apc\(^{fl/+}\) and VillinCreER\(^+\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) intestines show intense positive cytoplasmic expression for PTEN across epithelial and stromal cells, indicating that PI3K-AKT may not yet be activated.

The PI3K/AKT signalling pathway plays an important role in cell proliferation, tumour growth and metastasis and activation of PI3K/AKT pathway promotes cancer formation through a variety of mechanisms (Liu et al., 2009; Xue et al., 2012; Engelman, 2009). PTEN is a negative regulator of PI3K/AKT signalling, and its inactivation is a common cause of increased PI3K activity in cancers (Di Cristofano and Pandolfi, 2000). As described previously, intense PTEN expression by PTEN immunostaining was detected in tumours of both control VillinCreER\(^+\)Apc\(^{fl/+}\) and VillinCreER\(^+\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) intestines, suggesting that PI3K/AKT pathway may not be activated. Sections were then stained immunohistochemically against activated Phospho-AKT\(^{ser473}\) (pAKT) to assess the activation status of the PI3K/AKT pathway. Low levels of cytoplasmic pAKT staining were detected at the crypt regions in the normal intestines and across tumours of both control VillinCreER\(^+\)Apc\(^{fl/+}\) and VillinCreER\(^+\)Apc\(^{fl/+}\)Cbx3\(^{fl/fl}\) intestines, consistent with the suggestion that PI3K/AKT pathway may not yet be activated in the tumours.

The TGF-β signalling pathway has dual roles in exerting tumour suppressor effects by regulating cell growth, apoptosis and immortalisation in normal cells and early carcinomas and exerting prometastatic effects by promoting cancer progression, invasion, and tumour metastasis in more advanced tumours (Yingling, Blanchard and Sawyer, 2004; Siegel and Massague, 2003; Dumont and Arteaga, 2003). Given that no distant metastasis
was observed in both of control VillinCreER$^+$Apc$^{fl/+}$ and VillinCreER$^+$Apc$^{fl/+}$Cbx3$^{0/0}$ animals, evaluation of the tumor suppressive activity of TGF-β would be more relevant in this case. Since accumulated evidences suggests that the phosphorylation of Smad2/3 at its C-terminal region correlates with the growth inhibition mediated by TGF-β pathway, the phosphorylated Smad2/3 levels were analysed in control VillinCreER$^+$Apc$^{fl/+}$ and VillinCreER$^+$Apc$^{fl/+}$Cbx3$^{0/0}$ intestines to evaluate the TGF-β growth inhibitory effects. Immunohistochemical staining of phospho-Smad2/3 was highly expressed and predominantly located in the nuclei of all normal intestinal epithelium and tumour cells in control VillinCreER$^+$Apc$^{fl/+}$ and VillinCreER$^+$Apc$^{fl/+}$Cbx3$^{0/0}$ intestines, suggesting a role of Smad complex in transcriptional regulation. This ubiquitous phospho-Smad2/3 staining indicates that tumour repressive arm of TGF-β pathway is activated in Wnt-activated intestines.

5.3.3. Summary

In summary, the findings presented in this chapter demonstrated for the first time that Cbx3 loss accelerates Wnt-driven tumour progression and reduces survival of mice, particularly in male mice, with heterozygous loss of Apc.

These data also indicate that the advanced Wnt-driven tumorigenesis in Cbx3 deficient intestines do not show increased activation of Wnt, Notch, PI3K and TGF-β compared to the Cbx3 proficient Wnt-activated intestines. The exact nature and mechanism by which Cbx3 deficiency influences tumour formation and progression remains unclear, but it is clear that the phenomenon is influenced by gender, as Cbx3 deficiency is strongly associated with shortened survival and more aggressive phenotypes in male but not female mice.
Chapter Six

General Discussion
6. General Discussion

“The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.”

Sir William Bragg 1862 – 1942

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide (International Agency for Research on Cancer 2014). It is estimated that 110 new cases of CRC are diagnosed each day in the U.K. alone and the 5-year survival rate is as low as 50% (Cancer Research U.K. 2013).

The stepwise model of CRC initiation and progression implicates the requirement for the accumulation of multiple genetic and epigenetic mutations to drive the transformation of the normal epithelium to invasive adenocarcinoma (Fearon and Vogelstein, 1990). The first mutation in this process is often the inactivation of the tumour suppressor gene, Apc.

Although genetic mutations have been the focus of cancer research for many years, epigenetic alterations have been increasingly recognised as playing an important role in tumorigenesis. Members of the heterochromatin protein 1 (HP1) family have been implicated in various functions, including heterochromatin formation, gene silencing, RNAi mediated silencing, telomere stability, DNA replication and repair, and positive regulation of gene expression (Hediger and Gasser, 2006; Kwon et al., 2010; Dinant and Luijsterburg, 2009; Zeng et al., 2013). Compared to the wealth of studies conducted on HP1α and HP1β, much less is known about HP1γ.

Growing evidence suggest that HP1γ plays an important role in the epigenetic regulation of cancer development, via regulating cell proliferation, differentiation (Takanashi et al., 2009), telomere maintenance (Sharma et al., 2003) and DNA damage repair (Luijsterburg et al., 2009). What is more the level of HP1γ is frequently up-regulated in many cancer cell lines, including the colon (Takanashi et al., 2009). Loss of Cbx3, the gene encoding HP1γ, in selected cancer cell lines has also been reported to inhibit cancer cell growth and colony formation, while further evidence indicates that the deletion mutant of
HP1γ lacks tumorigenesis activity in a mouse xenograft model (Zhou et al., 2014; Sharma et al., 2003). These observations provide evidence that Cbx3 functions as a driver in cancer cell growth and the over-expression of HP1γ in cancer is therefore important in the process of tumorigenesis, at least in the tumour initiation stage.

Since these observations were made in cell lines and xenographs, the role of Cbx3 in the small intestinal epithelium has not yet been explored. Although not reported to be directly involved in intestinal homeostasis, Cbx3 has been implicated as a key regulator in cell differentiation via the molecular interaction with histone H4K20, and the loss of Cbx3 is required for cell differentiation to occur (Takanashi et al., 2009). Bearing in mind the role of Wnt pathway in intestinal homeostasis and intestinal stem cell maintenance, one could speculate that Cbx3 might be involved in regulating the trans-activation of Wnt target gene expression epigenetically.

In my PhD project, I set about investigating the effects of Cbx3 loss in normal intestinal homeostasis and in the context of aberrant Wnt signalling activation. Furthermore, I investigated the long-term consequences of Cbx3 deletion upon Wnt-driven intestinal tumour initiation and progression.

The main findings from this PhD thesis are:

- *Cbx3* in the intestinal epithelium is redundant with regards to intestinal homeostasis as *Cbx3* deficiency has no effects on intestinal homeostasis, cell lineage differentiation and survival
- *Cbx3* deficiency prolongs survival of mice with acute Wnt-activated intestines potentially via attenuation of Wnt activation at the receptor level and RNA splicing defects
- *Cbx3* deficiency promotes Wnt-driven tumour progression and reduces survival of male mice with heterozygous loss of *Apc* in the long term
- The multifaceted roles of HP1γ maintain a gene-repressive environment in heterochromatin for normal intestinal homeostasis, and provide a gene-permissive environment in euchromatin for carcinogenesis
Chapter 6: General Discussion

6.1. The redundant role of Cbx3 in normal intestinal homeostasis

The literature has indicated that Cbx3 has a role in stem cell maintenance and cell differentiation in adipocytes, male germ cells and cardiovascular system (Xiao et al., 2011; Brown et al., 2010). However, little is known about the nature of Cbx3 in intestinal epithelial cell differentiation and homeostasis. In Chapter 3, the effects of epithelial-specific deletion of Cbx3 from the murine small intestine were investigated.

The proposed role of Cbx3 on stem cell maintenance leads to the prediction that Cbx3 loss would result in diminished stem cell compartment and increased cell lineage differentiation in the small intestinal epithelium. In contrast to this hypothesis, histological and quantitative analysis of the small intestinal epithelium following Cbx3 loss has no dramatic effect upon homeostasis. No changes to the gross histology or structure of the intestine and survival up to 366 days following Cbx3 loss were detected, and no alterations in crypt and villus size, proliferative, apoptotic and cell migration were observed in the Cbx3 deficient small intestine. Furthermore, there was no enhancement in cell differentiation and no alteration in the majority of the stem cell markers in the intestinal epithelium in the absence of Cbx3. Despite the fact that Cbx3 deletion results in a significant increase in one of the CBC stem cell markers, Ascl2, which is a transcriptional target of Wnt signalling and is often upregulated in colorectal neoplasms (Jubb et al., 2006), intestinal homeostasis and cell differentiation were unaltered. This suggests that Cbx3-mediated up-regulation of Ascl2 alone might not be sufficient to alter intestinal stem cell maintenance and differentiation and therefore no effect on the overall intestinal homeostasis was observed. The findings presented in this study therefore indicate that homozygous loss of Cbx3 in the intestinal epithelium has no detrimental effect on the normal intestinal homeostasis, suggesting that Cbx3 acts redundantly in normal intestinal homeostasis and intestinal cell lineage differentiation.

There appears to be a discrepancy between the data presented here and in the literature on the proposed role of Cbx3 as a safety lock for the transition to cell differentiation and stem cell maintenance (Takanashi et al., 2009). In order to further understand the functional role of Cbx3 in maintenance of the small intestinal stem cells, the
Lgr5-EGFP-Cre transgene could be incorporated into the wild type and VillinCre<sup>Cre<sup>fl/fl</sup> mice for further analysis of the stem cell enriched populations of epithelial cells. Furthermore, single cell suspension from Lgr5-EGFP-CreER<sup>Cre<sup>ER</sup> Cbx3<sup>fl/fl</sup> small intestine could be enriched for GFP expressing cells and grown in organoid culture system in the presence or absence of tamoxifen to investigate if Cbx3 loss directly targets stem cells. Finally, genome-wide expression analysis of Cbx3 deficient small intestinal epithelium should be performed to further elucidate the role of Cbx3 in regulation of intestinal stem cell maintenance, differentiation and other potential physiological functions, by analysing the transcriptional effects of Cbx3 loss in the entire intestinal epithelium.

6.2. The role of Cbx3 in Wnt signalling and RNA splicing

Cbx3 has been previously reported to be up-regulated in human colorectal cancer tumours and multiple cancer cell lines. Furthermore, down-regulation of Cbx3 expression was previously reported to lead to growth inhibition of cancer cell lines (including colorectal cancer cell lines) suggesting a role of Cbx3 in carcinogenesis and as a therapeutic target for various types of cancers (Takanashi et al., 2009). It is well established that appropriate regulation of the Wnt signalling pathway is critical for intestinal homeostasis and active Wnt signalling has been extensively reported to play a pivotal role in intestinal tumorigenesis (Clevers, 2006). However, very little is known about the functional involvement of Cbx3 in Wnt signalling regulation and colorectal cancer development. Thus, it was of great interest to investigate the consequence of Cbx3 loss in the context of activated Wnt signalling. Activation of Wnt signalling was achieved by conditional deletion of both alleles of the Apc gene within the intestinal epithelium (Sansom et al., 2004).

Analysis of the effects of Cbx3 deficiency in Wnt-activated intestines revealed a significant increase in lifespan of mice with homozygous loss of Apc. The impact of Cbx3 deficiency on prolonged animal survival suggested that the loss of Cbx3 alleviates the oncogenic effects in the context of Wnt activation. The expression of Wnt target genes was examined to investigate the feasibility of Cbx3 as a potential therapeutic target in Wnt-activated intestines. Cbx3 loss slightly reduced the Wnt target gene up-regulation in Wnt-
activated small intestine but the attenuation of Wnt target gene expression did not reach statistical significance, it is possible that the slight reduction of Wnt target genes was sufficient to repress the enterocyte differentiation in Cbx3 deficient epithelium and therefore lower its oncogenic potential leading to an increase in survival. In order to further investigate the mechanism behind the attenuated Wnt target gene expression of Apc loss in Cbx3 deficient epithelium, luciferase assays were performed in HEK-293 cells by the use of Wnt-responsive Topflash system. Transient knockdown of CBX3 reduced ΔNLRP6–mediated Topflash activity by 41% and TCF4-VP16–mediated Topflash activity by 27%, suggesting that CBX3 mainly acted at the LRP6 receptor level. This suggestion is based on the observation that the loss of CBX3 blocked TCF-dependent transcription induced by a constitutively active LRP6 receptor, but a lower extent of blockage was observed by a constitutively active TCF-VP16 fusion protein, indicating that Cbx3 does function at the receptor level within the Wnt signalling cascade. Together these data provide evidence that Cbx3 deficiency attenuates Wnt signalling by suppressing its receptors and thus causes a suboptimal level of Wnt activation. This observation also explains that the loss of Cbx3 had little effect on Wnt target gene expression in Apc-deficient intestine, as the activation of Wnt signalling in the Apc mutants were downstream of the receptor. However, the exact mechanism underlying the regulation of Wnt signalling by Cbx3 remains to be elucidated. It is tempting to speculate that a positive feedback loop exists in the presence of Cbx3, in which initial Wnt pathway activation induces expression of a subset of gene products which in turn function in an autocrine or paracrine manner to induce further activation of Wnt signalling. In the absence of Cbx3, the positive feedback loop may be abolished and this resulted in a suboptimal level of Wnt activation. Whether such a feedback loop exists warrants future investigation. Furthermore, it is worth investigating further at which distinct levels Cbx3 is acting within the Wnt signalling cascade. Further Topflash assays could be performed using the mutant plasmids Axin-GID (which acts to titrate GSK-3 from the endogenous Axin complex) (Fraser et al., 2002) and ΔN-89-β-catenin (which is a non-degradable form of β-catenin) (Munemitsu et al., 1996).

Accumulated evidence indicates a role for Cbx3 in aiding in efficient RNA processing through the recruitment or stabilisation of the splicing machinery. Depletion of Cbx3 leads
to a decrease in recruitment of the RNA splicing factors SNRNP70 and SRSF1 to gene bodies, resulting in RNA splicing defects and subsequently leading to an increase in unspliced RNA species (Saint-Andre et al., 2011; Smallwood et al., 2012). It has been documented that the splicing factor SRSF1 regulates the expression of the tumour-related gene Rac1b, which is an alternative splice variant of Rac1 and it is frequently overexpressed in a subset of tumour samples (Goncalves et al., 2014; Schnelzer et al., 2000; Fiegen et al., 2004). Numerous studies have reported that Rac1b plays a critical role in tumorigenesis via augmenting Wnt signaling, promoting tumour cell survival (Matos et al., 2008) and increasing the invasive and metastatic properties of colorectal cancer cells (Esufali et al., 2007). However, the mechanisms of how changes in alternative splicing lead to Rac1b overexpression remain unclear. Gene expression analysis was performed to determine if Cbx3 affects the splicing of Rac1b gene. Following the loss of Cbx3 in the Wnt activated intestine, the splice variant Rac1b was significantly repressed. This was consistent with the hypothesis that loss of Cbx3 impairs RNA splicing and therefore leads to a reduced level of Rac1b in the small intestinal epithelium. This further confirmed the role of Cbx3 in influencing alternative splicing through its recruitment of the splicing machinery and splicing factors like SRSF1.

The deletion of Cbx3 in the Topflash assay resulted in marked repression of Wnt activity, together with the repressed alternative splicing of Rac1b gene in vivo, resulting in a prolonged lifespan of mice with homozygous loss of Apc. Collectively, the initial data presented portray Cbx3 as a positive regulator of Wnt signalling at the receptor level and as a positive regulator of alternative splicing in Rac1 gene. A more comprehensive insight into the role of Cbx3 could be gained by performing chromatin immunoprecipitation (ChIP) analysis of the Wnt-activated small intestinal epithelium to verify whether Cbx3 occupies promoter regions of Wnt target genes, as previous evidence suggested that Cbx3 binding is strongly correlated with the level of gene expression (Smallwood et al., 2012). Furthermore, we are planning to carry out a high-throughput RNA sequencing (RNA-seq) using total nuclear RNA of Wnt-activated small intestinal epithelium with or without Cbx3 loss to fully characterise the functional role of Cbx3 on alternative splicing.
6.3. *Cbx3* loss promotes Wnt-driven tumour progression

Previous work has established that the deletion of *Cbx3*, in the context of homozygous *Apc* loss in the intestinal epithelium, prolonged survival of mice with diminished level of Wnt activation and reduced expression of the tumour-related splice variant *Rac1b*. These data indicates the oncogenic potential of *Cbx3* on aberrant Wnt-activated intestines.

Homozygous loss of *Apc* alleles in the adult intestine resulted in rapid and catastrophic disruption of epithelial homeostasis and animals only survive up to 4 days post induction. In order to investigate the consequences of *Cbx3* deletion in Wnt-driven tumorigenesis, the delayed phenotype study was achieved through the deletion of one allele of the *Apc* gene within the intestinal epithelium, which has been previously reported as an intestinal tumour-prone model (Shibata *et al.*, 1997).

Although *Cbx3* deletion has been reported to inhibit the growth of various cancer-derived cell lines (Takanashi *et al.*, 2009), my data indicated that the survival probability of *Cbx3*-deficient mice was shortened in the context of *Apc* heterozygosity due to a dramatic increase in tumour number and size in the small intestine, particularly in male mice. In addition, the loss of *Cbx3* accelerated tumour progression, resulting in more advanced tumours compared to mice with heterozygous loss of *Apc* alone. The advanced Wnt-driven tumours in *Cbx3* deficient intestines showed no changes in the characteristic patterns of Wnt, Notch, PI3K and TGF-β signalling compared to the *Cbx3* proficient Wnt-activated tumours. Unprecedentedly, these observations suggest that *Cbx3* deletion confers cancer susceptibility and accelerate tumour progression in male mice with Wnt-activated intestines over long period of time. Previous studies have shown that deletion of *Cbx3* in adult male mice resulted in a dramatic reduction in hormone secretion that is associated with the reduced number of germ cells and a severe impairment of spermatogenesis (Brown *et al.*, 2010b). Together these findings not only suggest an important role for *Cbx3* in colorectal carcinogenesis and progression, but also add support to the accumulating evidence that sex hormones are relevant to the development of colorectal cancer (Slattery *et al.*, 2001).
The underlying mechanism for the long-term Cbx3 loss in the context of Apc heterozygosity on the intestinal epithelium is currently inadequately understood. Tumours arising from Cbx3-deficient mice demonstrated invasive potential, which is unprecedented in Apc<sup>fl/fl</sup> mice. It was unknown how Cbx3 deficiency may contribute to invasive potential of Wnt-driven tumorigenesis. However, an increasing number of investigations suggest the role of Cbx3 in cell differentiation and stem cell maintenance (Wang <i>et al.</i>, 2012; Xiao <i>et al.</i>, 2011). It is possible that Cbx3-deficient tumours became progressively more undifferentiated with higher proliferative, invasive and metastatic potential. Although this explanation, however, may be undermined by the results from previous chapters, which demonstrated Cbx3 deficiency resulted in no alteration to cell differentiation and stem cell compartment in intestines with or without homozygous loss of Apc. It is still worth investigating whether Cbx3 has a role in tumour cell differentiation, invasion and metastasis. Further insight into the nature of Cbx3 and cell differentiation could be gained by investigating the expression of tumour cancer stem cell markers (such as CD133, CD24 and CD44v6) in Apc<sup>fl/fl</sup> tumours with or without Cbx3 (Choi <i>et al.</i>, 2009; Todaro <i>et al.</i>, 2014). Furthermore, the accelerated tumour progression caused by Cbx3 deficiency in Wnt-driven tumorigenesis is being further investigated to address the ability of Wnt-activated tumours to progress and invade upon Cbx3 loss. A more invasive model of colorectal cancer is currently being generated, by incorporating with a PTEN gene deletion. By using Apc<sup>fl/fl</sup> mice with conditional deletion of the tumour suppressor PTEN in the epithelium with or without Cbx3, the role of Cbx3 in facilitating tumours to acquire increased invasiveness and metastatic potential could be further characterised.
6.4. **The functional versatility of HP1y in normal intestinal homeostasis and tumorigenesis**

Work to date has yet to clarify the multifaceted roles of Cbx3 in intestinal homeostasis and tumorigenesis. The findings of this study demonstrate the functional versatility of HP1y in different contexts (Figure 6.1).

![Diagram showing HP1y's multifaceted roles](image)

Figure 6.1: Simplified proposed multifaceted functional model of HP1y in intestine. We proposed that HP1y acts as a positive regulator of Wnt signalling at the LRP5/6 receptor level and a suboptimal level of Wnt activation was observed in the absence of Cbx3 (encoding the HP1y protein) *in vitro* and *in vivo*. Secondly, HP1y is proposed to aid efficient RNA processing through recruitment / stabilisation of the splicing machinery SRSF1 which mediates Rac1b splicing. Cbx3 loss resulted in a dramatic decrease in level of Rac1b in aberrant Wnt-activated intestine. Thirdly, H4K20me3 is proposed to serve as a docking site for HP1y, which in turn recruits histone methyltransferases Suv420h1 (to methylate H4K20me3) and Suv39h1 (to methylate H3K9me3), and results in heterochromatin spreading. HP1y is required to maintain a gene-repressive environment via formation and maintenance of heterochromatin marks in Wnt-driven tumourigenesis. Lastly, HP1y has a potential role in regulating DNA double strand break repair.
Normal healthy intestines do not seem to require Cbx3 for homeostasis, as long-term loss of Cbx3 did not perturb normal intestinal homeostasis and survival, suggesting the redundant nature of Cbx3 in intestinal homeostasis under physiological conditions. Upon aberrant Wnt-activation, Cbx3 deficiency suppressed Wnt-activation and reduced splicing of the tumour-associated gene Rac1b, leading to an increase in survival probability of mice with homozygous loss of Apc. In this context, Cbx3 is therefore thought to be a positive regulator of Wnt-signalling and RNA splicing. Consistently, a number of studies have shown that Cbx3 is widely associated with euchromatin and localized to active genes (Vakoc et al., 2005; Smallwood et al., 2007; Mateescu et al., 2008) and evidence has suggested a role for Cbx3 in alternative splicing (Saint-Andre et al., 2011). Together, these observations suggest an oncogenic nature of Cbx3 in aberrant Wnt-activated intestine (Figure 6.1).

In the long-term, deletion of Cbx3 accelerated Wnt-driven tumour progression, leading to a reduced lifespan of mice, particularly in male mice with heterozygous loss of Apc. Exactly how Cbx3 loss is involved in tumour progression has yet to be determined. Previous evidence has shown that Cbx3 loss depleted hormone secretion in adult male mice resulting in a severe hypogonadism, leading a loss of germ cells and defeat in spermatogenesis (Brown et al., 2010; Cleveland et al., 2009). Furthermore, the female hormone estrogen have been widely regonised to play a protective role against CRC formation and progression via aiding efficient DNA mismatch repair mediated by estrogen receptor β (ERβ) (He et al, 2012; Jin et al, 2010; Kennelly et al, 2008). It is therefore tempting to speculate that the diminished level of DNA mismatch repair in male mice resulted in more severe tumour progression and decreased survival of male mice. Detailed immunohistochemical analysis of ERβ expression in both male and female intestinal epitheliums is essential to verify this speculation. Another potential way that Cbx3 deficiency leads to more aggressive tumours could be by abolishing apoptosis. A previously published report demonstrated that Cbx3 has a role in regulating apoptosis through binding and sequestering the pro-apoptotic Bax and GADD45a proteins (Zhou et al., 2014), suggesting a potential role of Cbx3 in assisting cancer cells to escape from apoptosis. It would therefore be interesting to investigate the level of caspase-3 cleavage using
immunohistochemistry and to assess the binding of Cbx3 to the apoptotic proteins by co-immunoprecipitation analysis in the Cbx3-proficient and deficient tumours, in both male and female cohorts. Another potential mechanism that Cbx3 loss leads to more severe tumour progression could be by disrupting heterochromatin formation and gene silencing (Maison and Almouzni, 2004; Haldar et al., 2011; Smallwood et al., 2012; Larson et al., 2012). Concordant with this notion, a reduced level of heterochromatic mark H4K20me3 was consistently observed following Cbx3 loss in the small intestinal epithelium, suggesting a role of Cbx3 as a “gatekeeper” of epigenetic gene silencing in establishing, maintaining and spreading of H4K20me3. Similar scenarios are reported in the literature proposing that HP1 protein is necessary to establish H4K20me3 in heterochromatin (Kourmouli et al., 2004; Schotta et al., 2004) (Figure 6.1).

Previous evidence has suggested that H4K20 trimethylation (H4K20me3) is essential for the maintenance of perinatal, non-cancerous cell states and confers heterochromatin maintenance capability (Fraga et al., 2005; Pogribny et al., 2006; Schotta et al., 2008). Trimethylation specifically requires the recruitment of the Sur4-20h enzymes by HP1 (Schotta et al., 2004). H4K20me3 has been shown to have a significant correlation with HP1y expression in normal tissue samples and a dissociation of the correlation between HP1y expression and H4K20me3 may reflect the malfunction of epigenetic control in cancer tissues (Takanashi et al., 2009). These studies from the literature are consistent with our observation that co-localisation of HP1y to H4K20me3, which is essential for heterochromatin maintenance, is observed in normal intestinal homeostasis. Whereas in Wnt-activated intestine the HP1y-H4K20me3 association is lost, caused by HP1α displacing HP1y to the H4K20me3 marks. The relationship between HP1α and H4K20me3 remains to be determined but some early evidence has shown a dynamic correlation between HP1α and H4K20me3 levels in ES cells (Wongtawan et al., 2011), suggesting an important mechanism required for epigenetic malfunction in Wnt-activated intestine. Our findings are in agreement with the previous conclusion that HP1y binding at H4K20me3 is implicated to maintain the transcriptionally repressed state of heterochromatin. It is possible that Cbx3 deficiency leads to the disruption of heterochromatin formation and therefore induces widespread genomic instability, which allows further DNA double stranded breaks and
mutations aiding tumour progression and malignant transformation in Wnt-driven tumorigenesis.

Further investigation is required to establish the mechanism behind the loss of Cbx3 on chromatin compaction and heterochromatin formation. This could be achieved by examining levels of other heterochromatin (H3K27me3) or euchromatin (H3K36me3 and H3K79me3) histone marks. However, altered heterochromatin levels may not be the only contributor to genome instability in Cbx3 deficient intestines. Given that Cbx3 has been implicated to play a role in DNA repair pathways (review in Luijsterburg and van Attikum, 2011), deletion of Cbx3 may result in higher levels of DNA damage. This could be investigated through examining levels of DNA damage markers, such as the histone variant γH2AX, as an indicator of DNA double-strand breaks. Preliminary immunohistochemical analysis of DNA double-strand breaks demonstrated that γH2AX expression level was markedly increased in Cbx3-deficient tumours (data not shown). It would also be interesting to analyse the level of γH2AX expression separately in male and female tumours to determine whether there is a male-specific susceptibility to DNA damage. Furthermore, it would also be imperative to investigate RNA splicing (e.g. level of Rac1b, the tumour-specific splice variant of Rac1) in male and female tumour samples to determine whether there is a male-specific sensitivity towards HP1γ-mediated Rac1b splicing in colorectal tumours.

In order to fully understand the relationships between nucleosome organisation, Cbx3 and gene expression in normal intestinal homeostasis and Wnt-activated tumorigenesis, nucleosome positioning could be performed in intestinal epithelial genome with or without Cbx3 to gain a detailed picture of how Cbx3 influence nucleosome positioning in euchromatin and heterochromatin and subsequently facilitate or inhibit gene transcription. To identify genes that are either up-regulated or down-regulated in the absence of Cbx3, total RNA from the intestinal epithelium could be extracted and subjected to transcriptome analysis using Next Generation Sequencing. Once the genes with altered expression in the absence of Cbx3 are identified, Cbx3-targeted chromatin immunoprecipitation sequencing (ChIP-seq) analysis could be performed to further investigate the evidence of direct regulation of target genes by Cbx3.
Despite the fact that the results of this study are preliminary, current data indicates for the first time that Cbx3 plays multifaceted roles in intestinal homeostasis and tumorigenesis. Cbx3 has been implicated to play a novel role in positive regulation of Wnt signalling and RNA splicing in aberrant Wnt-activated intestine and a role in heterochromatin formation and gene silencing in long-term Wnt-activated tumorigenesis. Further phenotypic and mechanistic investigations as outlined previously would provide an increased understanding of the multifaceted roles of Cbx3 in aberrant Wnt-activation, RNA splicing, tumour progression and invasion, and its interaction with histone marks in our mouse models of colorectal cancer. Such knowledge has the potential to bring about the development of novel prognostic marker or therapeutic agent targeted Cbx3 in different stages of colorectal cancer, leading to more effective treatment regimes and ultimately resulting in improved patient outcomes.

“Today we are learning the language in which God created life.”

*US President Bill Clinton*
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